TEXT IS FLY WITHIN THE BOOK ONLY

UNIVERSAL
LIBRARY

OU_156826

UNIVERSAL
LIBRARY

OUP—786—13-6-75—10,000.

OSMANIA UNIVERSITY LIBRARY

Call No. 576·3 B771 Accession No. 35190

Author

Title

This book should be returned on or before the date last marked below.

INTERNATIONAL
REVIEW OF CYTOLOGY

VOLUME IV

INTERNATIONAL

Review of Cytology

EDITED BY

G. H. BOURNE
London Hospital
Medical College
London, England

J. F. DANIELLI
Zoology Department
King's College
London, England

VOLUME IV

Prepared Under the Auspices of
The International Society for
Cell Biology

ACADEMIC PRESS INC., PUBLISHERS

NEW YORK · 1955

Copyright 1955, by
ACADEMIC PRESS INC.
125 East 23rd Street, New York 10, New York

All Rights Reserved

NO PART OF THIS BOOK MAY BE REPRODUCED IN ANY FORM, BY PHOTOSTAT, MICROFILM, OR ANY OTHER MEANS, WITHOUT WRITTEN PERMISSION FROM THE PUBLISHERS.

Library of Congress Catalog Card Number (52-5203)

PRINTED IN THE UNITED STATES OF AMERICA

Contributors to Volume IV

E. J. CONWAY, *Department of Biochemistry, University College, Dublin, Ireland.*

R. COUTEAUX, *Laboratoire de Biologie animale (P.C.B.), Faculté des Sciences, Université de Paris, Paris, France.*

DAVID P. HACKETT, *Biological Laboratories, Harvard University, Cambridge, Massachusetts.**

M. J. KOPAC, *Department of Biology, Graduate School of Arts and Science, New York University, New York, New York.*

N. B. KURNICK, *Department of Medicine, University of California, Los Angeles, California and Research Laboratory of the Paraplegia Service, Veterans Administration Hospital, Long Beach, California.*

ALFRED MARSHAK, *Marine Biological Laboratory, Woods Hole, Massachusetts.*

HARALD MOE, *Department of Anatomy, University of Copenhagen, Denmark.*

K. MUHLETHALER, *Institut für Allgemeine Botanik, E. T. H. Laboratorium für Elektronenmikroskopie, Zurich, Switzerland.*

R. VENDRELY, *Centre de Recherches sur les Macromolécules, Strasbourg, France.*

W. S. VINCENT, *Department of Anatomy, Upstate Medical Center, State University of New York, Syracuse, New York.*

L. E. WAGGE, *Zoology Department, Imperial College of Science and Technology, London, England.*

M. WOLMAN, *Department of Pathology, Hadassah Medical School, Hebrew University, Jerusalem, Israel.*

---

* Present address: Department of Biology, University of Buffalo, Buffalo, New York.

Foreword

Our intention is, over a period of years, to publish articles over the whole field of cytology (including cell physiology) to enable those interested in cell biology more easily to form a unified concept of the field. We shall include not only review papers which bring certain topics up to date, but also historical reviews where necessary, and original theoretical papers where such papers introduce a unifying concept. One of the problems which confronts cytologists today is that rapid progress is being made with a variety of very distinct disciplines, which tends to create isolated pockets of knowledge. Our aim will be to emphasize the unity of cytology.

In the present volume we include a review by M. J. Kopac of important technical developments in micromanipulation which permit the quantitative study of small parts of cells. M. Wolman writes on the general principles and problems of fixation. L. E. Wagge discusses the functions of amebocytes, primarily in molluscs, but also gives a brief comparative treatment.

A. Marshak contributes a general view of the main elements of bacterial cytology, and R. Vendrely examines the cytochemistry of bacteria in some detail. These two reviews, and the preceding reviews of bacterial cytology in earlier volumes, make it appear certain that many of the most fundamental problems in bacterial cytology can only be solved by cytochemical studies at the electron microscope level. D. P. Hackett contributes a comprehensive review of recent studies of plant mitochondria, which, at the biochemical level, are closely similar to animal mitochondria. K. Muhlethaler's article on chloroplasts emphasizes the importance of the "sandwich" membranes which, originally found as plasma membranes, have since been discovered as a major structural feature of nuclear membranes, mitochondria, the golgi material, and plastids. N. B. Kurnick gives a critical review of various cytochemical techniques for the study of nucleic acids. Vincent's comprehensive review of nucleoli includes the stimulating results which he has obtained by isolation of nucleoli in bulk.

H. Moe contributes a survey of goblet cells as part of our program of reviewing special cell types and finally, in relation to the field of ionic movement, R. Couteaux discusses cholinesterases at neuromuscular junctions and E. J. Conway contributes the second part of his discussion of redox pump mechanisms for ionic transport.

In future volumes the majority of contributions will continue to be made by invitation, but we are prepared to consider reviews and theoretical articles sent without invitation.

J. F. Danielli
G. H. Bourne

July 1955

CONTENTS

Contributors to Volume IV v

Foreword vii

Cytochemical Micrurgy

By M. J. Kopac, *Department of Biology, Graduate School of Arts and Science, New York University, New York, New York*

I. Introduction 1
II. Preparation and Handling of Cells 2
III. Determinations of Small Quantities of Cellular Materials 6
IV. Special Procedures and Instrumentation 12
V. Application of Cytochemical Micrurgy to the Study of Isolated Subcellular Particulates 22
VI. General Applicability of Cytochemical Micrurgy 25
VII Summary 25
Acknowledgments 26
VIII References 26

Amoebocytes

By I. E. Wagge, *Zoology Department, Imperial College of Science and Technology, London, England*

I Introduction 31
II The Cytology of Amoebocytes 33
III Amoebocytic Activity during Shell Repair 43
IV. Amoebocytic Activity in Molluscan Digestion 53
V. The Excretory Function of Certain Amoebocytes 59
VI. Amoebocytes in Phyla other than the Mollusca 61
VII. Discussion 68
VIII. Summary 74
IX. References 75

Problems of Fixation in Cytology, Histology and Histochemistry

By M. Wolman, *Department of Pathology, Hadassah Medical School, Hebrew University, Jerusalem, Israel*

I. Introduction 79
II. Chemical and Physical Effects of Fixatives on Tissue Constituents 80
III. Effect of Fixation on Living Protoplasm 87
IV. Effects of Fixation on Some Characteristics of Tissue and Cell Constituents 96
V. Conclusions 99
VI. References 99

Bacterial Cytology

By Alfred Marshak, *Marine Biological Laboratory, Woods Hole, Massachusetts*

I. Introduction 103
II. Nuclei 104
III. Centrioles 105
IV. Chromosomes 106
V. Mitochondria 107
VI. Vacuoles 107
VII. Membranes 108
VIII. Transverse Wall: Flagella 108
IX. Nucleus of Bacterial Spores 109
X. Formation of Giant and Dwarf Forms 110
XI. Comparison with Cells of Higher Forms 110
XII. Conclusions 112
XIII. References 113

Histochemistry of Bacteria

By R. Vendrely, *Centre de Recherches sur les Macromolécules, Strasbourg, France*

I. Introduction 115
II. The Envelopes of the Bacteria 116
III. The Bacterial Nucleus 120
IV. The Cytoplasmic Bodies 130
V. The Isolation of Structural Elements of Bacteria 134
VI. Conclusion 138
VII. References 138

Recent Studies on Plant Mitochondria

By David P. Hackett, *Biological Laboratories, Harvard University, Cambridge, Massachusetts*

I. Introduction 143
II. Definitions 144
III. Methods 146
IV. Structure 151
V. Origin and Distribution 154
VI. Cytological Observations on Isolated Mitochondria 155
VII. Chemical Composition 158
VIII. Respiration 159
IX. Phosphorylation 174
X. Biosynthesis 177
XI. Physiology 182
XII. Genetics, Evolution and Radiation Effects 185
XIII. Conclusion 188
XIV. References 188
Addendum 196

The Structure of Chloroplasts

By K. Mühlethaler, *Institut für Allgemeine Botanik E.T.H. Laboratorium für Elektronenmikroskopie, Zürich, Switzerland*

I. Introduction 197
II. Methods of Investigation 198
III. Shape and Size of Chloroplasts 199
IV. Composition of Chloroplasts 200
V. Microscopic Structure of Chloroplasts 201
VI. Results of Electron Microscope Investigations 203
VII. Molecular Structure of the Grana Lamellae 212
VIII. The Development of the Chloroplasts 215
IX. Continuity of the Chloroplasts and Heredity 217
X. References 217

Histochemistry of Nucleic Acids

By N. B. Kurnick, *Department of Medicine, University of California, Los Angeles, California, and the Research Laboratory of the Paraplegia Service, Veterans Administration Hospital, Long Beach, California*

I. Introduction 221
II. Requirements for Quantitative Histochemical Analysis and Sources of Error 222
III. Fixation of Tissues 229
IV. Staining Methods 231
V. Physical Methods 252
VI. Summary 260
VII. References 262

Structure and Chemistry of Nucleoli

By W. S. Vincent, *Department of Anatomy, Upstate Medical Center, State University of New York, Syracuse, New York*

I. Introduction 269
II. The Cycle of the Nucleolus 271
III. Interphase Morphology of the Nucleolus 272
IV. Chemistry of the Nucleolus 280
V. The Origins of Nucleolar Materials 289
VI. Nucleolar Function 293
VII. References 295

On Goblet Cells, Especially of the Intestine of Some Mammalian Species

By Harald Moe, *Department of Anatomy, University of Copenhagen, Denmark*

I. Introduction 299
II. Goblet Cells in General 300
III. Goblet Cells in the Intestine 304
IV. References 333

Localization of Cholinesterases at Neuromuscular Junctions

By R. Couteaux, *Laboratoire de Biologie animale (P.C.B.), Faculté des Sciences, Université de Paris, Paris, France*

I. Introduction 335
II. General Features of Neuromuscular Junctions 336
III. Junctional Cholinesterases and their Histochemical Localization 355
IV. References 373

Evidence for a Redox Pump in the Active Transport of Cations

By E. J. Conway, *Department of Biochemistry, University College, Dublin, Ireland*

I. Introduction 377
II. The Active Transport of Cations in Yeast 378
III. The Active Transport of Na^+ Ions through Frog Skin 385
IV. Secretion of H^+ Ions by the Gastric Mucosa 387
V. Further Observations on Active Transport in Plants 394
VI. References 395

Author Index 397

Subject Index 415

Cytochemical Micrurgy

M. J. KOPAC

Department of Biology, Graduate School of Arts and Science, New York University, New York, New York

		Page
I.	Introduction	1
II.	Preparation and Handling of Cells	2
	1. Free Cells	2
	2. Separation of Cells from Solid Tissues	3
	3. Tissue Cultures	4
	4. Centrifugation of Cells	5
III.	Determinations of Small Quantities of Cellular Materials	6
	1. Measurement of Cell Volumes	7
	2. The Diver Balance	8
	3. Torsion and "Fish-Pole" Microbalances	8
	4. Roentgen-Ray Absorption and Optical Methods	9
	5. The Volumetric Submicromanipulator	9
IV.	Special Procedures and Instrumentation	12
	1. Micrurgy	12
	2. Optical	16
	3. Manometric Methods	16
	4. Microchromatographic Methods	17
	5. Microdilatometric Methods	17
	6. Ultraviolet Micrurgy	20
V.	Application of Cytochemical Micrurgy to the Study of Isolated Subcellular Particulates	22
	1. Localization of Enzymes in the Cell	22
	2. Estimation of Enzymatic Activities of the Nucleus and Subcellular Particulates	22
	3. Comparison of Microsomal Activity with the Matrix	24
VI.	General Applicability of Cytochemical Micrurgy	25
VII.	Summary	25
	Acknowledgments	26
VIII.	References	26

I. Introduction

This review is limited to a discussion of methods and instrumentation available for quantitative cytochemical investigations on isolated cells or parts thereof. The methods required to handle and measure small volumes of cells or subcellular fractions must, in most instances, be micrurgical. Furthermore, the methods must disclose the cytochemical properties of small amounts of material. At present, the most feasible cytochemical determinations are those of enzymatic activities, since one may take advantage of the turnover properties of an enzyme. It is theoretically possible to identify a single enzyme molecule.

Future work may be focused on some aspects of inorganic cytochemistry by combining micrurgy with microincineration, nuclear track radioautography, or emission electron microscopy. It should be pointed out that with nuclear track radioautography, a single beta or alpha particle may also be identified (Fitzgerald *et al.*, 1953). Scott and Packer (1939a, b) applied the emission electron microscope for locating magnesium and calcium in striated muscle tissue. Further work on the intracellular distribution of elements with improved emission electron microscopes and accessory instrumentation may be fruitful.

There are now available several books dealing with various phases of histo- and cyto-chemistry. Especially useful for much of the background are the following: Glick (1949), Kirk (1950), Gomori (1952), Pearse (1953), and Danielli (1953). Other important sources include the articles cited in the text of this review.

II. Preparation and Handling of Cells

Many varieties of free cells are available of which the amoeba and marine invertebrate eggs are almost classical materials for micrurgical studies. To these may now be added the ascites tumor cells, a group of great importance and potential worth for cytochemical study. In addition, suspensions of free cells in most instances may be obtained from solid tissues.

One must stress the importance of recognizing the viability of the cells under investigation. If the cytochemistry of living cells is being studied, then one should at least know whether or not the cells are alive at the time samples are taken. With amoebas and eggs, this point can be established by observation, providing the investigator is familiar with the material.

On the other hand, cells obtained from solid tissues present a more difficult problem. Following the separation of cells, frequently after mechanical means or after subjecting the cells to unusual and possibly unphysiological media, there is always the chance that such cells may have been irreversibly damaged. Actually, the only unequivocal index of viability of such cells is to test their growth capacity in media suitable for tissue cultures.

1. Free Cells

1. *Amoebas.* Certain features pertaining to culturing, micrurgical procedures and treatment, handling, and cytological fixation of amoebas have been summarized by Kassel and Kopac (1953) and Kassel and Melnitsky (1951). Other important papers on micrurgical treatment of amoebas with special reference to nuclear transplantation are by Lorch and Danielli (1953a, b).

b. Marine Invertebrate Eggs. Much work on marine invertebrate eggs has been reported. A helpful reference is a small book written by Just (1939). Papers dealing with micrurgical studies on various eggs are those by Kopac (1940, 1943, 1950a), Mitchison (1953), and Lorch, Danielli, and Horstadius (1953).

c. Ascites Tumor Cells. Klein (1951), Goldie and Felix (1951), Hauschka and Levan (1953), Hauschka (1953a, b), and others have converted a number of solid tumors into ascites tumors in mice. A few rat tumors have also been converted into ascites tumors (Yoshida, 1952). These recent developments in experimental cancer research offer valuable material for cytochemical and micrurgical studies. These cells may be readily obtained from a tumor-bearing mouse by puncturing the abdominal cavity with a hypodermic needle and aspirating a suspension of cells in the peritoneal fluid with a syringe. Not only is there available a wide range of ascites tumors, but each tumor may present a spectrum of cell types, including euploid, aneuploid, polyploid, as well as cells in various stages of mitosis. Furthermore, certain cytologically variable tumors have now been converted into clones by single cell isolation techniques and inoculation into newborn mice (Hauschka, 1953b).

2. Separation of Cells from Solid Tissues

a. Mechanical Separation. Suspensions of single cells or clumps of 2–5 cells may be obtained by mild mechanical means from a number of tissues. Fragments of rat liver when forced through cheesecloth and then bolting cloth will yield separated cells (Schneider and Potter, 1943). Low speed homogenization has also been used (Harrison, 1953). St. Aubin and Bucher (1952) have obtained good yields of cells suitable for counting mononucleate and binucleate cells by agitating resting or regenerating rat liver tissue fragments with glass beads in a Kahn shaker. Hyaluronidase does not help in the separation of these cells. On the other hand, trypsin or other proteolytic enzymes facilitate the separation. Some of the cells, however, are digested by proteolytic enzymes. Suspensions of cells from human ovarian neoplasms have also been prepared, frequently within a few minutes, by mild mechanical methods (Mateyko, 1954).

b. Calcium-Free Media. The dispersive action of Ca-free media on the hyaline layer of sea urchin eggs was reviewed by Kopac (1940) in connection with studies on extraneous coats. The addition of a calcium binder (oxalates, citrates, or chelating agents) accelerates the dispersion of hyaline layers which possess many of the characteristics of intercellular cements. Anderson (1953) reported on the isolation of cells from livers by perfusing the liver with either Ca-free media or media containing cal-

cium binders, followed by breaking the liver fragments with a loose-fitting lucite pestle in a Pyrex homogenizer tube. Northcroft (1951) separated cells from carrot tissues cultured in a medium containing ammonium oxalate. Cells from chick embryos may be dissociated by immersing the tissues in chick Ringer's solution buffered to a pH of 9.0–9.2 (Mookerjee, 1953).

c. Enzymatic Hydrolysis of Intercellular Cements. Another method for obtaining suspensions of cells from solid tissues or tissue cultures is based on the enzymatic hydrolysis of intercellular cements by proteolytic enzymes or hyaluronidase. Moscona (1952) has used a combination of trypsin, hyaluronidase, and Ca-free media for separating neuroblasts and other cells from grasshopper larvae. In most instances, trypsin will weaken intercellular cements sufficiently so that cell suspensions can be obtained (St. Amand and Tipton, 1954). Northrop (1947) reported that blastomeres, developing from fertilized *Arbacia* eggs in media containing papain, become separated, indicating a dispersion of the hyaline layer.

Testicular tissues growing in tissue culture may be converted into cell suspensions by incubating the cultures with trypsin (Frisch and Jentoft, 1953). Dulbecco (1952), Dulbecco and Vogt (1954), and Youngner (1954) have successfully used trypsin to obtain cell suspensions from kidney tissues grown in tissue culture. Suspensions of cells obtained with trypsin are adequate for the quantitative estimation of cell numbers. Moreover, the cells remain viable and will continue to grow in tissue culture from which replicate cultures may be established (Youngner). Commercial grades of trypsin (Bacto-Trypsin T:250 or Nutritional Biochemicals' Trypsin 1:300, for example) are satisfactory. Fischer (1949) and Morgan and Parker (1949) have reported that soybean trypsin inhibitor prevents the digestion of tissue culture clots by trypsin and, in addition, prevents the liquefaction of plasma clots by rapidly growing cells.

3. Tissue Cultures

This review does not describe tissue culture techniques except to point out that these procedures may be of value in providing many varieties of cells for cytochemical studies and also in determining the viability of cells that may have been exposed to unusual media or manipulations. Details covering tissue culture techniques may be found in the books by Cameron (1950) and Parker (1950). An extensive bibliography on tissue culture and applications has been recently published (Murray and Kopech, 1953).

From the aspect of micrurgy, certain tissue culture techniques may be important. For example, the perforated cellophane techniques (Evans and Earle, 1947) and mass, replicate cultures (Evans, 1951; Youngner, 1954)

with cells growing in liquid media provide cells as useful and convenient as amoebas to micrurgists.

One should note also the development of single cell techniques for establishing clones of cells in tissue culture (Sanford, Earle, and Likely, 1948-9; Likely, Sanford, and Earle, 1952). Important advances have been made on fluid culture media as recently reviewed by Stewart and Kirk (1954). With improved media and procedures, the micrurgist should be able to test a cell for its growth capacity in tissue culture even after the cell has been subjected to micrurgical and other experimentation.

4. Centrifugation of Cells

The centrifuge is an extremely serviceable instrument for cytochemical research. The classical application of the centrifuge by Harvey (1941, 1946, 1951) was to induce a rearrangement of cellular inclusions within the cell in accordance with their densities (Costello, 1939). With this technique, *Arbacia* and other eggs may be split into "halves" and "quarters." Other cells, including amoebas and amphibian liver cells may also be centrifuged with the result that intracellular layers of cellular inclusions are produced.

The centrifuge should provide accelerations up to 25,000 $\times$ g or higher with provisions against heating (preferably refrigeration). It is important to suspend the cells in a density gradient (pyknotic cushion) to avoid crushing. One must have a liquid medium with a high density and negligible osmotic activity to produce satisfactory pyknotic cushions.

Marine ova have not presented too much of a challenge since an isosmotic solution of sucrose has a sufficiently high density. By producing a gradient ranging from *sucrose* to *sucrose* + *sea water* to *sea water*, one can readily centrifuge cells to the point of producing "halves" and "quarters" providing the extraneous coats are not too tough.

Fresh water organisms offer a more difficult problem. Concentrations of sucrose high enough to give the right density are hypertonic. Accordingly various colloids have been used, including soluble starches, gum acacias, etc. Solutions of such substances, although providing adequate densities, have rather negligible osmotic properties.

Somatic cells from vertebrates present the most difficult problems. The densities of these cells are frequently high, thus requiring a medium with a high initial density in order that a gradient can be established to give a pyknotic cushion. After investigating a variety of polymers, including polyvinylpyrrolidone, polyglucoses, methyl celluloses, polyethylene glycols, dextrans, etc., Mateyko (1954) concluded that a 30% Colloidal Silica (DuPont) provides a sufficiently high density (>1.2). Moreover, it is

entirely harmless and osmotically inactive. This medium, following slight modifications, including an adjustment of pH, can be safely used with various vertebrate cells, including those from human ovarian neoplasms.

It is interesting to note that the inclusions in amphibian liver cells (*Rana, Ambystoma, Amphiuma*) can be readily stratified (20,000 × *g* for 60–90 minutes), as shown in Fig. 1. On the other hand, liver cells

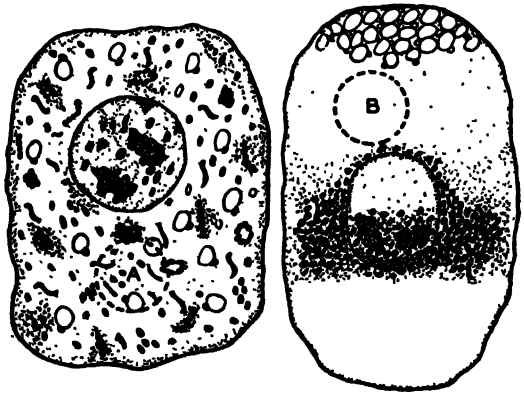


FIG. 1. Diagrams of optical sections of liver cells (*Amphiuma*) before and after centrifugation (from Kopac, 1953). Left diagram shows a liver cell with its normal complement of inclusions. Right diagram shows a similar cell after centrifuging (20,000 × g. for 90 minutes) with cytoplasmic particulates rearranged to form the following layers: I. (top) lipids, II. matrix, III. mitochondria, secretory granules, nucleus, matrix, IV. glycogen. The circles represent the diameter of a sphere (to scale) with a volume of 1 μμl. (diameter of sphere = 12.4 μ).

from rodents (mouse, rat, guinea pig) show no appreciable intracellular stratification even when centrifuged at 25,000 × *g* for 3–4 hours. Similarly, certain cells from human ovarian neoplasms (Mateyko and Kopac, 1954) will become stratified, although the layers are not always the same. Other cells, from the same neoplasms, will show no stratification even though subjected to 25,000 × *g* for periods up to 4–5 hours. There is, as yet, no satisfactory explanation why some cells can be stratified and others not. This in itself poses an interesting and possibly important problem.

III. Determination of Small Quantities of Cellular Materials

The enzymatic activities of a cell or its fractions should be expressed in terms of the amount of cell substance. A convenient unit is 1 μμl. (1,000 μ^3), which represents approximately 1/300th of the volume of a sea urchin egg, for instance. Since the activities of small amounts of enzyme-contain-

ing substances are low, such values can be conveniently expressed as $\mu\mu$M./hour/$\mu\mu$l. The symbols, μl., μM., and μg. are defined as 10^{-6} of the unit involved, and are called microliter, micromole, and microgram, respectively. Similarly, the symbols, $\mu\mu$l., $\mu\mu$M., and $\mu\mu$g., are 10^{-12} of the unit, and these may all be designated as micromicroliter, micromicromole, and micromicrogram, respectively.

In all enzymatic determinations, the reactions should be set up so that the activities become proportional to the amount of enzyme present (zero order reactions). One requirement, therefore, is that enough substrate be present so that the rate of the reaction becomes limited solely by the turnover capacity of the enzyme.

In cytochemistry, a reasonable first approximation is that the amount of a given enzyme in a sample is proportional to the volume of the sample. For this, it is essential that methods be provided for measuring either small volumes, or small masses (and densities). Several procedures are now available so that fair estimates can be made of the amounts of cellular substances contributing the enzyme under investigation.

1. *Measurement of Cell Volumes*

The volumes of spherical cells, as well as of cells that can be safely converted into spheres, may be readily calculated from diameters measured with an ocular micrometer. One precaution, however, must be emphasized. Eggs with weak vitelline membranes or other extraneous coats will become flattened rather easily. If one is measuring cells in a shallow hanging drop, there is a good possibility that some of the cells may become flattened and, obviously, the volumes as based on measurements of diameters will be too high. Accordingly, only those cells in the deeper portions of the hanging drop, where there is no danger of flattening, should be measured.

The measurement of irregularly shaped cells and subsequent calculation of volume is much more intricate. Chalkley (1929) and Mast and Fowler (1935) constructed a volumescope for measuring the volumes of amoebas. The method consists essentially of drawing an amoeba into a narrow capillary tube and calculating the volumes from the length of the amoeba in the tube and the cross sectional area. This method, however, may severely stimulate the organism with consequent alterations in volume. There is also the danger that small fragments of a cell may become pinched off and stuck to the glass tube during the manipulations.

Mention should be made of Chalkley's (1943–4) multiple point pattern method for measuring volumetric ratios of cells and tissues. The method is based on the principle that the number of random point hits on structures will be proportional to the volume of the structure. Unfortunately,

this method cannot be used for single determinations. Cell and nuclear volumes of motor horn cells were measured and reported by Mickelwright, Kurnick, and Hodes (1953).

Holter (1945) devised a colorimetric method for measuring the volumes of large amoebas. Holter estimated that the probable error of a single measurement by his method was about ± 5%. Lumsden and Robinson (1953) described a method in which an amoeba is partially flattened between two plates, the area occupied by an amoeba being measured with a planimeter and calculations of volume then made from the area and distance (thickness of cell) between the two plates. This method is probably less damaging to the amoeba than the volumescope. The reported precision corresponds to a standard deviation of 0.0005 μl., representing about 3% for small and 1% for large amoebas (*Chaos chaos*).

2. *The Diver Balance*

Zeuthen (1948) described his "diver balance", whereby the reduced weight (R.W.) of cells can be measured with errors no greater than ±0.01–0.02 μg. The R.W. is defined as the total mass of the object minus the mass of the volume of medium displaced by the object, *viz.*: R.W. $= V_c\ (d_c - d_m)$, where V_c = volume of cell, d_c = density of cell and d_m = density of the medium. Absolute weights (A.W.) may also be determined providing the same cell is measured in media of different densities. Thus, A.W. = R.W., if d_m is essentially zero (air, for example).

This method is based on the principle of the Cartesian diver and is sufficiently sensitive to measure the R.W. of large amoebas which may range from 1.06 to 2.3 μg. (Holter and Zeuthen, 1948). The method has the advantage that the cells can be measured repeatedly since no harm apparently is done to the cells either during weighing or handling. Furthermore, R.W. determinations may be made in a medium that is physiologically safe to the cells.

3. *Torsion and "Fish-Pole" Microbalances*

Lowry (1953) described simple torsion and "fish-pole" microbalances that have high sensitivities. The simpler, "fish-pole" balance, consisting of a delicate quartz fiber and pan plus a low-power microscope can be constructed to give a sensitivity and reproducibility of 0.01 μg. This type of balance is suitable for measuring the dry weights of tissue samples (frozen and dried, *in vacuo*) as small as 100 × 100 × 20 μ, corresponding to a wet weight of approximately 0.2 μg. It is possible that Lowry's microbalances and procedures may prove to be extremely valuable for weighing, under dry conditions, larger cells or aggregates consisting of a few smaller cells.

4. *Roentgen-Ray Absorption and Optical Methods*

Engstrom (1946) has employed soft X-rays to determine the concentrations of certain elements in histological materials. The dry weight of cytological objects may also be estimated by X-ray absorption techniques (Engstrom and Lindstrom, 1949, 1950). A microradiogram of the sample is produced from soft X-rays (8–12 Å), from which the X-ray absorption is obtained in terms of photographic density. From the absorption (estimated by certain calibration procedures) and the area of the object, the mass of the object can be calculated with an uncertainty ranging from 5 to 25%, depending on the material (Engstrom, 1953). With improved methods and better information on the elemental content of cell samples, a greater precision in determining dry weights should be expected. Fitzgerald and Engstrom (1952) have shown that nuclei of salivary glands (*Chironomous* larvae) and of ganglion cells (*Helix*, olfactory ganglia) have a lower specific mass than the cytoplasm.

By employing interference microscopy, Barer (1952) and Davies and Wilkins (1952) have been able to calculate the equivalent dry mass of proteins in a cell (31 $\mu\mu$g. hemoglobin per red blood cell). Mellors (1953a, b, c) and Mellors, Kupfer, and Hollender (1953), using multiple beam interference microscopy, determined the thickness, volume, and anhydrous organic mass of cells. With similar procedures, the hydrous protoplasmic mass of nuclei (240 $\mu\mu$g.) in living sarcoma cells has been calculated. These techniques and instruments will undoubtedly achieve wide application in cytological research as the skills required to operate them are acquired.

5. *The Volumetric Submicromanipulator*

None of the previously described methods is sufficiently sensitive to measure fractions that can be obtained from cells micrurgically. For example, following centrifugation, layers of particulate-free matrix may be obtained (Fig. 1). Samples of matrix may be removed with a micropipette, but the amounts available in most instances are low. Except for certain somatic cells of *Amphiuma* or other amphibians or occasionally large cells obtainable from human ovarian neoplasms, most cells are so small that not more than 1 $\mu\mu$l. of matrix can be removed without contaminating the sample with other particulates. At best, the largest samples that may be obtained will generally be less than 10 $\mu\mu$l.

For these reasons, the volumetric submicromanipulator was designed and constructed. With this new instrument, volumes of cytoplasmic substances of the order of 1 $\mu\mu$l. can be removed from a living cell and transferred to substrate mixtures where the enzymatic activity of the sample may be tested.

The pilot model, as developed by Kopac and Harris (1951) and Kopac (1953), has two micromanipulator units, integrated with volumetric controls, mounted in front of and on either side of an inverted microscope. In the improved model (Fig. 2), a second pair of micromanipulator units

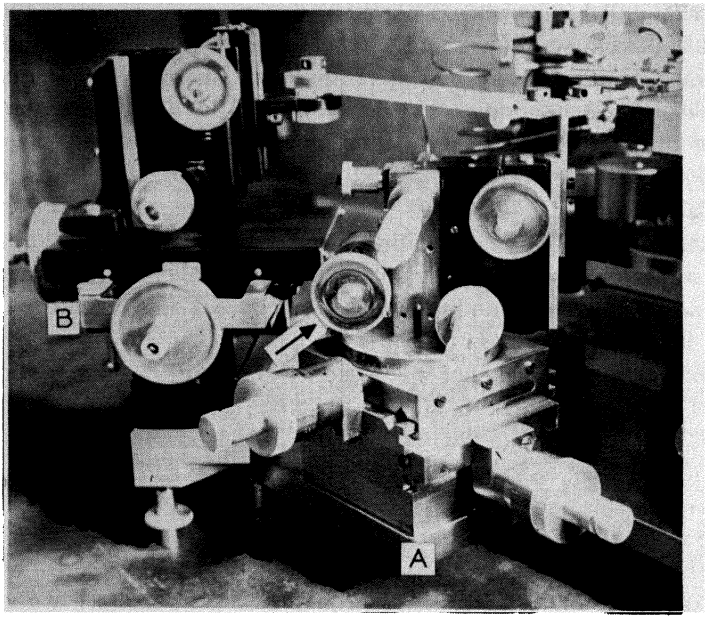


FIG. 2. Volumetric submicromanipulator (Kopac and Harris, 1951) showing left side of the four unit instrument. The volumetric controls are integrated with a three-way micromanipulator, A. The movements of the fine piston are regulated by a mechanism consisting of a feed screw and worm-gear drive equivalent to a screw with 125,000 threads/inch. One full turn of the volumetric control knob (indicated by arrow) moves the piston 8 microinches (volume = 2.4 μμl.). Another micromanipulator, B, is adapted for controlling a microneedle. The micropipette and microneedle clamps are mounted in front of the stage of an inverted microscope. All four micromanipulators have ball-bearing slides activated by precision micrometer screw heads. Vertical movements are modifications of coarse-fine focusing mechanisms.

has been added. It was found that, although there was no difficulty in penetrating a cell with a micropipette and removing a measured volume of cell substance, it was not always easy to displace the cell which became impaled on the tip of the micropipette. The best way to separate the impaled cell from the pipette is to flick it off with a microneedle.

The positions of the microneedles and micropipettes are shown in Fig. 3. Another important advantage now afforded by the addition of the two microneedles is that the centrifuged cells may be easily oriented in order

to be in a more favorable position with respect to the micropipettes. This is especially important if one wishes to obtain, for example, an uncontaminated sample of matrix, or a specific sample of cytoplasm with the nucleus.

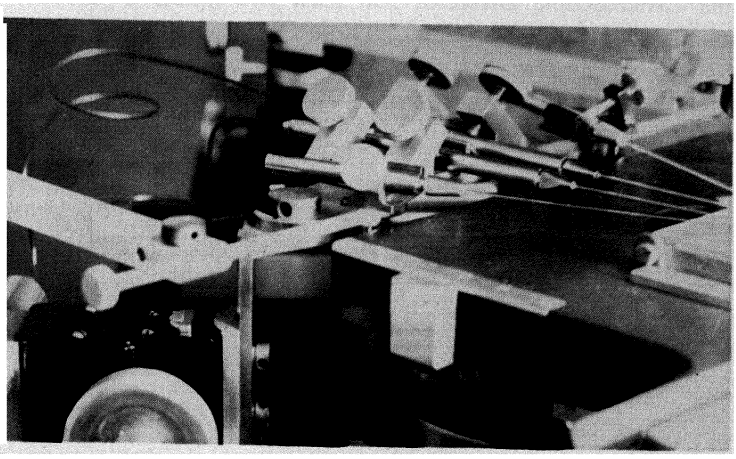


FIG. 3. Arrangement of microneedles and micropipettes. The micropipettes are flanked on either side by a microneedle. Note flexible copper tubes leading from pipette holders to volumetric controls. The pipette clamps and volumetric controls are mounted on opposite micromanipulators. The pipette tip may be inserted into the cell by manipulating the positioning controls with one hand and, at the same time, the volumetric controls can be manipulated with the other.

The volumetric submicromanipulator has two pistons, with diameters of 0.005 inch and 0.0158 inch. The smaller piston permits volume measurements down to 0.03 $\mu\mu$l. Samples of cell substance ranging from 0.3 to 10 $\mu\mu$l. can be measured and removed from living cells. The larger piston, which gives 10 times the volume of the smaller piston for the same linear displacement of the piston, is employed mainly for adding activators, inhibitors, or enzymes to reaction mixtures as required for microdilatometry (described in the following sections).

This instrument was adapted to the Gamma inverted microscope equipped with several types of objectives. An excellent objective is of the reflecting type (American Optical Company) which has a magnification of 50 $\times$ and a working distance of 3.8 mm. The Newton long working distance objectives are also suitable.

A special moist chamber was constructed (Fig. 4) for holding two square cover glasses. The top of the chamber can be raised to permit insertion of cover glasses from the rear position without disturbing the microneedles or micropipettes, which are inserted through the front opening.

IV. Special Procedures and Instrumentation

1. *Micrurgy*

a. Micromanipulators. Since 1859 when Schmidt developed and applied his "Microscopic Dissector", at least two hundred different micromanipulators have been described. These instruments are roughly of two types: those which require the manipulation of feed screws to provide control of the microinstruments, and those which operate on the lever or "joy-stick" principle.

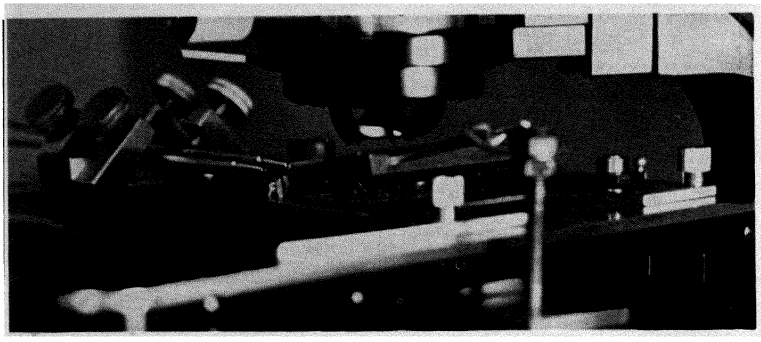


FIG. 4. Special moist chamber, adapted for the inverted microscope, opened from the rear position to permit insertion of two cover glasses which may be seen on the floor of the chamber. Micropipettes and microneedles (not shown) enter the chamber through an opening in the front. Relatively large "lying drops" are used with the inverted microscope so that the cells come to rest on the upper surface of the cover glass.

The most successful instruments in the first category are those of Chambers (1922), Peterfi (1923), Taylor (1925), and Kopac (1950b). These instruments consist of either spring-hinge bars, slides, or ball-bearing slides activated by feed screws. In the second category are several modifications which are currently meeting with favor, especially among beginners in micrurgy. The joy-stick controlled, pneumatic micromanipulators are those of de Fonbrune (1932) and Cailloux (1943). May (1953) described a joy-stick controlled, hydraulic micromanipulator. Emerson (1931) developed a joy-stick or lever control directly coupled to sliding components. A sliding micromanipulator (Reinert, 1938) and the Singer, single-control sliding micromanipulator (Barer and Saunders-Singer, 1948) have also been described and are available commercially. Bush, Duryee, and Hastings (1953) constructed an electric, joy-stick controlled micromanipulator which can handle up to four microneedles or micropipettes.

Although the lever or joy-stick controlled micromanipulators are easier

to operate, all are handicapped by limited loading capacity so that it is frequently awkward to mount micropipettes and other devices needed for microinjection. For such requirements, the massive, feed-screw operated micromanipulators are more satisfactory and frequently indispensable as shown in the volumetric submicromanipulator. The ability to mount microinjection units directly onto the micromanipulator more than compensates for the occasional inconveniences experienced in manipulating a feed-screw.

Most of the procedures involving micrurgy discussed in this review were established with feed-screw type micromanipulators. If the cells are nonmotile, there is rarely any need for rapidly maneuvering a micropipette. Indeed, many of the manipulative procedures are accomplished simply by employing the vertical movements on the instrument.

b. Microscopical Equipment. The conventional microscope equipped with a mechanical stage having a smooth movement and a range of 50 × 75 mm. plus a long working distance substage condenser will suffice for most micrurgical work. The disadvantages of hanging-drop preparations are frequently compensated by the ease with which one can change objectives.

On the other hand, the great advantage afforded by the inverted microscope is in the lying drop with the valuable result that cells rest on the supporting cover glass. It is, therefore, not necessary to hold the cells with a microneedle in order to accomplish an injection or extraction with a micropipette. The major objection to inverted microscopes is the inconvenience in changing objectives since the conventional revolving nosepiece cannot be used. Under such conditions, it is advisable to resort to the centering objective changers so that all objectives will have coincident optical axes. Furthermore, long working distance objectives, most of which are of the reflecting type, are indispensable.

It should be emphasized that no microscope, conventional or inverted, is entirely satisfactory for micrurgical work. It is true that most of the micromanipulators can be adapted to any of the microscope stands providing their stages are not involved in focusing. One has, however, the frequent impression that the tail is wagging the dog. A new design in microscope-micromanipulator instrumentation is needed. Specifically, the microscope stand should be redesigned and integrated with a stable micromanipulator that will accommodate at least four microneedles or micropipettes. Such an instrument is being developed by Kopac and Harris (1954).

c. Pipette Controlling Devices. For controlling volumes ranging from 0.0005 μl. to several microliters, the drop-retraction unit (Kopac 1943,

1950a), or some modification of it, is entirely satisfactory for qualitative microinjections or crude extraction of cytoplasm from cells. One of the preferred types, illustrated in Fig. 5, consists of coarse and fine volumetric controls. Micropipettes are filled by the coarse control. The fine control

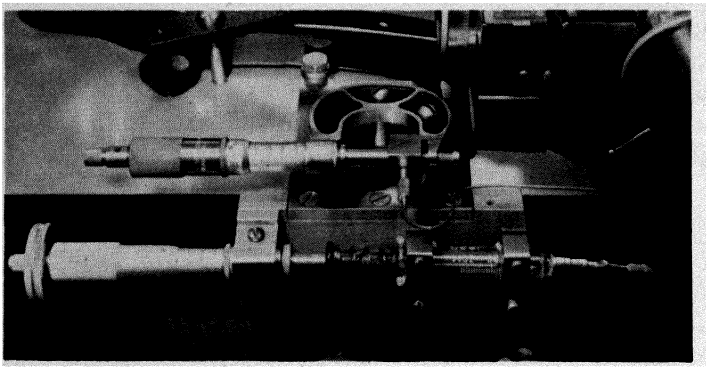


FIG. 5. Drop-retraction unit (Kopac, 1950a, b). The coarse volumetric control consists of a 2 ml. Luer syringe with a spring-loaded piston activated by a micrometer head. The fine volumetric control includes a modified micrometer (Scholander, 1942). The micrometer spindle was replaced by a No. 80 drill rod, which displaces mercury in the steel jacket. With this arrangement, two full turns of the micrometer drum displaces the same volume as produced by 0.01 turn of the micrometer-syringe unit.

is adapted for producing smaller volumes, or for enlarging a drop of aqueous solution suspended in oil to the required diameter, as measured with a filar ocular micrometer.

The drop-retraction unit is connected to the micropipette holder (Chambers and Kopac, 1950) with either flexible copper or polyethylene tubing. Volumes of the order of a micromicroliter can be handled efficiently with the volumetric submicromanipulator.

d. Moist Chambers. The moist chambers must be modified for the specific problem after the basic patterns described by Chambers and Kopac (1950). This is especially true when the inverted microscope is used (see Fig. 4).

Other variations might include provisions for controlling the gases in the chamber, ranging from complete anaerobiosis to aerobic mixtures of different oxygen content. The earlier work on such chambers was done by Cohen, Chambers, and Reznikoff (1928) in their studies on intracellular oxidation-reduction potentials. An improved chamber for such work is being developed by Diacumakos (1954) in my laboratory.

The "oil-chamber" of de Fonbrune has been used by Edstrom (1954)

for his studies on the nucleic acid content of single cells. For many problems, this chamber has a great deal of merit since, among other things, evaporation of aqueous media is strikingly curtailed.

A refrigerated chamber has been built (Kopac, 1951) in which micrurgical operations can be performed at temperatures limited only by the freezing point of the medium in which the cells are suspended.

e. Fabrication of Microneedles and Micropipettes. The trained and experienced micrurgist should be able to fabricate microneedles or micropipettes by hand using a simple gas microburner and glass rods or tubing. Complete instructions for making needles and pipettes by hand are given by Chambers and Kopac (1950). During recent years, several mechanical devices for pulling micropipettes have been described. In this group are Livingston's needle puller (made by Otto Hebel, Swarthmore College, Swarthmore, Pennsylvania), and also the instruments designed by Benedetti-Pichler and Rachele (1940) and by Alexander and Nastuk (1953). In the description of the latter, there is included an electron micrograph of a micropipette with a tip 0.19 μ in diameter. These pipette pullers are excellent for making standardized micropipettes.

Micropipettes with unusual tips or shapes can be fabricated with de Fonbrune's (1949) microforge. Powell (1952) has devised a simpler microstage (see also, Hilson, 1952). There is no question about the value of microforges for bending micropipettes near the tip or for fire polishing their openings.

An important innovation regarding micropipettes is to siliconize the inner and outer surfaces (Kopac, 1953). This treatment reduces the adherence of cytoplasmic residue to the pipette. Siliconized micropipettes may be readily cleaned since aqueous fluids do not stick to the glass. Several commercial preparations containing chlorosilanes in suitable solvents are available (Gilbert, 1951).

f. Micrurgical Techniques. It is not feasible to outline all the correct micrurgical procedures. Such procedures usually have to be worked out in accordance with the material and problem under investigation. The investigator, however, should become proficient in certain basic procedures which frequently may be directly applied, following slight modifications, to the specific problem.

Those basic procedures include: setting up of micromanipulator, microscope, and light source; preparation of moist chamber; rapid replacement of damaged or dirty microneedles or micropipettes; centering of microinstruments in optical field of microscope; proper vertical placement of microinstruments in relation to depth of hanging drop; preparation of hanging drops of the same or similar material to be used in the research;

mounting cover glasses on moist chamber; practice in manipulating the needles or pipettes in the field of the microscope; changing magnification from low to higher powers and vice versa; practice in manipulating micro-injection equipment; etc. Basic suggestions for these procedures are fully described by de Fonbrune (1949) and Chambers and Kopac (1950).

2. *Optical*

Most of the pertinent information concerning microphotometric procedures, including the application of ultraviolet light, has been discussed by Glick (1953). To this, one should also add the recent review on microscopy by Mellors (1953a). Blout (1953a) has summarized ultraviolet microscopy and microspectroscopy. The Ultraviolet Color Translating Microscope (Land *et al.*, 1949) is now being produced commercially and ten instruments will soon be available. It is not possible, at present, to evaluate this instrument nor to predict all the applications. The Color Translating Microscope cannot be synchronized with the micromanipulator in the ultraviolet range, although there is no reason why the results of micrurgy cannot be visualized immediately afterwards.

Blout (1951), Blout and Bird (1951), Blout *et al.* (1952) presented methods for infrared microspectroscopy using samples of organic solutes ranging from 1 to 10 μg. Cole and Jones (1952) described a reflecting microscope for infrared spectrometry. Adequate spectra for identification purposes can be obtained from 1–2 μg. samples. It was pointed out that "more convenient micromanipulator techniques" for sample transfer must be developed before further reduction in sample size is justified. Coates, Offner, and Siegler (1953) designed an infrared microscope attachment which requires as little as 0.1 μg. of sample. Some spectra were published of tissues obtained from sections, 25–50 μ thick, and at least 650 $\times$ 220 μ in area. Blout and Lenormant (1953) have been able to apply infrared spectroscopy to biological materials in aqueous media. A few spectra of proteins, nucleic acids, nucleoproteins, and bacteria were published. With the new advances in infrared spectrographic techniques, and the development of infrared microscopes as well as improvements in handling small samples, it is to be expected that new cytochemical data may be obtained (Blout, 1953b). Procedures are now available that permit analyses of a few cells, and possibly even single cells may be analyzed.

3. *Manometric Methods*

Zeuthen (1943) modified the Cartesian diver techniques so that he could use divers with a volume of 0.1 μl. These divers, with a stability of 0–20 $\mu\mu$l./hour and a sensitivity of about 20 $\mu\mu$l., were sufficiently sensitive to

measure the oxygen consumption of individual *Ophryotrocha* eggs and of individual *Difflugia*. The oxygen consumptions of individual, half, whole, and syncytial eggs of an ascidian (*Ciona*) were also measured (Holter and Zeuthen, 1944).

More recently, Scholander, Claff, and Sveinsson (1952a) described a technique which employs a reference diver permitting a sensitivity of about 0.2 $\mu\mu$l. and a stability of 0–10 $\mu\mu$l./hour. The method has now been employed for measuring oxygen consumption of protozoa (Scholander, Claff, and Sveinsson, 1952b), and of various invertebrate eggs (Claff, 1953).

This reference or micromicroliter diver promises to be an exceedingly valuable tool for measuring enzymic reactions in which oxygen is consumed. At the present time, it also represents one of the most sensitive, yet stable, manometric methods.

4. Microchromatographic Methods

An ingenious adaptation of chromatography, involving micrurgy, has been described by Edstrom (1953a, b) which permits determinations of the total quantity of ribonucleic acid from single nerve cells. Even more exciting is the report (Edstrom, 1954) that nucleotide analyses can be made of the ribonucleic acids obtained from individual cells. A sample of hydrolyzed ribonucleic acid (100–1000 $\mu\mu$g.) is placed on an alkali-treated, copper silk fiber with the aid of a micropipette. Platinum microelectrodes are led to the fiber and a direct current voltage (12V/mm.) is applied. After about 2 hours, the fiber is mounted in paraffin oil between two quartz slides and photographed with monochromatic light at wavelengths of 257 and 275 mμ. Four nucleotide fractions may be seen in the photograph of the fiber.

Edstrom stated that the method not only is a million times more sensitive than conventional methods, but it is sufficiently sensitive to give the nucleotide analysis of ribonucleic acid fractions obtained from single nerve cells which contain from 200 to 1,000 $\mu\mu$g. of RNA. This is a beautiful and potentially valuable technique that promises to give a great deal of information on the nucleotide content of cells and parts thereof.

5. Microdilatometric Methods

The estimation of enzymes in small cytoplasmic samples requires methods sensitive enough to measure activities of the order of $\mu\mu$l./hour. Probably the most sensitive method available is the dilatometric method set forth by Linderstrøm-Lang and Lanz (1938) and Linderstrøm-Lang (1952). Certain enzyme-substrate reactions produce volume changes if the substrate on hydrolysis yields ions or dipoles. The resulting electro-

striction effect reduces the volume of the reaction mixture and, if the mass remains constant, there will be a corresponding increase in density.

The dilatometric method, based on the fall of a reaction drop (increase in density resulting from hydrolysis of a dipeptide) in a density gradient column, was sufficiently sensitive to measure the hydrolysis of 30 $\mu\mu$M. of alanylglycine (Linderstrøm-Lang, 1939). This amount of substrate could be hydrolyzed by one sea urchin egg within 20 seconds, indicating that the sensitivity of the method is sufficient to measure the peptidase activity of 1/50th of a sea urchin egg.

Kopac (1953) described procedures, based on Linderstrøm-Lang's dilatometer, for measuring the hydrolytic activities of small samples of cytoplasm. By reducing the reaction volume from 0.1 μl. to 0.01 μl., the sensitivity could be increased by another order of magnitude. For example, the alanylglycine peptidase activity of pelomyxan cytoplasm is about 4 $\mu\mu$M./hour/$\mu\mu$l. The time required for 1 $\mu\mu$l. of cytoplasm acting on 0.1M L-alanylglycine to increase the density by 0.000001 (a quantity that can be measured in the present microdilatometer) in a reaction volume of 0.1 μl. is 159 minutes, whereas in a reaction volume of 0.01 μl. the required time would be 15.9 minutes.

In addition to certain improvements in measuring the positions of the reaction drops in the gradient columns, and hence the changes in density, there are also refinements in the technique of preparing the enzyme-substrate reaction mixtures. Part of the microdilatometer assembly with the gradient tubes and the horizontal measuring microscope is shown in Fig. 6.

As pointed out by Kopac (1953), the new procedure permits one to add measured amounts of enzyme (as removed from a cell with the volumetric submicromanipulator) to substrate volumes as small as 0.0005 μl., although one rarely uses reaction volumes smaller than 0.01 μl. The reaction mixtures are prepared by introducing a measured volume of substrate mixture into a drop of gradient fluid previously placed on a cover glass. It should be emphasized that all reactions are run in duplicate. These volumes are prepared with the drop-retraction unit (Fig. 5) and the diameters of the reaction drops are measured with filar micrometer ocular.

The cover glass with two substrate drops is transferred to the moist chamber (Fig. 4) mounted on the stage of the volumetric submicromanipulator. The cells are placed on another cover glass along side the substrate-containing cover glass. Here a measured volume of cytoplasmic substance (0.3 to 10 $\mu\mu$l.) is transferred from the cells to each of the two substrate drops. Then the cover glass with the two enzyme-substrate reaction mixtures is transferred to the loading micromanipulator (Fig. 6)

on the microdilatometer. On lowering the cover glass, the reaction volumes are conveniently placed into the gradient column.

If activators or inhibitors are required, they may be incorporated with the substrate, or else small amounts may be added to the enzyme-substrate mixture with the larger volumetric micropipette. Enzymes may also be added with the larger micropipette which delivers volumes ranging from 3 to 100 $\mu\mu$l.

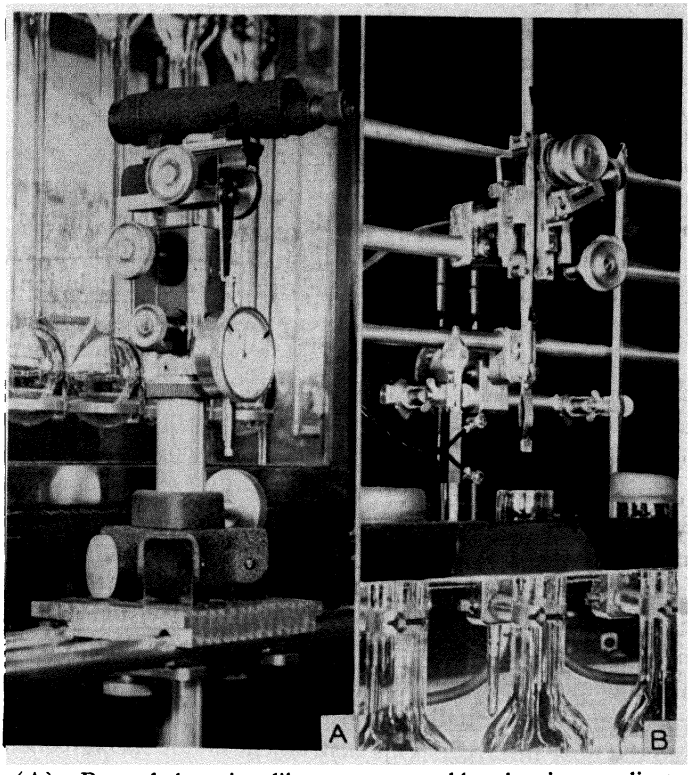


FIG. 6. (A) Part of the microdilatometer assembly, showing gradient vessels in water bath and the special horizontal measuring microscope (Kopac, 1953). The vertical movement of microscope (35 × with a working distance of 75 mm.) is synchronized with a dial micrometer. On the dial micrometer, 1 scale division (0.01 mm.) corresponds to a density difference of 0.000001 in a properly equilibrated and calibrated gradient column (density gradient = 0.001/cm.). Thus, a reaction drop with a volume of 0.01 μl., containing 0.1 *M* L-alanylglycine and 1 $\mu\mu$l. of pelomyxan cytoplasm will change its position by 1 scale division approximately every fifteen minutes (activity = 4 $\mu\mu$M./hour/$\mu\mu$l.). Finer changes in position of the reaction drop are read on the scale ocular in the microscope. (B) Upper part of microdilatometer assembly showing the loading micromanipulator for introducing a cover glass preparation with a pair of reaction drops into the gradient column.

Enzymatic activities that may be measured by microdilatometry include all proteinases, peptidases, phosphatases, pyrophosphatases, and nucleic acid depolymerases. The only requirement is that neither the substrate nor its products are soluble in the organic mixtures which make up the gradient column. Similarly, measured volumes of cytoplasm may serve as substrates for ATPase, with or without myokinase, nucleic acid depolymerases, with or without proteinases, and for other enzymes. Synthetic reactions may also be measured, including synthesis of peptide bonds, nucleotide polymerization, phosphorylation, etc. In these reactions, there would be an increase in volume and, accordingly, a decrease in density.

6. *Ultraviolet Micrurgy*

For many years, the feasibility of performing micrurgical operations in ultraviolet light has been considered. Up to recent times, there were no practicable ways of employing ultraviolet light for simultaneous viewing. Schemes for visualizing ultraviolet images by exciting fluorescent screens have been tested. This method is inadvisable since resolution on such screens is generally poor and rather intense ultraviolet light is required to produce images bright enough to see. Moreover, prolonged exposure to high intensity ultraviolet light is frequently injurious to living cells.

The solution of the problem became possible when the Television Microscope (Flory, 1951) was produced in the RCA laboratories. A new Vidicon tube, especially sensitive to ultraviolet light, has been developed in the David Sarnoff Research Center at Princeton, New Jersey (Zworykin, Flory, and Shrader, 1952). By picking up the image from the microscope, as with a camera for instance, the ultraviolet image is instantly translated into an excellent visible image on the screen of a television monitor. Magnifications with good resolution and contrast are possible up to 4000 ×.

A total instrument has been designed by Kopac (1954) which will permit micrurgy under essentially monochromatic light ranging from the ultraviolet to the near infrared. This instrument is now being constructed.

The following problems can be profitably investigated with ultraviolet micrurgy. The list is only a partial one, since nearly all micrurgical work done hitherto on living cells should be repeated with either ultraviolet or essentially monochromatic visible light.

A number of interesting problems involving nucleic acids can be investigated. For example, the action of RNase or DNase, following microinjection, on nuclear, nucleolar, and cytoplasmic structures may be effectively studied under ultraviolet light. Localized extractions of nucleic acids from cytoplasmic inclusions may also be demonstrated by injecting 0.3 *M* trichloracetic acid or 1 *M* NaCl.

These studies not only can be combined with microdilatometry for measuring RNase and DNase activities of cytoplasmic and nuclear structures, but they may also help validate various microchemical staining methods combined with nucleases.

The micrurgical determinations of intracellular pH levels will be facilitated with the aid of monochromatic light. One of the better methods for estimating protoplasmic pH is by the injection of sulfonphthalein indicators. Specifically, phenol red on injection into cytoplasm becomes yellow, while bromocresol purple becomes bluish red. The acid color of phenol red is yellow, indicating a pH less than 6.8 and the alkaline color of bromocresol purple is bluish, indicating a pH greater than 6.6. From these two colors and the pK values of the indicators, the pH of cytoplasm is therefore bracketed between 6.6 and 6.8.

A more objective scheme for estimating color changes is to illuminate the cell with a light complementary (determined by spectrophotometric analyses of the indicator solutions at several pH levels) to the expected color change of the injected dye. Thus, a dark zone would appear on the television screen on injection of an indicator which becomes yellow in the cytoplasm, if the cell is illuminated with blue light of the proper wavelength. Even more important, it is possible to avoid the interfering action of pigmented inclusions which are frequently present in cells.

At 530 mμ, one can estimate the reduction of cytochrome c introduced by microinjection into a cyanide poisoned cell. Since cytoplasmic inclusions can be stratified by centrifugation, it should be possible by combining monochromatic illumination and microinjection of reduced cytochrome c to determine unequivocally whether cytochrome oxidase is associated with mitochondria or with the matrix. Reduced cytochrome c, for example, would be oxidized, if microinjected into a region of a cell containing active cytochrome oxidase and oxygen.

The application of rapid image translation by the television principle to enzymatic and other cytochemical problems should yield valuable data. Coupled with this new tool is the volumetric submicromanipulator which permits the injection of known volumes of material into cells. Enzymes of known activity may be added to the cytoplasmic samples which serve as substrates. Especially effective will be the application of the ultraviolet micromanipulator to the study of cytoplasmic structures rich in nucleic acids.

V. Application of Cytochemical Micrurgy to the Study of Isolated Subcellular Particulates

1. *Localization of Enzymes in the Cell*

The following account illustrates the basic principles involved in establishing the distribution of enzymes in the cell. The procedures require the centrifuge, volumetric submicromanipulator, and the microdilatometer (Kopac, 1953). In Fig. 1 are diagrams of optical sections of isolated liver cells, before and after centrifugation.

A measured volume of cytoplasm can be removed with the volumetric submicromanipulator from the uncentrifuged cell and transferred to a substrate mixture, the substrate depending on the enzyme being investigated. This sample, A, contains various particulates (with exception of the nucleus) in accordance with their average distribution in the cell. From the centrifuged cell, exactly the same volume of matrix, B, may be similarly removed and transferred to the same substrate mixture as used with A. The activities of the enzyme from these sources are then measured in the microdilatometer.

If both samples produced the same activity, with the same substrate concentrations and conditions, then one would expect that both samples must have essentially the same amount of enzyme. Suppose, however, that the activity of fraction B were higher than that shown by A. This would indicate that the enzyme is probably not associated with the larger subcellular particulates since such particulates are not present in the matrix fraction. On the other hand, if the activity of A were higher than B, there might be a real reason to consider that the enzyme under investigation is primarily associated with the larger particulates. The matrix fraction, as previously discussed, is a sample of cytoplasm entirely free of those subcellular particulates visible under the microscope. In fact, it is the only fraction that may be obtained uncontaminated by other cell components.

2. *Estimation of Enzymatic Activities of the Nucleus and Subcellular Particulates*

The following procedures may be used to estimate the enzymatic activities of the nuclei and other subcellular particulates (Kopac, 1952a, b). Let p equal the activity, in $\mu\mu$M./hour, of V_1 $\mu\mu$l. of cytoplasmic matrix. Since the matrix volume, V_m, equals V_A, then the specific activity of the matrix, A_m, equals p/V_A. If another sample (total volume, V_B $\mu\mu$l.) consisting of matrix plus nucleus is used as a source of enzyme, the activity, q, equals $(V_B - V_n)\, A_m + V_n A_n$. From this, it follows that the specific activ-

ity of the nucleus, A_n, equals $[q - (V_B - V_n)\, A_m]/V_n$. The volumes, V_A and V_B in $\mu\mu$l. are measured with the volumetric submicromanipulator; V_n, is calculated from the diameter of the spherical nucleus; activities p and q are calculated from microdilatometric data. A_m is determined from measurements of the matrix fraction alone.

The nucleus, accompanied by measured amounts of matrix of known activity, is maintained in its normal cytoplasmic environment until transferred to the substrate mixtures. It is, therefore, not subject to irreversible, post-mortem changes such as may occur during the centrifugal fractionation of homogenates.

Since the matrix fraction may be obtained uncontaminated by subcellular particulates, whereas all other particulates will be contaminated to some extent by the matrix, it is clear that the activity of the matrix becomes a key to the estimation of the activities of all other subcellular particulates. For example, let r equal the activity of V_C $\mu\mu$l. of a sample rich in mitochondria. Here, r equals $(V_C - V_P)\, A_m + V_P A_P$, where $(V_C - V_P)$ equals the matrix volume of the sample; V_P equals the volume occupied by the mitochondria in the sample; and A_m and A_P represent the specific activities of the matrix and mitochondria, respectively.

The activity, r, is also determined from microdilatometric measurements while the volume V_C is measured with the volumetric submicromanipulator. If V_C equals V_1 and if p is greater than r, then the mitochondria must have a lower specific activity than the matrix.

The volume, V_P, must be evaluated indirectly by using an enzyme system in which A_P equals zero. By removing identical volumes from the cell with essentially the same particulate content, then $(V_C - V_P)$ will be constant. Accordingly, r equals $(V_C - V_P)\, A_m$ from which V_P equals $(V_C A_m - r)/A_m$ (Kopac, 1952b).

These procedures for establishing the distribution of a given enzyme in either the matrix or subcellular particulates are valid only if the complete enzyme system (for example, enzyme + coenzyme) is exclusively carried by one fraction or another. Situations may arise where one component of an enzyme system may be carried by the matrix and another integral component by one of the subcellular particulate fractions. In this instance, the specific activity of the matrix would be essentially zero (even though it carries the enzyme) while the specific activity of the particulate fraction should be proportional to the amount of that fraction present in the sample. The combination of matrix + particulates contains the complete enzyme system.

If the enzyme is carried by subcellular particulates and is not dependent on some component in the matrix fraction, then A_P equals $[r - (V_C -$

$V_P)A_m]/V_P$. And if, for this particular enzyme, A_m equals zero then A_P equals r/V_P.

3. *Comparison of Microsomal Activity with the Matrix*

Homogenates, and fractions therefrom, now represent a major source of material for biochemical and enzymological research (Schneider and Hogeboom, 1951). The localization of enzymes in the various fractions has been severely and justifiably criticized (Bradfield, 1950; Glick, 1953; Holter and Linderstrøm-Lang, 1951; Holter, 1952; Kopac, 1950c; and others). The question is still to be answered: are the properties of the various isolated particulates identical with the properties of the same particulates as present in living cells? In other words, are the isolated mitochondria, for example, identical with the mitochondria in the intact cell? Unless these queries can be answered in the affirmative, then any conclusions based on the properties of isolated mitochondria or other particulate fractions must be held in doubt.

Some of the methods discussed in this review may shed light on the entire problem of isolated particulate fractions. At present, one of these fractions, the microsomes, is being investigated. The reasons for studying the microsomes, as a first step, are clear.

In the living cell, on centrifugation, there is formed the matrix layer, free of all microscopically visible subcellular particulates which include, *inter alia*, mitochondria, secretory granules, plastids, lipid bodies, nuclei, etc. These particulates make up, presumably, the nuclear fraction and the large granule fractions which can be isolated by the centrifugal fractionation of homogenates. It would seem that the microsomal fraction would have to come from the matrix since both are free of the larger particulates.

Accordingly, the enzymatic activities of the matrix and microsomal fractions should be compared. A thorough investigation of the phosphatase activity of microsomes from frog liver cells has been undertaken (Sonnenschein and Kopac, 1953a, b; Sonnenschein, 1953). This investigation has already shown that optimal pH levels for a given substrate may depend on the concentration of the substrate. Data are now available for five physiologically significant substrates with each substrate showing an optimal pH at various concentrations. These data give a fairly complete characterization of phosphatase activity in the form of a spectrum consisting of substrates, substrate concentrations, and optimal pH.

If it should turn out that the matrix fraction, micrurgically removed from living frog liver cells, gives precisely the same spectrum of phosphatase activities as the microsomes, then it is reasonably safe to conclude that the microsomes have been derived from the matrix and not as disintegration

products of the larger particulates. On the other hand, if striking discrepancies become evident, then the origin and identification of microsomes with any native cell fraction would be open to justifiable doubt. The comparisons of microsomes with matrix fractions in reference to phosphatase activities are now in progress.

The study of cytolytic phenomena in a variety of cells provides many reasons why one might question the identity of isolated particulates with particulates present in living cells. In the first place, the homogenizing media now used certainly do not in any way simulate the environment these particulates enjoy in the living cell. The testing of homogenizing media by microinjection (Kassel and Kopac, 1950, 1953) leaves no doubt about their unphysiological nature. Furthermore, observations on cytolysis as induced in starfish oöcytes, for example, makes one wonder how any of the particulates isolated from homogenates could retain even a trace, let alone all, of their native properties. A case in point is the demonstration of the Devaux effect on cytolysis (Kopac, 1950a).

VI. General Application of Cytochemical Micrurgy

It is evident that many of the procedures described in this review will not find wide application due to the expensive instrumentation and considerable skill that is required to handle such instruments properly. It would be highly unlikely that these methods could ever be designated as routine procedures.

On the other hand, these methods even though complicated and arduous by virtue of their precision may evaluate controversial methods that can or are being practiced routinely. For example, the controversy now going on in reference to phosphatase activity in nuclei may be settled. The reliability of the nuclease-staining techniques for localizing nucleic acids may also be established. In addition, conclusions based on isolated subcellular particulates may be unequivocally validated. Better fractionation media may be one of the products of these investigations.

All of the above problems, now actively investigated, may be placed on a firm foundation insofar as the more commonly employed procedures are concerned. On that basis, critical, even though difficult procedures, may in the long run be the most practical.

VII. Summary

Improvements in procedure and instrumentation based on microdilatometry together with the development of the volumetric submicromanipulator now provide a methodology whereby the activities of certain enzymes may be measured not only in single cells but also in parts of cells. The centrifu-

gation of living cells from which certain fractions may be removed and tested for activity makes possible the localization of a variety of enzymes in the cell. These methods are, therefore, specialized and critical techniques designed to confirm the validity of simpler methods which have a much greater application.

Acknowledgments

The reviewer is deeply indebted to his associate, Mr. Jack Harris, whose skills as a designer and instrument maker have brought to realization such instruments as the volumetric submicromanipulator and a horizontal measuring microscope for the microdilatometer. An ultraviolet micromanipulator and a new electrically controlled micromanipulator are now being constructed. The investigations in the reviewer's laboratory were supported in part by Grants C-843 and C-2018 from the National Cancer Institute, National Institute of Health, Public Health Service, and Grants DRG-193 and DRG-271 from the Damon Runyon Memorial Fund for Cancer Research, Inc

VIII. References

Alexander, J. T., and Nastuk, W L. (1953) *Rev. Sci. Instr.* **24**, 528.
Anderson, N. G. (1953) *Science* **117**, 627.
Barer, R. (1952) *Nature* **169**, 366.
Barer, R., and Saunders-Singer, A. E. (1948) *Quart. J. Microscop Sci.* **89**, 439.
Benedetti-Pichler, A. A., and Rachele, J. R. (1940) *Ind Eng. Chem. Anal. Ed.* **12**, 233.
Blout, E. R. (1951) *Instrument News* **2**(3), 1.
Blout, E. R. (1953a) *Advances in Biol. and Med. Phys.* **3**, 285.
Blout, E. R. (1953b) *Trans N.Y. Acad. Sci.* [2] **15**, 280.
Blout, E. R., and Bird, G. R. (1951) *J. Opt. Soc. Amer.* **41**, 547.
Blout, E. R., and Lenormant, H. (1953) *J. Opt. Soc. Amer* **43**, 1093.
Blout, E. R., Parrish, M., Jr., Bird, G. R., and Abbati, M. J. (1952) *J. Opt. Soc. Amer.* **42**, 966.
Bradfield, J. R. G. (1950) *Biol. Revs.* **25**, 113.
Bush, V., Duryee, W. R., and Hastings, J. A. (1953) *Rev. Sci. Instr.* **24**, 487.
Cailloux, M. (1943) *Rev. can. biol.* **2**, 528.
Cameron, Gladys (1950) "Tissue Culture Technique." Academic Press, New York.
Chalkley, H. W. (1929) *Physiol Zool.* **2**, 535.
Chalkley, H. W. (1943-4) *J. Natl. Cancer Inst.* **4**, 47.
Chambers, R. (1922) *Anat. Record* **24**, 1
Chambers, R., and Kopac, M. J. (1950) "McClung's Handbook of Microscopical Technique," 3rd ed., pp. 492-543. Hoeber, New York
Claff, C. L. (1953) Trans. *N.Y. Acad. Sci.* [2] **15**, 281
Coates, V. J., Offner, A., and Siegler, E. H., Jr. (1953) *J. Opt. Soc. Amer.* **43**, 984.
Cohen, B., Chambers, R., and Reznikoff, P. (1928) *J. Gen. Physiol.* **11**, 585.
Cole, A. R. H., and Jones, R. N. (1952) *J. Opt. Soc. Amer.* **42**, 348.
Costello, D. P. (1939) *Physiol. Zool.* **12**, 13.
Danielli, J. F. (1953) "Cytochemistry: A Critical Approach." Wiley, New York.
Davies, H. G., and Wilkins, M. H. F. (1952) *Nature* **169**, 541.
Diacumakos, E. (1954) Personal communication.

Dulbecco, R. (1952) *Proc. Natl Acad. Sci.* **38**, 747.
Dulbecco, R., and Vogt, M. (1954) *J. Exptl. Med.* **99**, 167.
Edstrom, J. E. (1953a) *Nature* **172**, 809.
Edstrom, J. E. (1953b) *Biochim. et Biophys. Acta* **11**, 300.
Edstrom, J. E. (1954) *Biochim. et Biophys. Acta* **12**, 361.
Emerson, J. H. (1931) U.S. Patent 1,828,460.
Engström, A. (1946) *Acta Radiologica* Suppl. 63.
Engström, A. (1953) *Physiol. Revs.* **33**, 190.
Engström, A., and Lindstrom, B. (1949) *Nature* **163**, 563.
Engstrom, A., and Lindstrom, B. (1950) *Biochim. et Biophys. Acta* **4**, 351.
Evans, V. J. (1951) *in* "Methods in Medical Research" (Visscher, ed.), Vol. 4, p. 221. Yearbook Publishers, Chicago.
Evans, V. J., and Earle, W. R. (1947) *J. Natl. Cancer Inst.* **8**, 103
Fischer, A. (1949) *Science* **109**, 611.
Fitzgerald, P. J., and Engstrom, A (1952) *Cancer* **5**, 643.
Fitzgerald, P. J., Simmel, E., Weinstein, J., and Martin, C. (1953) *Lab. Invest.* **2**, 181.
Flory, L. E. (1951) *Cold Spring Harbor Symposia Quant. Biol.* **16**, 505.
Fonbrune, P. de (1932) *Compt rend* **195**, 603.
Fonbrune, P. de (1949) "Technique de Micromanipulation." Masson et Cie, Paris.
Frisch, A. W., and Jentoft, V. (1953) *Proc Soc. Exptl. Biol. Med.* **82**, 323
Gilbert, P. T., Jr. (1951) *Science* **114**, 637.
Glick, D. (1949) "Techniques of Histo- and Cyto-chemistry." Interscience, New York.
Glick, D. (1953) *Intern. Rev. Cytol.* **2**, 447
Goldie, H., and Felix, M. (1951) *Cancer Research* **11**, 73.
Gomori, G. (1952) "Microscopic Histochemistry. Principles and Practice." Univ. of Chicago Press, Chicago
Harrison, M. F. (1953) *Nature* **171**, 611
Harvey, E. B. (1941) *Biol. Bull.* **81**, 114.
Harvey, E. B. (1946) *J. Exptl Zool* **102**, 253.
Harvey, E. B. (1951) *Ann. N.Y. Acad Sci.* **51**, 1336
Hauschka, T. S. (1953a) *J Natl Cancer Inst.* **14**, 723
Hauschka, T. S. (1953b) *Trans. N.Y. Acad. Sci.* [2] **16**, 64.
Hauschka, T. S., and Levan, A. (1953) *Exptl Cell Research* **4**, 457.
Hilson, G. R. F. (1952) *J. Gen Microbiol.* **7**, 175
Holter, H. (1945) *Compt. rend. trav lab Carlsberg Ser chim.* **25**, 156
Holter, H. (1952) *Advances in Enzymol.* **13**, 1
Holter, H., and Linderstrom-Lang, K (1951) *Physiol Revs.* **31**, 432
Holter, H., and Zeuthen, E. (1944) *Compt rend. trav. lab. Carlsberg Ser. chim.* **25**, 33
Holter, H., and Zeuthen, E. (1948) *Compt. rend. trav. lab. Carlsberg Ser. chim.* **26**, 277.
Just, E. E. (1939) "Basic Methods for Experiments on Eggs of Marine Animals." Blakiston, Philadelphia.
Kassel, R., and Kopac, M. J. (1950) *Anat Record* **108**, 85 (abstr.)
Kassel, R., and Kopac, M. J (1953) *J. Exptl. Zool.* **124**, 279.
Kassel, R., and Melnitsky, I. (1951) *Stain Technol.* **26**, 167.
Kirk, P. L. (1950) "Quantitative Ultramicroanalysis." Wiley, New York.

Klein, G. (1951) *Exptl. Cell Research* **2**, 291.
Kopac, M. J. (1940) *Cold Spring Harbor Symposia Quant. Biol.* **8**, 154.
Kopac, M. J. (1943) "Micrurgical and Germ-Free Methods" (J. A. Reyniers, ed.), pp. 26-71. C. C Thomas, Springfield, Ill.
Kopac, M. J. (1950a) *Ann. N.Y. Acad. Sci.* **50**, 870.
Kopac, M. J. (1950b) *Sci. Amer.* **183**, 48.
Kopac, M. J. (1950c) *Ann. Revs. Physiol.* **12**, 7.
Kopac, M. J. (1951) *Anat. Record* **111**, 117 (abstr.)
Kopac, M. J. (1952a) *Anat. Record* **113**, 584 (abstr.)
Kopac, M. J. (1952b) *Anat. Record* **113**, 585 (abstr.)
Kopac, M. J. (1953) *Trans. N.Y. Acad. Sci.* [2] **15**, 290.
Kopac, M. J. (1954) Ultraviolet Micrurgy Project. DRG-271. Damon Runyon Memorial Fund for Cancer Research, Inc.
Kopac, M. J., and Harris, J. (1951) *Anat. Record* **111**, 116 (abstr.)
Kopac, M. J., and Harris, J. (1954) Unpublished.
Land, E. H., Blout, E. R., Grey, D. S., Flower, M. S., Husek, H., Jones, R. C., Matz, C. H., and Merrill, D. P. (1949) *Science* **109**, 371.
Likely, G. D., Sanford, K. K., and Earle, W. R. (1952) *J. Natl. Cancer Inst.* **13**, 177.
Linderstrøm-Lang, K. (1939) *Harvey Lectures Ser.* **34**, 214.
Linderstrøm-Lang, K. (1952) "Proteins and Enzymes," pp. 9-13. Stanford Univ. Press, Stanford.
Linderstrøm-Lang, K., and Lanz, H., Jr. (1938) *Compt. rend. trav. lab. Carlsberg Ser. chim.* **21**, 315.
Lorch, I. J., and Danielli, J. F (1953a) *Quart. J. Microscop. Sci.* **94**, 445.
Lorch, I. J., and Danielli, J. F. (1953b) *Quart. J. Microscop. Sci.* **94**, 461.
Lorch, I. J., Danielli, J. F., and Horstadius, S. (1953) *Exptl Cell Research* **4**, 253.
Lowry, O. S. (1953) *J. Histochem. Cytochem.* **1**, 420.
Lumsden, C. E., and Robinson, C. V. (1953) *Compt. rend. trav. lab. Carlsberg Ser chim.* **28**, 358.
Mast, S. O., and Fowler, C. (1935) *J. Cellular Comp. Physiol.* **6**, 151.
Mateyko, G. M. (1954) Personal communication
Mateyko, G. M., and Kopac, M. J. (1954) *Proc. Am. Assoc Cancer Research* **1**(2), 31 (abstr.)
May, K. R. (1953) *J. Roy. Microscop. Soc.* **73**, 140.
Mellors, R. C. (1953a) *Cancer Research* **13**, 101.
Mellors, R. C. (1953b) *J. Histochem. Cytochem.* **1**, 396.
Mellors, R. C. (1953c) *J. Opt. Soc. Amer* **43**, 326.
Mellors, R. C., Kupfer, A., and Hollender, A. (1953) *Cancer* **6**, 372.
Mickelwright, H. L., Kurnick, N. B., and Hodes, R. (1953) *Exptl. Cell Research* **4**, 151.
Mitchison, J. M. (1953) *J. Exptl. Biol.* **30**, 515.
Mookerjee, S. (1953) *Nature* **171**, 796.
Morgan, J. F., and Parker, R. C. (1949) *Proc. Soc. Exptl. Biol. Med.* **71**, 665.
Moscona, A. (1952) *Exptl. Cell Research* **3**, 535.
Murray, M. R., and Kopech, G. (1953) "A Bibliography of the Research in Tissue Culture." Vols. 1 and 2. Academic Press, New York.
Northcroft, R. D. (1951) *Science* **113**, 407.
Northrop, J. H. (1947) *J. Gen. Physiol.* **30**, 375.
Parker, R. C. (1950) "Methods of Tissue Culture," 2nd ed. Hoeber, New York.

Pearse, A. G. E. (1953) "Histochemistry: Theoretical and Applied." Little, Brown, Boston.
Peterfi, T. (1923) *Naturwiss. Jahrg.* **6**, 81.
Powell, E. O. (1952) *J. Roy. Microscop. Soc.* **72**, 214.
Reinert, G. (1938) *Arch. f. exptl. Zellforsch. Gewebzücht.* **22**, 681.
St. Amand, G. S., and Tipton, S. R. (1954) *Science* **119**, 93.
St. Aubin, P. M. G., and Bucher, N. L. R. (1952) *Anat. Record* **112**, 797.
Sanford, K. K., Earle, W. R., and Likely, G. D. (1948-9) *J. Natl. Cancer Inst.* **9**, 229.
Schmidt, H. D. (1859) *Am. J. Med. Sci.* **73**, 13.
Schneider, W. C., and Hogeboom, G. H. (1951) *Cancer Research* **11**, 1.
Schneider, W. C., and Potter, V. R (1943) *J. Biol. Chem.* **149**, 217.
Scholander, P. F. (1942) *Rev. Sci. Instr* **13**, 32.
Scholander, P. F., Claff, C L., and Sveinsson, S. L. (1952a) *Biol. Bull.* **102**, 157.
Scholander, P. F., Claff, C. L., and Sveinsson, S. L. (1952b) *Biol. Bull.* **102**, 178.
Scott, G. H., and Packer, D. M. (1939a) *Anat. Record* **74**, 17.
Scott, G. H., and Packer, D. M. (1939b) *Anat. Record* **74**, 31.
Sonnenschein, N. (1953) Doctoral Thesis. New York University.
Sonnenschein, N., and Kopac, M. J. (1953a) *Anat. Record* **117**, 611 (abstr.)
Sonnenschein, N., and Kopac, M J. (1953b) *Anat. Record* **117**, 612 (abstr.)
Stewart, D. C., and Kirk, P. L. (1954) *Biol. Revs.* **29**, 119.
Taylor, C. V. (1925) *Univ Calif Publ. zool.* **26**, 443.
Yoshida, T. (1952) *J. Natl. Cancer Inst.* **12**, 947.
Youngner, J. S. (1954) *Proc. Soc Exptl. Biol. Med.* **85**, 203.
Zeuthen, E. (1943) *Compt rend trav lab. Carlsberg Ser. chim.* **24**, 479.
Zeuthen, E. (1948) *Compt rend. trav lab. Carlsberg Ser. chim.* **26**, 243.
Zworykin, V. K., Flory, L. E., and Shrader, R. E. (1952) *Electronics* **25**, 150.

Amoebocytes

L. E. WAGGE

Zoology Department, Imperial College of Science and Technology, London, England

Page

I. Introduction 31
II. The Cytology of Amoebocytes 33
1. The Cytology of Amoebocytes in *Helix aspersa* 33
2. The Cytology of Amoebocytes in Other Molluscs 39
III. Amoebocytic Activity during Shell Repair 43
1. The Origin of Amoebocytes, and Importance of the Mantle 43
2. Calcium Reserves in the Mantle 48
3. Amoebocytic Activity in Relation to Theories of Shell Formation 49
4. Phosphatases within Amoebocytes 50
5. Investigations using Radioactive Materials 51
IV. Amoebocytic Activity in Molluscan Digestion 53
V. The Excretory Function of Certain Amoebocytes 59
VI. Amoebocytes in Phyla Other than the Mollusca 61
VII. Discussion 68
VIII. Summary 74
Acknowledgments 75
IX. References 75

I. Introduction

Cells capable of independent movement play an important part in a variety of functions in all animals. Amoebocytic cells, described by various workers, even within one phylum, are not necessarily cells that a student of tissue culture would always regard as true amoebocytes. Indeed, since the origin of many of these cells was unknown to the worker, it was necessary when describing them to make use of a general term. The present article aims at studying the cytology and physiology of some of these freely moving cells. The phylum Mollusca has been chosen for a detailed review, and further interesting examples have been drawn from other parts of the animal kingdom. Willmer (1945) suggests there are three fundamental types of cells that he has described as epitheliocytes, mechanocytes and amoebocytes. The epitheliocytes form a skin, contain keratin, and readily "keratinize" if starved. They are not phagocytic, produce much mucin as a binder and tend to liquefy the substrate with a gel $\rightleftharpoons$ sol reaction. They may originate from the ectoderm (e.g. outer epithelium), from the mesoderm (e.g. kidney tissue), or from the endoderm (e.g. intestine). The mechanocytes or fibrocytes are supporting cells that may be contractile. They are capable of a gliding movement aided by pseudopodia, are nonphagocytic, and in tissue culture occupy a three

dimensional arrangement. They are a mixed collection of mesenchyme cells that may owe their morphological appearance to their surroundings. The amoebocytes or wandering cells do not adhere together in culture, being capable of independent movement by means of pseudopodia. They contain collagen, not keratin, and seldom produce mucus. They are markedly phagocytic and in consequence take up dyes. They are considered to be true blood cells, although many investigators believe that fibroblasts and wandering cells are interconvertible, both being regarded as a different functional state of the same cell. Willmer (1935) points out that in a tissue culture all the cells are tending to behave like a primitive organism, and one cannot necessarily consider that, under these conditions, the cells are carrying out their normal functions. For example, the shape of a blood cell in culture may be spherical, but the same cell, moving along a blood vessel wall may be very variable in shape. There is also the added difficulty that even within the same phylum, cells performing the same function may not be of similar type or origin. Fretter (1939) refers to the same difficulty with regard to organs, when she emphasizes the impossibility firstly of adopting vertebrate nomenclature for invertebrate organs, and secondly of even maintaining one name for homologous organs in the same phylum; the so called esophagus or stomach of *Tectibranchs,* for example, does not function as do these organs in the Vertebrate or even in the Gastropod.

Using Willmer's three fundamental cell types as a basis, the term amoebocyte in the Molluscs will be found to have been used for all three types of cells. Considering an amoebocyte as a cell capable of independent movement, irrespective of origin, this term embraces cells differently named by various authors. For instance, "leucocyte" implies generally that the cell is known to be a true blood cell, whilst "phagocyte" denotes the cell's ability to enclose within itself a foreign body, often of nutritive value. In general throughout this article the term that the original author applied will be used when reference is made to his work.

Further discussion of the classification of cell types, especially in the invertebrate groups, is of interest. Liebman (1946), regarding a leucocyte as a blood cell capable of active or passive migration throughout the body, considers there are two types of leucocytes in the invertebrates: *lymphoidocytes,* phagocytes dealing mainly with excretory material, and *trephocytes* dealing with the transport and release of nutritive materials. The latter move into other cells, and in animals where there is a low content of dissolved nutrients in the blood they appear to be the main, or sole carriers of trophic material. They are primarily anabolic and their function is most conspicuous in various manifestations of growth; for

example, wound healing and regeneration, moulting and metamorphosis, and the growth of ovarian eggs. In the Mollusca, Liebman (1947) regards the granular, spherular, and signet-ring leucocytes as trephocytes. They develop (Takatsuki, 1934) from cells Liebman classifies as lymphoidocytes, the transitional stage being amoeboid and phagocytic, characters not persisting in the mature cells. George (1941) divides into three main types the free cells of mesenchymatous origin that have been satisfactorily investigated. In some groups they are more or less confined to lymphatic vessels and in others they wander freely through the tissue spaces. The three types he recognizes are as follows:

Type 1. Relatively undifferentiated cells that have the capacity to differentiate to other types of blood cells and in some groups into any cell of the body.

Type 2. Phagocytic cells that serve the function of consuming foreign materials and dead or damaged tissue of the animal itself.

Type 3. A greater or smaller number of various types of more specialized granular and vacuolated cells that contain nutritive materials and give evidence of enzyme activity. In many low forms these constitute an important part of the food handling and digesting mechanisms. In other forms, including the vertebrates, their original function has been taken over largely by the digestive glands of the gut tract.

Dividing the leucocytes of the Lamellibranchs into these three types, George (1941) briefly describes their structure in the following manner: Type 1 are small cells, with large nuclei and little hyaline cytoplasm. Type 2 are phagocytic cells with more cytoplasm than type 1, and type 3 are cells with granules in their cytoplasm. This division, particularly regarding types 2 and 3 is far too generalized, although it is obviously dangerous to assume that the cells of different genera are necessarily identical, especially since no single author has made an exhaustive study of the subject. Table I gives some idea of the range of size and extent of movement of some of the Molluscan amoebocytes that have been described by various authors.

II. The Cytology of Amoebocytes

1. *The Cytology of Amoebocytes in* Helix aspersa

Type 1 cells are very numerous in the Gastropod *Helix aspersa* in a form similar to that described by George in the Lamellibranchs. In *Helix* they were examined in those places particularly concerned with the regeneration of the shell, notably the digestive gland, the kidneys, the intestine, the mantle, and the regenerating shell (Wagge, 1951). They were referred to as small amoebocytes, type *A*, and were 10–20μ in diameter,

according to the extension of the cytoplasm. The nucleus was around 10μ in diameter and the cytoplasm, which was hyaline, was often closely contracted around the nucleus. The nuclei of these cells absorbed much calcium, staining markedly with gallamine blue, and were found to be

TABLE I
MOLLUSCAN AMOEBOCYTES

Genera	Size of cell	Size and shape of nucleus[a]	Movement[b]	Reference
GASTROPODA				
Tectibranchia				
Philine	6–12μ	o	E	Fretter (1939)
Nudibranchia				
Various species	4–10μ	s, o, or c	I.2	Millott (1937a)
Jorunna and others	5–24μ	o	E	Millott (1937b)
Pulmonata				
Helix	40–50μ	o 15μ × 7μ	I.1	Crawford and Barer (1951)
	10–20μ	o 10μ	E	Wagge (1951)
LAMELLIBRANCHIA				
Ostrea	9–13μ	o 3μ × 4μ	E	Takatsuki (1934)
	5–15μ	o 5μ × 15μ	E	Takatsuki (1934)

[a]Shape of nucleus: s, spherical, o, ovoid; c, crescentic

[b]Movement: E, Extensive, throughout the body. I.1, Limited, but may be due to the author's specialized study I.2, True limitation of movement of the amoebocyte within the animal.

rich in alkaline phosphatases (Fig. 1). A feature of particular interest, recorded also by many other authors, was the very large number of these cells that might be operating in a particular area. Associated with the repair of the shell were larger amoebocytes, Type *B* (Wagge, 1951), with much more cytoplasm that could extend to 50μ in length and had nuclei of 10μ in diameter (Fig. 2). Whereas it appeared from sections, that these cells originated from the mantle epithelium (Wagge, 1951), further work with tissue cultures has revealed that two types of cells were grouped as Type *B*, the one coming from the mantle's epithelium and the other from its connective tissue (Crawford and Barer, 1951; MacGee-Russell, 1954). The cells from the mantle's connective tissue are among those that have consistently been referred to as "amoebocytes" (Figs. 3 & 4). Haughton (1934a) considered it likely that they come from the connective

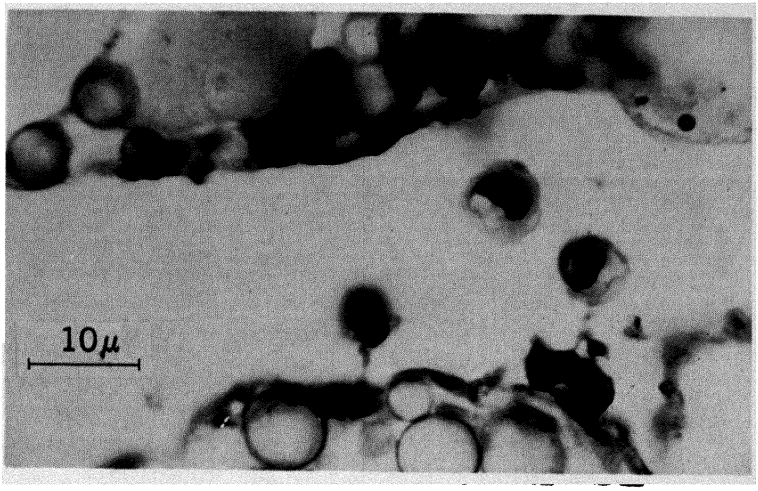


FIG. 1. Amoebocytes of *Helix aspersa* from a section of the digestive gland, stained to demonstrate the distribution of alkaline phosphatases. Some of the amoebocytes lie in a blood space and others within lime cells. The calcium spherules are almost circular and their remaining calcium is adsorbed on to the membrane. These amoebocytes are probably true blood cells.

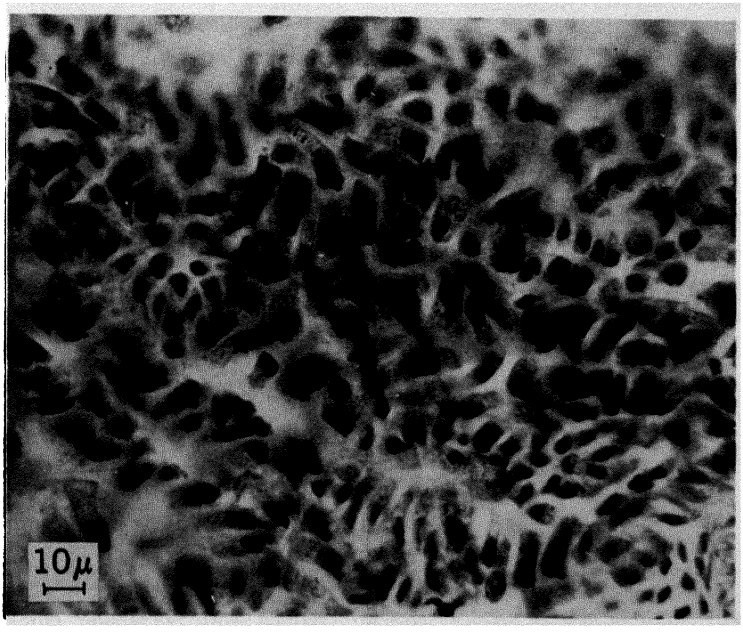


FIG. 2. Amoebocytes of *Helix aspersa* assisting shell repair. The dark areas are those containing calcium. Stain: gallamine blue (Stock, 1949).

tissue of many parts of the body and that such regions, as well as being their place of origin are, together with blood vessels, places where these cells remain dormant during periods of inactivity. When actively engaged in the movement of materials for shell repair, they moved along the blood

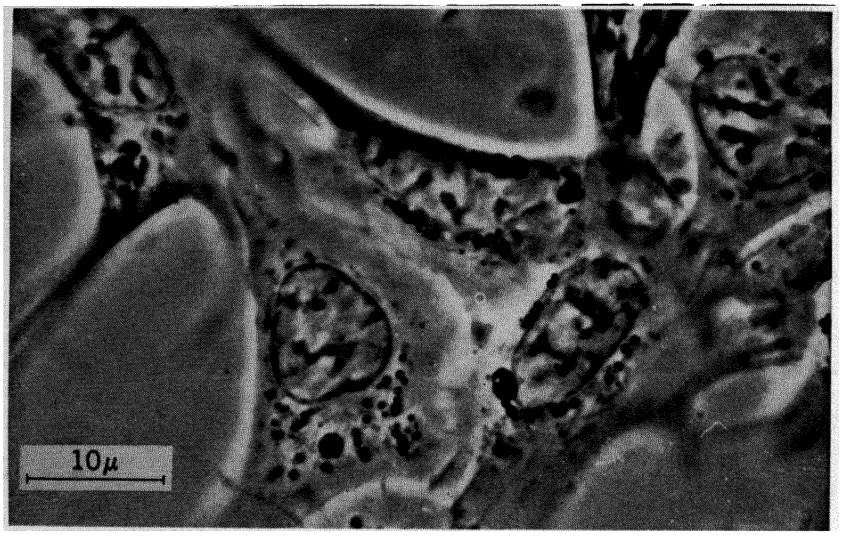


FIG. 3. Living amoebocytes from a culture of the mantle tissue of *Helix aspersa*. This phase contrast photograph was taken by Dr. G. N. C. Crawford during his research (Crawford and Barer, 1951).

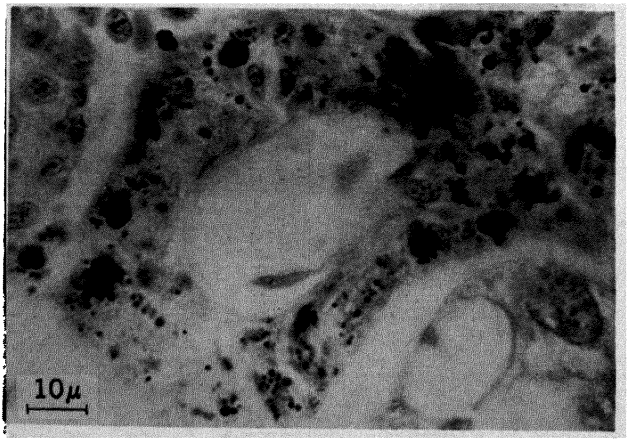


FIG. 4. Amoebocytes of *Helix aspersa* (stained with gallamine blue), in a blood space in the digestive gland. These amoebocytes appear to be similar to those in Fig. 3.

spaces of the digestive gland and mantle and passed through the epithelium to the damaged area (Wagge, 1951).

The most detailed cytological study of any Molluscan amoebocytes is being carried out at the moment by Crawford (1954). He is making a more exhaustive investigation than that already published in his account of the action of formaldehyde on living cells (Crawford and Barer, 1951). The amoebocytes of *Helix aspersa* that formed part of this work (Fig. 3) were considered by the authors to have originated from the connective tissue of the mantle. Working with living material, they drew attention (with accompanying photographs) to the extremely variable shapes and sizes that these cells could assume in life. Giving average measurements, the amoebocytes were usually 20μ in width, 40–50μ in length and had an oval nucleus, about 7μ wide and 15μ long. Within the nucleus, chromosomes were not, as such, visible and those opacities that did occur within the nucleoplasm could not be related to chromosomes with any certainty. They might represent the heterochromatic regions of chromosomes or be not at all related to them, particularly since the number, size, and shape of the opacities varied considerably. Yonge (1923) reported a similar difficulty in recognizing individual chromatin granules in the nuclei of the phagocytes of *Mya*. In the *Helix* amoebocytes both the nuclear boundary and the nucleoplasmic opacities tended to be more distinct in cells that had been in tissue culture for 48 hours or more. There were various inclusions within the cytoplasm; using phase contrast, numerous small dark granules of about 1μ in diameter were found, frequently arranged in rows. In the living cell some stained faintly with Janus green and, after fixation, could be stained with Altmann's acid fuchsin, suggesting they were mitochondrial in nature. Larger spherical bodies or vacuoles also occurred that stained with neutral red in the living material and, after fixation with formaldehyde, could be colored with Sudan black. They appeared, therefore, to be lipochondrial (Baker, 1950) and were frequently aggregated in mulberry-like structures in the cells in older tissue cultures. There was considerable variety with regard to the chemical structure of these vacuoles. Pigment granules were often found to be present, the large, compound, inclusion bodies being especially heavily pigmented. Apart from these various inclusions, the greater part of the cytoplasm appeared to be optically homogeneous, both with conventional illumination and with phase contrast. In the perinuclear region, especially, there might be more granular cytoplasm in the cells of old cultures.

The boundary of the cell, when alive, was very faint, even with phase contrast, probably due to the extreme thinness of the cell periphery. The thickness of the fixed and dried cells was investigated with the electron

microscope using "shadow methods" (Barer, 1951). The perinuclear cytoplasm was found to be approximately 0.13μ thick. The edge of the cell often had a raised rim (0.06μ thick). The delicate fibrils arising from the pseudopodia were only 0.015–0.025μ thick. These measurements were taken of dried cells in which shrinkage may have occurred. No measurements of living cells were taken with the electron microscope. Crawford's later work, when published, will give greater detail of the cytology of these cells. However, the description given above added much to the one given earlier by Gatenby, Hill, and MacDougald (1934). Both accounts described inclusions, and the "Golgi apparatus" (lipochondria) fragmenting into the cytoplasm as isolated granules. Bourne (1950) considers that as far as the present state of knowledge allows, the Golgi complex is probably to be regarded as the protective and storage region of the cell. The segregation of lipoprotein spheres, of which it consists, is associated with phospholipoid granules that may themselves form additional spheres. These spheres and granules are arranged in the cell according to diffusion currents and this allows the segregation of substances coming into the cell. An epithelial cell tends to have its Golgi apparatus compactly arranged to receive substances coming through the distal edge of the cell, whereas, in other cells where substances may enter in any direction, this apparatus is more generally distributed. The mitochondria are regarded as the respiratory and possibly synthetic centers of the cell. Many of their enzymes are constituents of the Krebs tricarboxylic acid cycle, which has been described as the meeting point of protein, fat, and carbohydrate metabolism. The mitochondria may, therefore, be concerned with the synthesis and breakdown of these three substances.

Amoebocytes from the connective tissue of the mantle of *Helix* (Fig. 3) appeared to be identical with those found in other areas associated with shell repair, for example the digestive gland (Fig. 4). Many of the granules and vacuoles within the amoebocytes which were actively engaged in shell repair contained calcium products, whereas others were brownish yellow proteinaceous spherules. These inclusions within cells that would absorb materials from all directions were more diffusely scattered than those in the cells arising probably from the mantle epithelium (Fig. 5), a feature already discussed concerning the distribution of the Golgi apparatus. Except for the arrangement of the granules, the main difference between the cells from the connective tissue and epithelium of the mantle was that of shape. The epithelial cells were more columnar and were without extensive pseudopodia, their shape being relatively constant. One of the most striking features of the amoebocytes from the connective tissue was their constant variation in shape, illustrated by Bourne (1935) and Crawford and Barer (1951).

2. *The Cytology of Amoebocytes in Other Molluscs*

Gresson (1937), culturing pieces of mantle of the Lamellibranch *Modiolus modiolus* described amoebocytes very similar to those examined by Gatenby and Hill (1934) and Crawford and Barer (1951). They moved

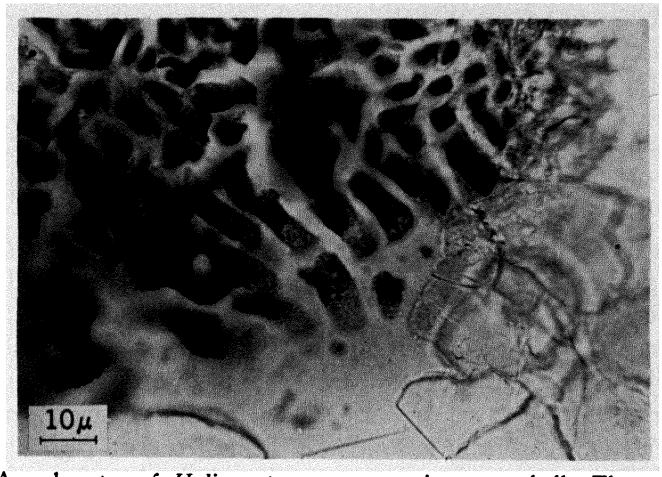


FIG. 5. Amoebocytes of *Helix aspersa* regenerating new shell. The majority of these cells are believed to come from the mantle epithelium.

away from the explant and the majority remained independent of it throughout their existence in the tissue culture. These cells were also believed to arise from connective tissue elements of the mantle. Movement was by means of pseudopodial processes, composed of hyaline cytoplasm. Gresson was not in agreement with Goodrich (1919) that the "pseudopodia" of Molluscan leucocytes (as well as those of other invertebrates) were either derived from optical sections of extensive, folded, membranous processes, or were due to changes taking place under abnormal conditions. [Gatenby, Hill, and MacDougald (1934) working with *Helix aspera* agreed with Goodrich, as did Takatsuki (1934) working with *Ostrea edulis*]. Gresson (1937) however, found that the amoebocytes of *Modiolus* had flat ectoplasmal processes as well as fine pseudopodia. The latter often stretched between two adjacent cells, or attached the cell to the coverslip. A flow of cytoplasm could be observed along these pseudopodia, occasionally with a few small granules. The cell, by virtue of the movement of these pseudopodia, underwent rapid changes of shape and position. Gresson considered their formation was not due to abnormal conditions, as there were no accompanying degenerative changes, and they did not occur in necrotic cells. Fretter (1939) reported

pseudopodia arising under normal conditions in the Tectibranch molluscs. Gresson found that some of the cells contained mostly finely granular cytoplasm with a few, or no, vacuoles or vesicles, but that others contained coarsely granular cytoplasm, with clear vesicles and sometimes more solid bodies. His observations on particular cells where these vesicles were being formed led him to believe that the cells take up material from the medium in the form of solid particles that are first visible as granules or larger bodies, and that these become dissolved and converted into vesicles. Within the more hyaline cells the vesicles moved in and out of focus as the cells themselves moved. In one instance, an irregularly shaped cell, when first observed, contained finely granular endoplasm and four small vesicles. After one and a half hours the vesicles were slightly larger and the granules had increased in number. In about an hour these vesicles had increased greatly in size and number, while granules and small vesicles were scattered through the endoplasm. The granules and vesicles varied in their reaction to stains, some were surrounded by a clear space, others had deeply staining rims. It appeared that the large bodies underwent changes prior to their disappearance, when small clear areas and later a clear central region were formed. The unstained central region tended to spread towards the periphery, producing a vesicle with a deeply staining rim; finally the rim disappeared, leaving a clear vesicle or vacuole. As a result of these observations Gresson concluded that the hyaline and finely granular amoebocytes were converted into coarsely granular cells by the accumulation of material engulfed from the medium. The nucleus, in life, was often obscured by granules. After fixation it was rounded or elongated. The chromatin of the rounded nuclei appeared to be in the form of granules, or small masses, clumped in the central region. Granules were also present on the inside of the nuclear membrane. In the elongate nucleus, the chromatin in the central region consisted of granules arranged in rows, or sometimes of threads or rods. The variation in the shape of the nucleus was considered due merely to the pressure of surrounding structures. Observations showed that the elongate cells, when watched for long periods, underwent rapid movements and changes of shape; they often became spherical and rounded and later were elongate again. It was evident, therefore, that only one type of amoebocyte was being examined. However, when old cells were packed with vacuoles and other bodies, they assumed a rounded position, and, although short pseudopodia were present, they did not appear to become elongate again. Such cells were often present among those situated farthest from the explant but might also be present in other parts of the media.

Fretter (1939) found the amoebocytes of *Philine aperta* might have

fine pseudopodial processes or be distended by large vacuoles. She was able to distinguish them from cells of neighboring tissues because the amoebocytes had smaller nuclei without a nucleolus, but with large chromatic granules. The granules within the cytoplasm might be filled with fluid or contain ingested particles, some of which were probably nutritious, whereas others had a brown granular appearance without apparent food value. Yonge (1926a) was similarly able to distinguish phagocytes from epithelial cells of the alimentary tract because of differences in the shape of the nucleus. Those of the epithelial cells were oval and lightly staining, those of the phagocytes were smaller, spherical, and with a great number of granules of chromatin that stained darkly with hematoxylin. Yonge (1926a) considers that although as many as seven different types of blood cells have been described for Lamellibranchs, some of these are probably different stages of the same type. In *Ostrea edulis*, Takatsuki (1934) found two main types of amoebocytes, granular leucocytes and more hyaline lymphocytes, the former having a more marked amoeboid movement. He examined the nature of their granules. Kollmann (1908) had found that, in *Carcinus*, the reserves in the amoebocytes diminished on starvation. This was not the whole story, however, in *Ostrea* where even after starvation, some of the granules were not appreciably reduced in number. Takatsuki got no positive result with stains for fat, although there was a slight reaction on the outer membrane. They dissolved in dilute acid or alkali. Acid fixatives in particular caused the granules to be destroyed. When fresh, the granules were yellow, or yellowish green, they were neutrophil with a tendency to become stained by basic dyes. These granules were not distinguishable in fixed and stained material. The yellow-green material in amoebocytes and other cells has been interpreted in a variety of ways. MacMunn (1900) suggested its presence was due to decomposition of ingested chlorophyll, and he called it enterochlorophyll. Galstoff and Whipple (1930) examined its properties and found it to be a green pigment, existing in a highly dissociated state, soluble in some organic solvents but not in others, and insoluble in fat solvents. Yonge (1926b) distinguished in *Ostrea edulis* between green or brown vacuoles and bright yellow or brownish concretions, the latter being found also in the cells of the digestive diverticulum. The vacuoles were found to be in the process of being intracellularly digested. The concretions remaining after starvation were indigestible remnants of food and would later be expelled.

The movement of amoebocytes has been examined in tissue culture by several workers. Gresson (1937) described in detail the behavior of the amoebocytes of *Modiolus modiolus*. Pseudopod formation usually took

place at one side of the cell only and was accompanied by a flow of cytoplasm. The pseudopodia became free from the coverslip and the cell contracted towards them. As pseudopodia were constantly being formed, movement in one direction did not continue for long. The end of the pseudopodium in contact with the glass spread out to form a broad terminal region, from which fine processes might originate. When tension became too great, that part of the cell rapidly contracted, but a blunt projection usually remained marking the place from which the pseudopodium originated. When several cells came into contact, the pseudopodia formed a loose network. One cause of the elongation of the cell was the terminal expansion brought about by pseudopodia being active simultaneously at the two opposite ends of the cell.

Movement through the tissues has been judged, in the main, by the positions the amoebocytes occupy when the material is sectioned. Fretter (1952) has augmented this work by studying the behavior of the amoebocytes with labeled phosphorus. The movement of cells through tissues has, as yet been more thoroughly studied in vertebrates. Simmonds (1953), for example, examined the rate and pathways of absorption of erythrocytes from the subarachnoid spaces in the rabbit by means of radioactive phosphate. The labeled cells were steadily absorbed into the circulation at the rate of about 1% of the injected dose per hour for the first 16 hours. Since ligation of the main cervical lymphatics did not affect the rate of absorption, the majority of the labeled cells must have been absorbed by some nonlymphatic pathway, presumably arachnoid villi. Absorption was found to be significantly slower in the anaesthetized animals kept in the head downward position.

Amoebocytes of a number of invertebrates have been used for the purpose of studying the behavior of single cells under varying conditions. Much of this work deals with the properties of protoplasm and does not concern the present article, at least directly, although points arising from such studies are fundamentally of great importance. The work of Crawford and Barer (1951) illustrated difficulties encountered in making cytological examinations with fixed material. According to the type and concentration of the fixative used, there may be a swelling or shrinkage, making the size of the cell in the fixed material quite different from that of the living organism. Similarly, artificial vacuoles or bubbles may appear, or the cytoplasm may change from hyaline to granular, or vice versa. Fauré-Fremiet, studying the action of certain elements on protoplasm, found that monovalent ions of lithium and sodium aided the amoebocyte's ability to adhere to glass and assisted the fluidity of the cytoplasm. Potassium worked in a similar manner, but more slowly. The ions of divalent sub-

stances caused the amoebocytes to behave differently. Thus magnesium ions reduced their power to adhere to glass and made the protoplasm more viscous. Strontium, barium, and calcium ions considerably reduced the stability of their cytoplasm. Fauré also examined the petaloid pseudopodia of amoebocytes. He found that they were exclusively at the edge of the cytoplasm, had considerable elasticity, and were continually changing, being only temporary formations. The surface area, and thus absorptive area, of amoebocytes with such petaloid pseudopodia was very great.

III. Amoebocytic Activity during Shell Repair

Amoebocytes play an important part in shell repair (Wagge, 1951; Wagge and Mittler, 1953). An understanding of their activity and origin is to be found both by the study of molluscs with damaged shells and by the study of suitable tissue cultures. Such work helps to throw light on the role of the mantle in shell repair and on the validity of the various theories concerning calcium transportation and the formation of the new shell.

1. The Origin of Amoebocytes and Importance of the Mantle

While working on shell repair in *Helix aspersa* (Wagge, 1951), the author found that amoebocytes were responsible for the secretion of the organic membranes as well as the crystals of calcium carbonate which are enveloped in protein spheres (calcospherites). During the course of this work it appeared that certain of these cells originated from the mantle, and further work has confirmed this suggestion. Some of the cells come from the mantle epithelium and others from the connective tissue of the mantle. The latter appear to be identical with the amoebocytes of *Helix aspersa* studied by Crawford and Barer (1951). Earlier work on tissue culture had in fact established that cells from these two areas were capable of independent movement. Gatenby and Duthie (1932) gave detailed descriptions of the behavior of such cells in *Helix aspersa* from their hanging drop tissue culture material. The mantle epithelial cells wandered freely from their cultures, they were columnar in shape, and were without pseudopodia. The wandering cells, originating from the connective tissue of the mantle and generally termed amoebocytes, appeared in considerable numbers where the space between the coverslip and the explant was deep. They moved by means of pseudopodia and contained much pigment. Within the culture, these two types of cells were found to be connected with the appearance of crystals, and Gatenby (1933) describing the migration of these mantle epithelial and connective tissue cells, stated that he considered that all the phenomena he had observed fell under the heading of

reparative changes and would normally occur when the snail was wounded. Bourne (1935) examined both the pulmonary wall and pieces of foot of *Helix* in tissue culture. He agreed with the majority of the results of Gatenby and his associates (1932, 1934) and added other interesting facts. Cytoplasmic buds were responsible for the formation of new cells from the epithelial layers. These cells consistently migrated with a flattened distal margin, and there was often an attenuation of the proximal portion to a mere thread. There was a tendency for these cells, particularly from the foot, to separate from the explant in masses. Koníček (1933), studying the migration of amoebocytes in explants of the mantle of *H. pomatia*, found that amoebocytes and mucoid cells were the most stable ones and that from their growth a connective tissue was formed.

Crawford and Barer (1951) employed a method of wounding the mantle of *H. aspersa* in order to obtain good supplies of these amoebocytes for their work on the effect of formaldehyde on living cells. Gatenby (1933) described the mantle epithelial cells as secreting a liquid from which, at a certain concentration in the blood, crystals were formed. Bevelander and Martin (1949) showed that small pieces of mantle could deposit pieces of shell, and recently much use has been made of this fact in work with radioactive materials. Hirata (1953) gained similar results from the tissue culture of the mantle of *Crassostrea virginica*. Pieces of the mantle of the oyster were able consistently to produce shell of the typical organic matrix and birefringent crystalline material, although at a slower rate than in the intact oyster. He found that all the parts of the mantle were capable of this type of shell formation, and was able to keep these isolated mantle-shell preparations in good condition for several days.

This fact, i.e. that certain mantle cells are capable of independent movement, helps considerably in the understanding of the part played by the mantle during shell formation, although whether there is as much independent movement of cells from the mantle during the formation of new shell, as there is during repair, is not known. During repair, such independent movement of these cells was borne out by their presence both in the new tissue and in the extrapallial fluid (between the mantle and the shell). Similarly, when pieces of old shell were placed over the damaged area, these cells were found "eating into" this material (Wagge, 1951). They lay in small groups in the depressions they had created (Fig. 6). The blood of the mollusc is without fibrin and the clotting of any wound is carried out by large numbers of amoebocytes. Similarly the massing of amoebocytes in an explant is considered to be for a protective purpose rather than for true tissue formation (Haughton 1934a). There is no loss of identity of the cells when they come together in this manner.

Drew (1910) described the very great increase in numbers of such cells when *Cardium norvegicum* was wounded. The cells migrating to the damaged tissue were connected by protoplasmic processes and as these contracted, the edges of the wound were drawn closer together. Again

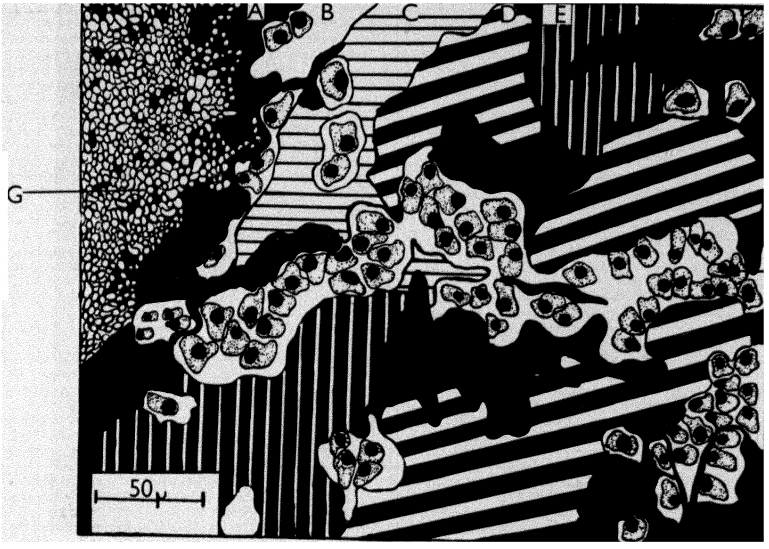


FIG. 6. Amoebocytes removing calcium and protein from pieces of the original shell, placed over a damaged area. A: Original shell. B, C, D, and E: Places where the original shell has been etched away to varying depths. F: Amoebocytes with their nuclei packed with calcium. Long lines of such amoebocytes are traceable from the pieces of original shell to areas where regeneration is taking place. G: New shell formed below an original shell fragment.

Takatsuki (1934) found agglutination in *Ostrea edulis* to be entirely the function of amoebocytes and not of the blood plasma.

To what extent do the mantle epithelial cells act *in situ* when they are laying down new layers of shell, or absorbing calcium from other parts of the shell? They could not lie in positions in which they were found in certain regenerates and at the same time have remained an integral part of the mantle epithelium (Fig. 5). Considering the ease with which they are able to migrate independently there is no need to assume that during the life of the snail they are less capable of moving from the mantle than they are in tissue culture material. Mantle epithelial cells that could not have remained *in situ* are, for example, those that were absorbing material from pieces of old shell and those that were found lying in very definite rows where pathways were left stretching through repaired areas to regions where shell deposition was still taking place (Fig. 7).

The part that the mantle plays in both shell formation and repair has been variously interpreted. Gray (1838) and Meckel (1846) believed that there were all sorts of glands in the mantle that secreted the shell. Leydig (1876), however, believed mantle glands played no important role in

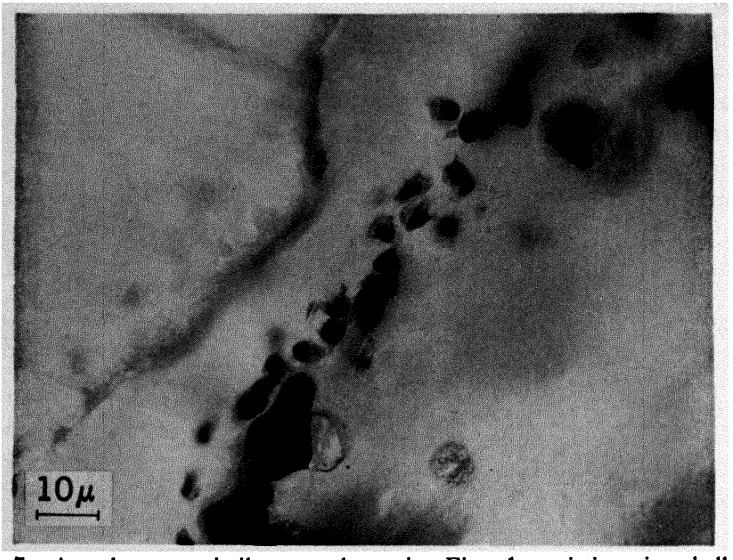


FIG. 7. Amoebocytes similar to those in Fig. 1 assisting in shell repair. Note their fine pseudopodia. Their nuclei are stained intensely for calcium (gallamine blue).

shell formation. Longe and Mér (1880) stated that the edge of the snail's mantle contained special glands that secreted calcium salts, but these were not connected with shell formation. This view was accepted by Villepoix (1892), Bierderman (1903), Reichert (1928), and Neuman (1928). Studying *Helix callizona,* Hayasi (1939) found calcium to be present in a great many types of gland cells, e.g. in the mantle, the "lung", the intestine, and the foot, but he could not recognize any discrete so-called calcareous glands within the mantle. He found that in the regenerating snail the mantle epithelium became active and calcium appeared to be secreted abundantly from it but, as stated, no special calcium glands were detected. The amount of calcium present in the mantle cells varies both seasonally and in different species. Manigault (1939) found that calcium could only be detected in the mantle of *Haliotis* at certain seasons, notably those times during the spring and summer when new shell was being formed. He found that although calcium was transported through it there was no storage of calcium in the mantle epithelium of the pulmonates he

studied. Using gallamine blue stain, the author was unable to find large calcium deposits in the mantle epithelial cells of *Helix aspersa* (Wagge, 1951), although there was plenty of calcium in the underlying connective tissue layers of these regenerating snails, and epithelial cells, when free,

FIG 8. Part of the mantle epithelium of *Helix aspersa* from a stained section demonstrating the distribution of alkaline phosphatases. Note the similarity of these cells with those in Fig. 5.

stained more readily for calcium than they did *in situ* (Fig. 8). Others have reported calcium in these cells, e.g. Sioli (1934) in *H. pomatia* and Bevelander and Benser (1948) in the following genera of Lamellibranchs: *Atrina, Pedalion, Codokia, Pinctada,* and *Venus.* Could this variability be due to the fact that, when required, this tissue can absorb calcium from the shell or from the underlying regions but at other times remains free of such stores? (All cells, of course, contain some calcium.) It is known that in *H. aspersa* these cells can absorb calcium from the fully formed piece of shell placed over a damaged area, and it is known that the amount of calcium that is moved when required to damaged or newly formed shell can be in excess of the total amount of calcium content of the animal's soft parts (Sioli, 1934). It is therefore possible that while they still remain *in situ,* the mantle epithelial cells can absorb calcium from the shell, although as stated earlier, these cells in the regenerated tissue, containing old pieces of shell, took up positions which only freely wandering cells could assume. Sioli (1934), working on *H. pomatia* found the mantle epithelial cells did not give up their calcium during shell repair. This fact was based on chemical analysis that showed no fall in the amount of calcium in this area during regeneration. If the epithelium is capable of ab-

sorbing calcium from the neighboring regions a fall would be detectable only if these cells were examined immediately after they had secreted their materials and before more absorption had occurred. Periodic absorption may explain the erratic presence of calcium and hence the failure at times to get positive results in these cells, using calcium staining techniques. Alternatively, Prenant (1927) showed two types of calcium carbonate present in the cells, react differently to stains. Amorphous calcium carbonate is very soluble in water, is slightly birefringent, and stains easily. Crystalline calcium carbonate is practically insoluble in water, is very birefringent, and is almost colorless when stained. This behavior of the two types of calcium carbonate was particularly evident with the stain gallamine blue (Stock, 1949).

Even if the mantle epithelial cells are able to absorb *in situ*, there is still the problem of getting the material to the appropriate region. Sioli (1934) found that during shell repair calcium was not carried in the blood plasma, or in the extrapallial fluid of *Helix pomatia*. The foregoing account has shown that mantle epithelial and connective tissue cells are able to: (1) absorb calcium, (2) move it to a damage area, (3) secrete the organic and crystalline framework of the new piece of shell. With freely moving cells the physico-chemical reaction can take place directly within the restricted area under repair, and not generally throughout the whole of the extrapallial fluid. Manigault (1933), in his experiments on the concentration of carbon dioxide necessary to obtain crystallization, found that only around the optimum concentration of 15% carbon dioxide were calcospherites formed. Greater or less concentration failed to give precipitation.

2. *Calcium Reserves in the Mantle*

The store of calcium that lies in the subepithelial mantle tissues has been examined in several molluscs. Hayasi (1939) found it possible to detect it much more easily by crystallization methods than by staining. Trueman (1942) using the ammonium oxalate method, followed by examination with polarized light, found that, in *Tellina tenuis*, the calcium which lay in the tissue immediately below the mantle epithelium extended from the tip of the mantle to the tissues nearer the visceral mass. Hayasi (1938) detected calcium in a similar position in the mantle of *Anodonta* with lesser amounts in the underlying tissues. Those reserves nearer the surface tended to change in form and decrease in size, and he assumed some change in the composition of the calcium took place in the connective tissue at the time of shell deposition. He found that calcium could be secreted from all parts of the muscle. There are reserves of this nature

in the connective tissues of *Helix aspersa* and here the author considers that they lie mainly within "resting" amoebocytes which, when required, will pass with their contents to the area where shell deposition is taking place. Manigault (1933) believed this subepithelial calcium was merely a waste product, not used for shell formation in *Haliotis*, *Anodonta*, *Anomia*, and *Pecten*; however, such calcium stores appeared to be utilized in *Helix aspersa* (Wagge 1951). When snails were fed on diets with very high concentration of calcium, they continued to use these calcium stores for additional shell thickening (Wagge, 1952). In *H. aspersa* the subepithelial calcium that does not lie within resting amoebocytes is probably absorbed by the mantle epithelial cells which in turn become amoebocytic. Trueman (1942) considers that in *Tellina tenuis* the calcium carbonate is secreted through the epithelial cells of the mantle from the subepithelial tissues. He notes that there appear to be no special cells near the mantle edge for secretion. The epithelial cells themselves showed no special modification either for the secretion of the periostracum or the secretion of calcium carbonate, except for some possible enlargement in size. Prenant's (1924) work on epiphragm formation in *H. pomatia* supports many of the points already discussed. He found subepithelial layers within the leucocytes that later formed calcareous glands. He described the development of such leucocytes: the nucleus first increased in size without cytoplasmic enlargement, then with no further nuclear change the cytoplasm enlarged and became filled with granules and much lipoid material. These calcareous glands, which are probably massings of connective tissue amoebocytes, were said to develop at the pallial disc. They secreted their contents across the mantle epithelium and calcospherites were subsequently formed from these secretions.

Variation in the amount of calcium in the mantle led Hayasi (1938) to suggest a rhythmic deposition, a condition not accepted by Trueman (1942) for *Tellina tenuis*, but one that the author would consider likely for *H. aspersa*. When snails were fed on synthetic diets that were given at controlled intervals, it was possible to trace rhythmic deposition of the materials that thickened the shell (Wagge, 1952). The texture of the organic membranes formed during feeding with synthetic diets was different from those formed with natural feeding, and one was therefore able to study shell deposition more readily.

3. Amoebocytic Activity in Relation to Theories of Shell Formation

The fact that the mantle cells can, when required, act amoebocytically, helps to bring together various theories that have been advanced regarding the formation of the shell. Secretion of the shell by the mantle has

been described by many workers, including Reaumur (1709), Villepoix (1892), Rawitz (1892), Davis and Fleure (1903), Dakin (1912), Crofts (1929), Prenant (1924) Kessel (1933), Trueman (1942), and Bevelander (1952). Some have attributed the function to the epithelial cells and others to the subepithelial tissues. In general, the calcium carbonate has been considered to be passed by these cells into the extrapallial fluid where crystallization occurs from a colloidal gel. De Waele (1930) refuted this theory of the function of the mantle cells and considered that shell formation was a truly physico-chemical process occurring in the extrapallial fluid, the necessary substances having been brought in solution from the blood. His theory was accepted by Manigault (1933) and Dotterwech and Elssner (1935). De Waele postulated the presence within the extrapallial fluid of a complex of proteins, calcium carbonate, and carbon dioxide which dissociated under optimum conditions within the immediate surroundings. Apart from the fact that such a complex is chemically unknown, the major difficulty regarding this theory is that under the conditions of De Waele's experiments there was a protein concentration of 58%, in contrast to the 4% of the shell. There is no evidence that the resorbtion of organic material from the new shell, necessitated by this theory does, in fact, take place. Whether mantle cells are concerned or not, both these types of theory imply a reaction involving a considerable region of the extrapallial fluid. Amoebocytic behavior of mantle cells overcomes the problem of diffuseness, since they mass at the appropriate region. At the same time, the final reaction will still be a physico-chemical one, but occurring under much more controlled conditions. The type of complex De Waele described is much more likely to occur within a living cell than in body fluid outside it. When studying the formation of pearls, Boutan (1923) had observed that they were formed by amoebocytic cells that passed through the mantle epithelium and secreted the calcium carbonate of the pearl.

4. Phosphatases within Amoebocytes

Enzymatic control, particularly that of the alkaline phosphatases, has been studied by Manigault (1939) and Wagge (1951). Manigault found that there was a marked increase in phosphatase activity in the liver, mantle, and blood during shell regeneration, and he considered that these enzymes played an important part in the mobilization of calcium salts during the initial stage of the process. The molluscs he studied were *Loligo, Anomia, Mytilus, Haliotis,* and *Helix pomatia.* Again, he found that under normal conditions these enzymes were most abundant when shell formation was at its peak. In *H. aspersa* cytological tests (Wagge, 1951) determined the position of these enzymes more precisely than had

Manigault's biochemical examinations. Neither the cells now known to be mantle epithelial nor mantle connective tissue cells were found to contain much of these enzymes. They were markedly present in the nuclei of small amoebocytes, believed to be true blood cells (Fig. 1). These latter cells can also absorb calcium and are found in blood spaces in many parts of the body (Fig. 7). During experiments on shell repair, they were very numerous in the areas under examination, notably the digestive gland, the mantle, the kidney, and regenerating tissue. There was also much extracellular alkaline phosphatase within the digestive gland and blood spaces of the mantle.

5. *Investigations Using Radioactive Materials*

Work with labeled calcium (Ca^{45}) greatly assists the understanding of the behavior of the mantle. Small pieces of mantle are used, eliminating the complexity of using the whole animal. Jodrey (1953) writes "the mollusc mantle has long been considered as the tissue immediately responsible for the secretion of the shell; and it was indeed surprising to find that the Ca^{45} of mantle tissue of whole oysters and mantle shell preparations in labeled sea water was extremely low, even though a relatively large amount of isotope was being deposited in the shell." Two mechanisms, he considered, could explain this low activity of the mantle and high activity of the shell: (1) the calcium deposited in the shell might not enter the mantle at all, (2) a very small amount of calcium might enter the mantle and pass quickly to the shell because of a rapid turnover rate. Jodrey (1953) tested these hypotheses on mantle shell preparations and found that in fact there was a rapid turnover rate. In one *Crassostrea virginica*, the estimated calcium renewed in the whole mantle would be 1.34 mg. per day. The estimated amount of calcium deposited in the shell at the same period, as measured from counts of radioactivity of the shell, was 1.27 mg. It would seem from these figures that the greater part of the calcium was being deposited in the shell. There is, however, a good deal of inert calcium normally within the mantle. About 2.4% was renewed every 24 hours, the turnover rate being of the order of 0.6 μg. of calcium per minute per gram of mantle. Shell deposition is apparently brought about by this small portion turning over at a very rapid rate. The inert calcium has been identified in several species as calcium phosphate (Hayasi, 1939; Bevelander and Benzer, 1948), or as conglomerates of calcium and protein matter (Hayasi, 1938). The decreased rate of deposition of mantle shell preparations as compared with the whole animal (Hirata, 1953) is due, Jodrey (1953) suggests, either to a less efficient transport system or to a diminished energy supply. Both of these could be due to less amoebocytic cells being available.

Bevelander (1952) found that both fresh water and marine molluscs could take up labeled calcium ions from the water and that shell deposition could be independent of the digestive and circulatory systems. In *Anodonta grandis* he found labeled calcium localized in the entire periphery of the mantle, mainly in the subepithelial layers corresponding to those described by Hayasi (1938) and Trueman (1942), but also within the epithelial cells. Again he found phosphatases within the subepithelial and epithelial regions of the mantle. He reported the enzyme within many amoebocytes in the mantle. The role of phosphorus and phosphatases in this region is not fully understood, although it is known that phosphorylation occurs as the result of the action of mantle phosphatase on mantle mucus (Bevelander and Krimsky, 1949). The significance of this phenomena in relation to mineralization is not understood. The deposition of labeled phosphorus in molluscs has been studied both by Bevelander (1952) and by Fretter (1952). In her work with species of *Helix*, *Arion*, and *Agriolimax*, Fretter found that the isotope was carried in amoebocytes that were present both in the digestive gland and in the regenerating tissue of *H. aspersa* and *H. pomatia*. In *Arion hortensis* groups of amoebocytes containing the tracer were seen against the anterior aorta where they may have been augmenting or depleting the supplies. In the mantle of *H. aspersa* and *H. pomatia* the cells accumulating the labeled phosphorus were at the edge of the mantle skirt. In *H. pomatia* much was found in the subepithelial glands, as described by Prenant (1924). Here the isotope lay within cells as postulated by Prenant and not within the blood as believed by Heaysman (1951). Fretter found that labeled phosphorus was stored within the lime cells of the digestive gland. Although in the author's work with *Helix aspersa* the calcium deposits were found to be a carbonate (Wagge, 1951), these lime cells will probably store a variety of elements that are important for the body's metabolism. The whole question of the relationship between calcium and phosphate ions, particularly in relation to shell formation, is very confusing and this problem is dealt with in some detail by Fretter (1952). The amount of phosphorus in the molluscan shell is very small (Pelseneer, 1906; Turek, 1933) or may be entirely absent (Heaysman, 1951). Bevelander (1952) considers that the phosphorus that is present in the shell is confined to its organic constituents. This would be in agreement with Danielli's (1953) theory concerning the behavior of phosphatases. He considers they represent the enzymatic center of a contractile protein. Two examples where there is a connection between protein synthesis and alkaline phosphatase activity are in the formation of the horny teeth of *Myxine* and in the formation of the horns of the deer.

IV. Amoebocytic Activity in Molluscan Digestion

It is well known that, in a large number of animals, freely moving cells assist in the absorption and digestion of food as well as helping to remove residual materials from the digestive tract and other parts of the body. Such cells, because of their ability to engulf foreign matter, are termed phagocytes. The role of phagocytes in digestion is at present disputed by various authors. Yonge (1926a) and Takatsuki (1934) consider that phagocytes, when present, play a far more important part than is believed by Sawano (1929), Mansour (1946a), Mansour-Bek (1946) and George (1952).

The origin of these phagocytes is not completely understood, but George (1952), assuming they are blood cells, discusses the view that they are probably playing a primitive nutritive role, one which is maintained in animals where the stomach and gut are weak in proteolytic enzymes. The blood cells, containing the necessary enzymes, ingest intracellularly and thus supplement the main digestive system. The close relationship between blood cells and digestion, George points out, is well illustrated in both ontogeny and phylogeny of blood formation. For example, the first blood in the vertebrate embryo is formed in the mesoderm of the yolk sac adjacent to the food bearing endoderm. Subsequently blood is formed in the liver, in the spleen, and other hemopoietic regions of gut tract origin. Even in the higher vertebrates where the marrow takes over the principal blood-forming function, the walls of the gut remain one of the important areas of leucocyte production throughout life. When leucocytosis occurs, George regards this as a primitive response of the blood-forming tissue to an excess of foreign matter of nutritive value. Yet in spite of his emphasis on the fundamental connection between blood cells and digestion, George found that in the Lamellibranchs he studied, those cells were of minor importance in the particular aspect of digestion he was studying, namely fat digestion. He agreed with Yonge (1926b) that they absorb and hydrolyze fat, but failed to find enough evidence of their passage, with ingested fat, back into the blood spaces and tissues to establish that they played a major role in digestion. This evidence does not, however, settle the issue, for the movement of the wandering cells may be periodic, and sections showing them in the blood spaces would then be, to some extent, a matter of chance. The movement of the amoebocytes carrying deposits from the lime cells of the digestive gland in *Helix aspersa* was certainly a periodic one (Wagge, 1951), and absence of such cells in some sections was no criterion for a judgment of the behavior of these amoebocytic cells. When the amoebocytes were actively engaged in transporting material, very large numbers of them were involved and were found in blood spaces and less

often between cells of neighboring tissues. Although George (1952) says that in the Lamellibranchs he studied he has never seen leucocytes containing fat in blood or tissue spaces, similar cells are certainly present in these positions in *H. aspersa* when calcium is being transported by them for shell repair. Millott (1937b), however, working on a Nudibranch, *Jorunna tomentosa*, reported that the phagocytes he studied were not found connected with the blood system and were confined to the lumen of the digestive gland and the midgut. Based on this distinction, he considered they were not comparable with the phagocytes Yonge and other workers had reported in the gut of Lamellibranchs, which wander freely in and out of the lumen of the gut and through all the tissues and are essentially blood cells. Millott's feeding experiments, however, he considered, did show beyond all doubt that these amoeboid cells were really phagocytes, taking up food particles in a fashion similar to the cells of the digestive gland. He described and carefully figured the origin of these phagocytes from the epithelium of the digestive gland. Studying still other Nudibranchs, Millott (1937a) described wandering cells in all respects similar to the phagocytes of the Lamellibranchs. Among the Nudibranchs he examined were species of *Acolidia*, *Ancula*, *Goniodoris*, *Acanthodoris*, *Polycera*, and *Archidoris*. This second type of wandering cell occurred in the blood spaces of the submucosa, and less commonly in the general vascular system. Millott considered that they were essentially blood cells, owing to the position in which they were found, particularly as they were found in considerable numbers in the anterior dorsal blood gland. He noted that they wandered freely through the more stable tissues. Millott considered that they were a special type of blood cell, whose main function was excretory, taking up effete matter from the hemocoeles and discharging it into the lumen of the gut.

From these accounts there would appear to be two distinct types of phagocytes concerned with absorption and excretion. The first, originating from the epithelium of the digestive gland and not migrating into blood spaces, and the second, a true blood cell, with wider areas of operation throughout the body. Yonge (1923–1946) has studied the behavior of the phagocytes of many Lamellibranchs. In *Ostrea edulis*, where he found the phagocytes were universally present in the entire alimentary tract and in the gills and the palps, he studied the intake of materials by these phagocytes, notably with regard to their absorption of fat, iron saccharate, and dogfish blood corpuscles. When *Ostrea* was fed with iron saccharate, the phagocytes were invariably present in the cells in which iron was being ingested. After four days, although there was no trace of iron in the tubules of the digestive gland, there were many phagocytes full of minute

granules of iron immediately around the tubules, in the connective tissue, and occasionally in blood vessels and gonads. A similar condition was found in *H. aspersa* (Wagge, 1951). When the snail was fed with calcium carbonate, the phagocytes behaved in a like manner. After the calcium products had been absorbed by the phagocytes from the lime cells, the blood spaces of the digestive gland and mantle were periodically packed with these amoebocytic cells. Yonge (1926b) found that in *Ostrea,* fine food particles were ingested and digested intracellularly in the tubules of the digestive diverticula. The products of digestion were carried away by the phagocytes, which in turn had the enzymatic complement for further intracellular digestion of fats and proteins. Whereas these phagocytes absorbed fairly large particles that were of nutritive value they did not absorb particles of any size that had no food value. India ink, for example, was passed into the lumen and was never ingested by the phagocytes. Often the phagocytes carried the food material to connective tissue cells where it was stored. There is good evidence for the belief that many of these connective tissue cells are in fact phagocytes in a resting state (Haughton, 1934).

In Yonge's (1926a) interpretation of intracellular digestion in the Lamellibranchs, phagocytes play an important part. He considered that they ingest directly particles of food value that would be too large to enter the cells of the digestive diverticulum. In the Nuculanidae (Yonge, 1939a), where phagocytes are absent, there are additional wide diverticula for the ingestion of complete diatoms. Although, in contrast to this, Millott (1937b) did not find the size of diverticula important in Nudibranchs where large and small particles were ingested both by the phagocytes and by the digestive gland cells. Millott (1937b) describes with drawings the process of digestion of a nucleate blood corpuscle within the phagocytes. The corpuscle lay in a large vacuole where it soon began to show signs of erosion. The edges became irregular and the contents were converted into oil-like droplets. The nucleus next showed signs of digestion and finally, also within it, everything was resolved into oil-like droplets which showed a marked affinity for safranin.

Those who consider that much or all the digestion in the Lamellibranchs is extracellular (Mansour, 1945; Mansour-Bek, 1945; and George, 1952), dismiss the digestive function of the phagocytes as something of little significance. Yonge (1946) considers that this apparent evidence of considerable extracellular digestion in Lamellibranchs is due mainly to the cytolysis of phagocytes and partly to the indigestible residue of intracellular digestion that is regularly passed from the cells of the diverticula into the gut, and could be confused with cell-droplet secretion. Graham

(1932), describes a similar process for the removal of residual material in *Patella,* Millott (1937a, b) in Nudibranchs and Morton (1951) in the Struthiolariidae. If digestion in the Lamellibranchs is truly extracellular, then Yonge (1946) points out that the style, being proteinaceous, would itself be in danger of digestion.

Mansour (1946b) challenged two of Yonge's statements. Firstly, that the digestion of protein and fat was exclusively intracellular, taking place either in the cells of the diverticula or in the wandering phagocytes, and secondly, that the digestive diverticula were not organs of secretion. Mansour (1945) found that filtered, phagocyte-free, stomach juice showed the presence of free proteolytic and lipolytic enzymes, as well as those of carbohydrates (Yonge points out that cytolyzed phagocytes would give similar results). Mansour (1945) starved certain *Unio* until both their digestive diverticula and feces were free of coloring matter and then, keeping them on colorless food, injected the foot with chlorophyll solution. Brownish green globules were found in the cell of the diverticula and later appeared in the lumen of the gut and finally in the feces. Mansour considered this passage of the coloring matter from the blood to the lumen of the gut, through the cells of the diverticulum, to be an indication of the secreting action of these cells. Yonge (1946) replied that it was not surprising to learn that in *Unio* injected chlorophyll solution later appeared in the cells of the diverticula. These, he said, must elaborate their intracellular digestive enzymes and obtain the necessary material from the blood. Subsequent appearance in the lumen of the gut of globules containing chlorophyll would be expected, since the indigestible residue of intracellular digestion is regularly passed from the cells of the diverticula to the gut. Yonge further pointed out that even where phagocytes are absent, extracellular enzymes may not be present; this is true for example in the protobranch Lamellibranchia (Yonge, 1939b), in certain herbivorous protobranch Gastropoda, e.g. *Patella* (Graham, 1932), and in *Pterocera* (Yonge, 1932a).

Mansour (1946a) claimed to distinguish between ripe holocrine cell fragments and true phagocytes. He argued that much material that Yonge called phagocytic was in fact cell fragments. He gave as one extreme example Yonge's (1936a) study of *Tridacna,* where he found 17 zooxanthellae (7μ each) ingested in one phagocyte, whose normal size would be 10μ. These Mansour judged to be a mass of secreting globules adhering together. The digestive gland cells fragment in the 12 species of Lamellibranchs that Mansour (1945) studied and these fragments, loaded with globules, reached the lumen of the stomach. He considered it not unlikely that these fragments, which sometimes resemble phagocytes, are the direct

source of the free enzyme in question. Much of this controversy will be settled when it is firmly established whether we have here the fragmentation of cells, as Mansour claims, or whether this process is in fact one method for the formation of phagocytes. Millott (1937b) describes and very carefully illustrates the breaking away of phagocytes in *Jorunna tomentosa* from cells of the epithelium of the digestive gland. These cells passed food to the midgut, but they were not to be found in blood spaces or generally throughout the tissues. Potts (1923) similarly described the origin of phagocytes from the epithelium of the digestive gland in *Teredo*. These phagocytes contained enormous quantities of wood, as did the epithelial cells from which they originated. One would assume that this process of phagocyte formation is quite distinct from fragmentation of digestive gland cells where egesta is passed directly to the intestine. Morton (1951) found that in *Struthiolaria* this was the method employed for the removal of waste matter from the diverticula. Apart from small amounts of extracellular enzymes that would be accounted for in this manner, the Struthiolariidae conformed entirely to Yonge's rule of the intracellular nature of digestion in style-bearers. Mansour-Bek (1945) supported Mansour (1945) in his observations on extracellular digestion. She doubted if phagocytes were responsible for the proteolytic and lipolytic enzymes present in the stomach. They would, she considered, on cytolysis, have to give off their intracellular enzymes more readily than when they were ground and extracted. In her opinion it is the fragmented parts of digestive gland cells that supply the enzymes. She does not discuss the origin of the phagocytes.

George (1952) re-examined digestion, and in particular fat digestion, in certain Lamellibranchs. He considers it is still an open question as to whether the tubules of the digestive gland are secretory or digest phagocytically. His own experiments were not conclusive and gave only circumstantial evidence of absorption in the form of bits of debris and green-brown granules of an organic nature in the cells. They might have been ingested, but were probably manufactured. He suggests that the cells of the digestive diverticula may be specialized for phagocytosis or absorption of some substances, but not of others. Certainly a large number of authors, in agreement with Yonge (1923–1946) have attributed an absorptive function to them, including: Enriques (1902); List (1902); Gutheil (1912); Vonk (1924); Peczennik (1925); Nelson (1925); Graham (1931, 1932); Millott (1937a, b); Fretter (1937); Coe and Fox (1944); and Morton (1951).

George (1952) found that hydrolysis of fat occurred in the ciliated epithelium of the gut, in the ducts of the digestive diverticula, and in the

phagocytes. Emulsions injected into regions other than the digestive tract, as for example the heart, were ingested by phagocytes. There was no evidence of free lipase in blood plasma that would have been secreted by phagocytes or from some other source. George considered that, although the phagocytes contained their own lipase and definitely hydrolyzed fat, their assistance in digestion was strictly limited. The species he mainly studied were *Crassostrea virginica* and *Modiolus demissus*. He considered that extracellular fat digestion definitely occurred, the lipase present in the stomach juice coming from the crystalline style. In species of *Crassostrea, Modiolus, Venus, Ensis*, and *Atriona*, hydrolysis was demonstrated by the use of nile blue sulphate as indicator. This main source of lipase might be supplemented in some species by a secretion from the glandular tissue of the digestive diverticula. Normally, neutral fat fed to these Lamellibranchs was reduced to fatty acids in the lumen of the gut and absorbed as independent particles through the ciliated epithelium of the gut and ducts of the digestive diverticulum. The reserve material found in the large wandering cells in the connective tissue was not provable as fat in the form of neutral fat or fatty acid, but might have been a chromolipid, perhaps mixed with other substances. Storage, probably as fatty acid, occurred in large polygonal, vacuolated cells in the connective tissue. In *Mya*, Yonge (1923) had found that glycogen and fat were important storage products and were just those things for which the phagocytes were enzymatically equipped to deal. As has been discussed, George considered the amount of fat carried by phagocytes to be insignificant in total nutrition. At most, these phagocytes might be acting as an auxiliary system, transporting small amounts to the blood and other tissues. Morton (1951) studying digestion in the Struthiolaridae, likewise considered that their wandering phagocytic cells were performing an accessory digestive function. He questioned whether they were primarily nutritive or were more concerned with the removal of waste particles. Among the materials of no food value that they ingested were neutral red particles that were found in the phagocytic cells of the veliger larva. The presence of a very thick zone of these cells in the proximal intestine of *Struthiolaria* would point also to a rejectory function. Yonge (1923) had reported similar results for *Mya* and Takatsuki (1934) for *Ostrea*. Millott (1937b) suggested the significance of the phagocytes he studied in the Nudibranch *Jorunna* might be that they formed a convenient means of extending the ingestive surface of the digestive system into the lumen of the midgut, particularly if they returned to the region of the digestive gland epithelium from which they arose.

Zachs and Welsh (1953), studying the behavior of the phagocytes of

Venus mercenaria, found that they contained both lipases and cholinesterase (hydrolizing acetylcholine). In fact, the greatest lipase activity was present in two places: in the amoebocytes and in the intestinal epithelium. Variation in the amount of lipase and cholinesterase activity in the region of the intestinal epithelium of *Venus* seemed to lend support to the concept of extracellular digestion of fat in this region. In many Gastropods, digestion is at least in part extracellular. Fretter (1939), studying digestion in Tectibranch molluscs (species of *Philine, Scaphander, Haminea,* and *Actaeon*), found that, although the main bulk of digestion was extracellular and took place in the esophagus, small food particles were ingested by certain cells of the digestive gland and by the amoebocytes, and there underwent further digestion. In the mid-intestine, whose main function was the elaboration of waste material, amoebocytes continued to absorb some food. Fretter remarks that the numbers of these cells in any part of the gut was considerably increased in the presence of particles of food value. They were acting mainly as scavengers in the intestinal regions.

V. The Excretory Function of Certain Amoebocytes

The possibility that certain of the phagocytes have an excretory function has been discussed by many workers. Janssens (1891) and de Bruyne (1896) had noted the similarity in appearance of the contents of certain of the blood cells of the Lamellibranchs and those of certain excretory cells in the organ of Bojanus. Again Dakin (1909) noted the similarity of the contents of the lymphocytes of the gut wall with those of the excretory pericardial cells in *Pecten*. Yonge (1923) found that the phagocytes of *Mya* sometimes ingested substances of no food value and considered they were probably excretory, whereas Graham (1931) working on *Ensis*, described cells, filled with masses of brown granules within spacious vacuoles, in the epithelium, or free in the lumen of the gut and in the submucosa. He considered the cells were possibly excretory. Excretion through the gut wall was described by Darboux (1899, 1900) in Annelids. Working on Aphrodite, he described the abstriction of spherical cells, rich in uric acid, and its derivatives from the lining of the intestinal caeca. The view that these cells are excretory was supported by Fordham (1925). From the appearance of the secretion of injected specimens of *Archidoris brittanica*, it appeared that the wandering cells, loaded with particles of iron saccharate, were migrating from the blood stream, through the epithelium, and into the lumen of the gut where they appeared to be discharging their load. Carriker (1946) described amoebocytes in *Lymnaea stagnalis* that contained fecal vacuoles that were so large that they forced the cell into

a peripheral, lobate ring. When precautions were taken to prevent iron saccharate from entering the gut directly, Millott (1937b) found that there was a very small amount of iron saccharate in the gut of the Nudibranchs he was studying and a relatively much greater amount of iron in the wandering cells and blood spaces of the submucosa. It seemed highly probable, therefore, that the wandering cells were carrying iron saccharate from the blood stream and not in the reverse direction. The evidence was not, however, absolutely conclusive and he cited the following considerations. Firstly, it was possible that some of the suspension of iron saccharate was chemically changed on entering the blood, and the solution, in a diffusible form passed through the gut wall and was reprecipitated in the lumen of the gut. He pointed out that there was, however, no evidence at all to show iron in a soluble form diffused through the epithelium. The epithelial cells, apart from the mucous glands, did not show any patches of staining due to iron. Any stained areas in the intercellular spaces were found to be due to the wandering cells. Secondly, the absorption or ingestion of iron saccharate by the wandering cells was not necessarily an indication of their excretory function, since secreting cells also tended to take it up, as shown by the staining of the mucous cells. There was, however, no evidence to suggest that the wandering cells were secretory. In the hind gut of the specimens fixed one and one half hours after injection, the amount of iron present in the wandering cells in the epithelium of the gut was greatly in excess of that present in the lumen. This fact supported the idea that all the iron saccharate found in the gut had been passed through the gut wall from the hemocoele, into which it had been experimentally injected, and that none had reached the gut directly by accident. The wandering cells, in their excretory capacity, were responsible for carrying this iron saccharate from the blood stream into the lumen of the gut. The experiments indicated that the iron saccharate began to appear in the gut after one and one half hours and was still being discharged by the wandering cells into the lumen of the gut after 19 and one half hours. Fretter (1939) describes a similar function of the amoebocytes in *Venus fasciata*. Materials that would be harmful to the mollusc are also excreted in this way. Orton (1923, 1928), studying the mortality of oysters, found amoebocytes absorbed minerals that were present in poisonous quantities such as copper, zinc, tin, and arsenic. Herdman and Boyce (1899) had reported copper in the amoebocytes of the American Green Oyster suffering from leucocytosis.

VI. Amoebocytes in Phyla Other than the Mollusca

To attempt to review the work on amoebocytes in the whole of the animal kingdom, or even in the invertebrates, is a task beyond the scope of this article. A few general examples will, however, be examined and discussed. Again the amoebocyte will, for convenience, be considered as a cell capable of independent movement, irrespective of origin, more particularly since the origin of some of these cells is unknown. Haughton (1934a) points out that independent movement is characteristic of many cells, not all of which are capable of true amoeboid movement, which is reversible, in contrast to thigmotaxis, which is irreversible.

In the invertebrates the principal functions undertaken by amoebocytes or wandering cells are those that aid digestion, excretion, growth, regeneration, and repair, as has been shown in the foregoing detailed review of the amoebocytes of the Mollusca. In the Porifera, wandering cells of the parenchyma are the principal participants in the nutrition of the organism. In some sponges, they share with the porocytes and choanocytes the function of capturing and digesting food, whereas in other sponges the function of ingestion and digestion, which is largely intracellular, may be taken over entirely by the amoebocytes of the parenchyma. Hemocytopoietic tissue first appears in the Metazoa. In the sponges this is replaced by large and small lymphocyte-like cells and granulocytes. There are primitive nucleolate cells that may differentiate into any other type of sponge cell.

In the Coelenterates the part the wandering cells play in regeneration is chosen for review. Liebman (1946) studied the wandering cells in *Tubularia*. Those that assisted in regeneration, or accumulated in the region of a new prospective polyp, arose from the endoderm of the enteric cavity. They also accumulated in great numbers in the sexual individuals. Moore (1952a, b) described the accumulation of interstitial cells in the tips of the developing outgrowths of *Cordylophora lacustris*, where they were situated in the endoderm and were particularly rich in ribonucleic acid. She found good evidence that they had migrated to this position rather than having entirely proliferated within it. She reviewed earlier evidence on the disputed role such cells play in the process of budding. She considers that certain apparent conflicts are resolvable if the function of the interestitial cells is not to differentiate into new ectoderm or endoderm but to lay down the rudiments of the adult apical structure. Interstitial cells do not accumulate in the regions of growth (e.g. stolon tips) nor in induced outgrowths that do not contain the rudiment of an oral cone. Kanajew (1930) similarly found that interstitial cells were essential for the regeneration of the head of *Hydra* but not for the regeneration of

the foot. Again, in *Pennaria*, Puckett (1936) showed that where the interstitial cells had been removed by x-radiation, hydranths were not regenerated, but stolonic growth continued. Zawansin (1929) showed that irradiation caused the death of interstitial cells and loss of the power of regeneration. Kirchner (1934) described the function of the interstitial cells in the *Cordylophora* colony, including the part played in sexual reproduction and nematocyst formation. He also noted their accumulation in the endoderm at the tip of the rudimentary hydranth, but assumed, as did most previous workers, that they differentiated into new ectoderm and endoderm.

Stolte (1936), in his general review, shows that unspecialized cells are important in regeneration in many animals. Wolff and Dubois (1948) showed their importance in this respect in planarians. In the acoelomate phyla, blood elements are still diffusely scattered throughout the mesenchyme, except for those within a primitive vascular system in the Nemertines. Amoeboid cells with acidophil granules and phagocytic cells assist in nutrition. Prenant (1922) described in the Platyhelminthes certain cells that were morphologically and functionally similar to the mesenchyme cells (hematocytoblasts) of the vertebrate embryo. They were large round cells that lay in the intercellular spaces of the parenchyma, with large basophilic nuclei and a thin covering of basophilic cytoplasm. They could fuse with each other and with the surrounding parenchyma and become connective tissue cells. Alternatively, by cell division, they could become lymphocyte-like cells. Certain cells within the parenchyma had rhabdites and erythrophil bodies with affinities for acid stains. Within the vascular system of the Nemertines, Prenant (1922) found two types of cells: small lymphocytes and large erythrocytes. He considered they could differentiate one into the other according to the environment.

In the segmented worms a considerable evolution in blood cells has occurred (Liebman 1942a, b, c). Blood evolution in the Gephyrians and the Glycerids, which includes anucleation, has progressed to an extent comparable with that in the higher vertebrates (Jordan, 1933). The leucocytes of certain Oligochaetes are also highly advanced. Kindred (1929) described five types present in *Pheretima indica* which he names: lymphocytes, monocytes, granulocytes, lamprocytes, and linocytes. The lymphocytes arise from embryonic mesoderm cells (hemocytoblasts) and can differentiate into any type of blood cell. They are 4–5μ in diameter and the nucleus is ovoid, coarsely granular and without a nucleolus. The cytoplasm is finely granular and contains minute vacuoles, and from it small filiform pseudopodia may be formed. Both nucleus and cytoplasm are basophilic and many of the cells contain chloragogen globules that have

been ingested from the perivisceral fluid. These cells are strongly phagocytic and often aggregate in large numbers. The monocytes are 7–10μ in diameter and the nucleus is ovoid and coarsely granular. The cytoplasm, which is finely granular, has two distinct zones with Giemsa's and Wright's stains, an inner acidophilic zone and a peripheral zone filled with irregular basophilic granules and minute vacuoles. The cells may contain two nuclei, suggesting amitotic division, and are both phagocytic and amoeboid. The granulocytes are 4–12μ in diameter and the nucleus, which is usually spherical and without a nucleolus, is coarsely granular and shows signs of amitosis. The cytoplasm contains small spherical granules, alkaline in nature, mostly eosinophilic. Pseudopodia, when present, are petaloid and the cells are nonphagocytic. The lamprocytes are much larger cells of 22μ, and the nucleus, which is usually hidden by globules, is small and spherical. The cytoplasm is filled with large yellowish globules, the contents of which have not yet been fully determined, but which is not fat. These cells do not form pseudopodia and degenerate in the perivisceral cavity. The linocytes, when fully developed, are equally as large as the lamprocytes. They differ from all the other leucocytes in having cytoplasm filled with either a single large vacuole, a number of irregularly sized vacuoles, or vacuoles and coiled threads. The nucleus is spherical and granular and the cells are very elastic and compressible. Other cells that are free in the perivisceral fluid are detached chloragocytes and parietal peritoneal cells.

Kindred (1929) considers that such an Oligochaete as *Pheretima* has a leucocyte differentiation and production comparable with that of fishes. He suggests a phylogenetic history of hemocytopoietic organs, starting at the Platyhelminthes level where the blood is largely mesenchymatous and the lymphocytes are wandering mesenchyme cells. There is here a generalized proliferation of leucocytes similar to that found in the embryological mesenchyme of annelids and vertebrates. In the next higher stage, the Nemertines, the lymphocytes (though produced in the same way as in the Platyhelminthes) enter the closed vascular channels and may differentiate into hemoglobin containing erythrocytes which act as reservoirs for oxygen. In worms, only a few leucocytes enter the blood vessels, the remainder functioning in the perivisceral cavities. This condition contrasts with the vertebrates where the majority of these cells go into the blood system, the red blood cells performing their function entirely within it. The leucocyte producing organs are, however, much more localized in the vertebrate than in the annelid. Whereas in *Pheretima* there are a series of segmentally arranged leucocytopoietic organs necessitating little movement of cells, the blood system of the vertebrate is a vitally important means of distributing the leucocytes throughout the body.

In the Insecta, Mellanby (1939) describes many forms of blood cells, although some may be one form with different appearances. The least differentiated of these are the free immature mesenchyme cells. Basophil phagocytes help to break down obsolescent tissues, especially during the pupal period; they appear to digest and then set free their constituents, which are then reconstructed into other tissues. They also serve to protect the insect's body against invasion of foreign organisms which they appear to ingest and encapsulate. Other cells are the eosinophilic non-phagocytic oenocytes that discharge substances into the blood and play an important part in the insect's growth.

Within the extensive systems of lacunae and sinuses of the Echinoderms, numerous wandering cells are found. George (1941) describes the free cell types that are universally distributed throughout the phylum. There are leucocytes with nucleolate nuclei and petaloid and membranous pseudopodia. They are phagocytic and granular, giving a positive oxidase reaction and having vacuoles whose contents stain with neutral red. Kindred (1924) describes their increase in numbers during regeneration. Other migratory cells with spherules are variously evolved and probably act as food carriers. There are several other types of wandering cells of more limited distribution. Vibratile cells, with cilia, serve to keep the body fluids agitated. Vesicular and compartment cells have brownish granules in their vacuoles. Red cells with hemoglobin occur in certain Holothurians and are correlated with the development of a highly muscular body. Small spherical lymphocytes occur that have a large nucleus with a nucleolus, and have little cytoplasm. In the Asteroidea fine food particles are taken up and transported by certain of these amoebocytes, intracellular digestion occurs within them (Van der Heyde and Oomen, 1924).

Liebman (1950) has studied in detail the leucocytes of *Arbacia punctulata*. He divides them into two main types, trephocytes and phagocytes. There are three kinds of trephocytes, green, red, and colorless. The green are the smallest, being 6–8μ in diameter, with a nucleus that is small, rounded, and anucleolate and with minute chromatin granules. The cytoplasm is uniform and usually greenish yellow in color. The red trephocytes are the most numerous, are 6–12μ in diameter, and their nucleus is anucleolate and may be fragmented into two portions; the cytoplasm is brownish red with echinochrome. The colorless trephocytes are 6–13μ in diameter and have a minute anucleolate nucleus, their cytoplasm is basophilic and contains colorless inclusions. None of these trephocytes are known to ingest foreign material; they move by means of blunt lobopodes and are often associated with oöcytes. It is considered that they disintegrate and provide food for the oöcyte, the echinochrome of the

latter arising from this source. The phagocytes of *Arbacia,* on the other hand, are able to engulf granules and have true amoeboid movement. They all have a single nucleus which is relatively large and has a nucleolus. Different developmental and functional stages are distinguished of what is probably the same type of cell. Flagellated forms are the youngest; they are spherical and move rapidly with the aid of one or two flagella. The amoeboid form has lost the flagella and this develops into the fibroblast which has fibrillar cytoplasm. Finally, these cells become petaloid forms that are extremely common in the perivisceral fluid. The presence of flagella at the early stage is probably due to the origin of the phagocyte from the ciliated coelomic epithelium.

The functions of the wandering cells in the invertebrates are often more extensive than in the Chordates. Particularly in the invertebrates they play a more important role in digestion and excretion. In the Chordates, as far as their primitive function is concerned, such cells may often be regarded as archaic survivors that have been largely outmoded by the process of evolution. In this sense they are vestigial. On the other hand, they may have taken over special metabolic functions related in nature to their original function. Thus the formation of bile, formerly credited to the liver cells, is the function of primitive reticulo-endothelial cells (Lemberg, 1938). Such a high degree of organization, however, although true for most vertebrates, is not to be found in the Protochordates, whose complement of wandering cells are less advanced than in many invertebrates, particularly the segmented worms. The lymphocytes of the Protochordates are small spherical cells with large, nucleolate nuclei, surrounded by a small amount of basophil cytoplasm and with few or no granules or vacuoles. They are capable of giving rise to all the other organs during budding and regeneration (Berrill, 1935). Macrophages, probably playing an important part in digestion, have a more abundant cytoplasm that contains vacuoles enclosing ingested material. In addition to these lymphocytes and macrophages, George (1939) describes three other types of cells, and finds that many of these are nursing cells for other tissues. He describes other vacuolated cells with some nutritive inclusions and cells with colored granules, sometimes carotenoids. Lastly there are nephrocytes with vacuoles containing purine bases.

In *Perophora viridis,* Liebman (1946) describes three kinds of trephocytes that might represent three stages of the same cell. The youngest are 6–8μ in diameter and have resistant yellow-orange granules. The next stage are 6–10μ in diameter and may have a fragmented nucleus. They are called green cells because of their large yellowish or dark green granules that contain both fat and glycogen. The oldest cells contain very

large inclusions that are colorless and their nucleus is often fragmented. Only the youngest of these cells are amoeboid, all of them are believed to aid respiration and pigmentation, as well as digestion.

Jordan (1933) tracing the development of blood-forming tissues in the vertebrates examined the evolution of the lymphocyte. The most primitive state he found in the Hagfishes where there are only lymphoid cells with typical hemocytoblast features. Extravascularly they differentiate into granulocytes and intravascularly into erythrocytes, thrombocytes, and peculiar fusiform cells. In the Dipnoi both hematocytoblasts and lymphocytes occur in the peripheral blood and undergo further differentiation, the larger into erythrocytes, the smaller into thrombocytes. In the Ganoid and Elasmobranch fishes the lymphocytes begin to outnumber the hemocytoblasts, and this process continues in the Teleosts, Amphibia, and Reptilia. In the Aves and Mammalia there are normally no hemocytoblasts. The lymphocytes are able to differentiate into granulocytes and erythrocytes, though this occurs only pathologically in the spleen after fetal life. The phylogenetic history of blood-forming tissues indicate the primary importance of the spleen, with renal and bone marrow erythrocytopoiesis a secondary adaptation. In the Hagfish the spleen is a diffusely scattered, perivenous myeloid tissue found throughout the submucosa of the gastrointestinal tract. In the lamprey the spleen is aggregated in the spiral valve. In the Dipnoi, it is no longer diffuse but still lies within the gut, in the wall of the stomach. In the higher forms the spleen lies outside the gut and, with the development of hollow bones, the bone marrow plays a more and more important part in the formation of the various types of blood cells.

There would appear to be a direct line of lymphocyte development from Annelids to Cyclostomes (Jordan, 1933). The blood of certain Polychaetes (e.g. *Amphitrite*) contain peculiar spindle cells representing morphologically modified lymphocytes. These are similar to the fusiform lymphocytes found among the Vertebrates only in the Hagfishes and the Lampreys. The coelomic fluid of certain Gephyreans and Glycerids contain genuine erythrocytes similar to those of the blood of Vertebrates, and several Echiuroid genera (*e.g. Thalassema and Magella*) have actual erythroplastids very similar to the red blood corpuscles of mammals. There is in fact no real gap between the bloods of the various groups. Throughout them all, the lymphocyte-like cell represents the primordial element from which differentiate erythrocytes, granulocytes, and monocytes. The primordial blood cell is originally a peritoneal derivative and constitutes a portion of the cellular content of the coelomic fluid. The lymphocytes and their derivatives may migrate into the primitive vascular

channels. Normally they maintain the capacity for elaborating hemoglobin. In the vertebrates, where hemoglobin is almost universally present in the erythrocytes, it is of interest that there are certain South Georgian fish inhabiting very cold waters that are without erythrocytes or any blood pigment (Mathews, 1931: Rudd, 1954). Leptocephalous larvae are without blood pigments until they develop into the elver, and *Petromyzon* has a blood pigment slightly different from the other vertebrates. Apparently other fishes when swimming quietly get enough oxygen from that dissolved in the blood plasma and need their hemoglobin only to provide additional oxygen during greater activity (Nicloux, 1923; Munro-Fox, 1954). From the standpoint of evolution the presence of South Georgian fish without blood pigment is of considerable interest, since their Antarctic environment is believed to have been isolated and washed by cold seas throughout the whole of the Tertiary period (Regan, 1914). Echinochrome, with its important respiratory functions, lies within the trephocytes of certain Echinoderms, but in the majority of the invertebrates respiratory pigments do not occur within cells but are found in the coelomic fluid or blood plasma. This is true whether the substance concerned is hemoglobin, hemerythrin, chlorocruorin, or hemocyanin.

Vertebrate monocytes appear to be comparable, but not necessarily identical, in structure and function to the phagocytes of the invertebrates. They assist in the disposal of foreign bodies and senile and necrotic tissues of the organism. Lewis (1937) showed they have membranous processes similar to those of the phagocytic leucocytes of the invertebrate (e.g. in the Echinoderms) and that they play an important part in the using up of the body fluids. Lewis believes these cells exercise a digesting, modifying action on the body fluids. The granular leucocytes of the vertebrates, like those of the invertebrates, are both neutral red positive and oxidase positive and have the power of digesting substances, a variety of enzymes having been obtained from them (Stern, 1932). There is some support for the suggestion that the leucocytes play an important part in the formation of plasma proteins in the hyperproteinemia observed in myeloid leukemia (Luck, 1938).

Liebman (1946), who classifies wandering cells in the invertebrates as trephocytes with a nutritive function, and lymphoidocytes with an excretory function, finds the condition more complicated in the vertebrates, particularly because the wandering cells usually represent both numerically and functionally only a vestigial cell system. The basophil mast cells most closely resemble the trephocytes of the invertebrates. They are poorly amoeboid, nonphagocytic, and nutritive, giving off portions of their cytoplasm or completely disintegrating. Their granules contain fat, protein,

and mucin substances also contained in the trephocytes of the invertebrates. Ribonucleic acid is suggested as the cause of their basophilia. In the Urodele *Triturus viridescens*, Liebman (1945) described the function of its various leucocytes, particularly with reference to their activity around the growing egg. Lymphocytes, acting mainly as trephocytes, were found in the interovular spaces; they passed into the egg and there underwent fragmentation and were assimilated; their function was not, however, solely trophic. In the interovular spaces, they ingested yolky material and sometimes pigment. The last remains of the corpus atreticum were phagocytosed and removed by them, primarily in the monocytoid or macrophage stage. Neutrophils were more abundant as the egg degenerated and yolk platelets were actively phagocytosed. The neutrophils themselves rapidly disintegrated within the egg and phagocytosis was continued by the follicle cells. Eosinophils were the only leucocytes remaining in the egg after the neutrophils had disappeared, and their numbers and rate of increase remained high for a long time. They appeared to be concerned with the breakdown of protoplasm (i.e. protein). These conditions are similar to those that Liebman (1927, 1931) found in the Oligochaetes, but whereas elements of the lymphoid type appeared to be the only phagocytes in the blood of the invertebrate, polymorphonuclear neutrophils took their place beside them in the vertebrates and superseded them in several respects. They first appeared in the degenerating mature ovum, a condition which parallels their early appearance during injury. They are the only phagocytic blood element to be found within the egg. Their phagocytic activity is intense, but not lasting, since they soon die out when their function is taken over by follicle cells. The differences between lymphocytes and neutrophils within the ovary, as far as phagocytic activity was concerned, appeared to be of a quantitative nature. This was not the case between neutrophils and eosinophils. The catabolic functions were fundamentally dissimilar and appeared to be brought about by quantitative, probably chemical differences.

VII. Discussion

Research into the cytology and physiology of amoebocytes has not only been made by the more conventional methods of sectioning and staining but also by a study of living cells in tissue cultures and of their behavior towards radioactive materials. Such a variety of approach has helped to establish more clearly certain facts regarding the origin, structure, and behavior of these cells. Particularly in the Invertebrates, the breaking away of cells from a more or less stable tissue may be a common and normal occurrence within the living animal. Certainly in the tissue cultures of some invertebrates (Bourne, 1935) there is far less tendency

for the migrating cells to show degeneration or de-differentiation than there is in the vertebrates (Horning and Richardson, 1929). Migration, therefore, might not be an unnatural phenomenon for cells from "stable" tissues in many more invertebrates than vertebrates. Wandering cells, or amoebocytes have, in the Mollusca, been described as originating from many tissues as well as the blood. Epithelial layers have been the origin of such cells, particularly those of the digestive tract, mantle, and foot. Millott (1937b) described the budding of phagocytes from the digestive gland of *Jorunna tomentosa* and Potts (1923) similarly described the origin of such cells in *Teredo*. Gatenby and Duthie (1932) showed that cells from the mantle epithelium in *Helix aspersa* freely wander off both in tissue culture and regeneration. Bourne (1935) found that epithelial cells of *Helix* both from the mantle and the foot gave off cytoplasmic buds. Horning (1932) has described such budding in degenerate cells in tissue culture and has assumed that under these conditions this was a means of restoring the nucleo-cytoplasmic ratio. Bourne, however, observed this budding in normal healthy tissue. The connective tissue, particularly of the mantle, is the site of origin of many amoebocytes. In *Helix aspersa* such cells have been described by Gatenby and Hill (1934), Haughton (1934a), Bourne (1935), and Crawford and Barer (1951), and in *Modiolus modiolus* by Gresson (1937). Many of the phagocytes are, however, regarded as true blood cells, a few examples are: the Tridacnidae (Yonge, 1936a); the Nudibranchs (Millott, 1937a); *Anodonta* (Gutheil, 1912); and *Helix* (Gatenby and Duthie, 1932; Hill, 1934). Other amoebocytes are considered to have developed from simpler forms or, in other words, to pass through various stages. In particular, simple hyaline types alter, especially with the acquisition of granules (Kollman, 1908; and Gresson, 1937). By virtue of their function and movement, alteration in shape is very common among amoebocytes, except when they become so densely packed with vacuoles or granules that they remain spherical.

It is known that under certain circumstances, e.g. wounding, the numbers of amoebocytes in a particular area is greatly increased (Gatenby, Hill, and MacDougald, 1934; Wagge, 1951). This is most probably at first due to the migration of cells from other areas and from resting positions. Haughton (1934b) found that this large increase in numbers occurred as soon as 15 minutes after wounding a *Helix*, a time that ruled out either division or budding and was best explained by migration. Millott (1937a), studying the excretory function of certain wandering cells of Nudibranchs supported this theory of migration. An increase in the number of amoebocytes discharging particles of iron into the lumen of the gut was associated with a reduction in number of wandering cells in the

epithelium and subjacent tissues. There is also, however, a considerable increase in the actual number of amoebocytes in the snail's body if damage to the shell occurs repeatedly. In those snails that had pieces of regenerated tissue removed for 10 successive days the "amoebocyte count" was much higher than in the normal snail (Wagge, 1952). It is of interest to appreciate that amoebocytic cells are capable of passing both into other cells and through stable tissues, mainly through intercellular spaces that the amoebocytes form themselves as they squeeze along. Fretter (1952) has shown the value of radioactive materials to trace the path of amoebocytes in Molluscs and others have studied the behavior of blood cells in vertebrates. The presence of living cells within others has definitely been established in tissue cultures. Lewis (1925) saw frog mononuclear and epitheloid cells from tubercular rabbits engulf their own kind but stresses the great rarity of such cases and of their probable pathological nature. Fischer and Dolschansky (1929) repeatedly observed small cells wandering in and out of the stroma cells of spleen cultures. They did not observe any digestion of these elements, which they consider as derivatives of stroma cells. Liebman (1950) believes that these wandering cells are lymphocytes and their often fragmented and pycnotic condition does not exclude the possibility that they are occasionally digested by their hosts. Erythrocytes have been used to study the passage of cells through other tissues. Simmonds (1953) showed that rabbit erythrocytes probably passed through arachnoid villi when the main cervical lymphatics were ligatured. However, Courtice, Harding, and Steinbeck (1953) found that erythrocytes in the rat, rabbit, and guinea pig passed normally by lymphatic absorption from the peritoneal cavity. The right lymph duct contained as many as 5,500,000 red cells per cubic millimeter whereas local phagocytosis and removal in the mediastinal lymph nodes was insignificant compared with this lymphatic absorption.

The type of cell division that occurs in these freely moving cells is not always certain. Bohuslav (1933a, b) believed that both amitosis and mitosis occurred in his Molluscan cultures. Gatenby (1932), Gatenby and Hill (1934), Gatenby, Hill, and MacDougald (1934), and Bourne (1935) described amitosis in their cultures of the mantle of *Helix*. Gatenby and Duthie (1932) stated that in culture, normal regeneration, and probably normal growth, these cells of the snail multiply by amitosis, and Bourne (1935) described cytoplasmic fragmentation as another form of division, that might be due to the formation of unusual pseudopodia. Gresson (1937), when studying *Modiolus*, did not observe definite stages of either mitosis or amitosis but found nuclei that appeared to be in the early prophase stages. Fretter (1939) found examples of amoebocytes undergoing nuclear division in *Philine aperta*.

The reduction in numbers of amoebocytes is equally interesting; most work has been done on blood cells. George (1941) suggests that this reduction is often due to a scarcity of nutrient materials in the body fluids, which cause an atrophy of lymphopoietic tissue. Improved feeding restores the balance. Kollmann (1908) has noted the reduction in numbers of the blood cells of crabs during moulting, at a time when they do not feed. Jordan (1938) obtained very interesting results with Urodeles when he starved some for four months. Among the reduction of leucocytes which occurred, the eosinophils disappeared completely and erythrocytic transformations included changes in the structure of both cytoplasm and nucleus and fenestration of the nuclear membrane. The neutrophils and basophils lost their granules and the production of granulocytes in the liver ceased. The spleen was reduced in size. After two weeks of good feeding there was a complete restoration to the normal condition in the blood, liver, and spleen. Variation in size and shape both of the nucleus and cytoplasm in differing types of amoebocytes is to be expected considering their many places of origin; whereas the type of granule or vacuole present is a functional character dependent on whether they assist excretion (lymphoidocytes), or help in the transport and release of nutrients (trephocytes). Similarly the presence and type of pseudopodia is largely connected with the origin of the cells. Those that come from epithelial layers have less well developed pseudopodia than those from connective tissues. Few authors uphold Goodrich's (1919) view that in the invertebrates in general true pseudopodia do not exist and those so named are merely folds of a continuous membrane forming petaloid extensions; fine pseudopodia are lateral views of such petals, or are artifacts. Among those who dispute this statement are Liebman (1950) for Echinoderms and Annelids, and Gresson (1937) and Fretter (1939) for Molluscs. Petaloid cytoplasmic extensions, whether they are true pseudopodia or not, certainly greatly increase the surface area of the cell and have the power of great elasticity (Fauré-Fremiet, 1929). Long filopodia are common in amoebocytes from the connective tissue of *Helix* (Bourne, 1935). They serve for anchorage; and, when the cells are massed together, they form an interlocking network over damaged tissue, which is particularly valuable as a temporary plug in an animal whose blood is unable to clot due to the absence of fibrin. Lobose pseudopodia also occur, particularly in the cells with true amoeboid movement.

The results of work on shell repair in Molluscs have in the past appeared conflicting. The mantle epithelium, or its subepithelial layers have each been claimed as solely responsible for deposition of the regenerated tissue. Others believed these layers play a far less important role, the main

reaction being a purely physico-chemical one within the extrapallial fluid between the mantle and the shell. These facts, and many others have already been discussed in the appropriate section where reference is made to the authors concerned. One of the most important points resulting from the review of the general field of this work, is that many apparent differences are more easily understandable if it is true that during the repair of the shell of a Mollusc, epithelial and connective tissue cells of the mantle move to the damaged area with the same facility of independent action that they display in tissue culture. Repair does not then rely on the behavior either of a limited area of the mantle, or of physico-chemical reactions going on in a wide area of the extrapallial fluid. Amoebocytic cells, mainly originating from the mantle can then concentrate their activity strictly within the damaged area. Other amoebocytic cells will be brought into action from more remote regions of the body and their numbers will increase not only by resting cells becoming active, but also by amitotic division and cytoplasmic budding. Some of the facts that have already been discussed supporting this hypothesis are now summarized. Amoebocytic cells form the repaired shell and they transport the necessary materials from the digestive gland and shell. Many of these cells originate, it is believed, from the epithelium or the connective tissue of the mantle. Their numbers increase considerably both in the damaged area a very short time after injury by migration, and in the whole activated region of the animal following a series of damages to the shell. Mantle epithelial and connective tissue cells have, in tissue culture, the power of becoming amoebocytic (freely moving) as have connective tissue and epithelial and blood cells from other parts of the body. Many of these cells can pass through other tissues and are known to mass in large numbers both for protection as well as for true growth. Some contain phosphatases that appear to assist shell calcification or the protein synthesis associated with it. Under certain circumstances the mantle tissues contain calcium but there appears to be a very rapid turnover rate of the calcium that is actually used for shell repair.

There is visualized here a primitive transport system that operates in emergencies, allowing a speedy organization of those processes within the body that control repair. To what extent does a similar primitive system operate in invertebrates to assist digestion? In Molluscs results for digestion are as conflicting as are those for repair. Is it true that, in the Lamellibranchs, phagocytes play an essential role in intracellular digestion, or can they be regarded as insignificant and vestigial in a digestion that is mainly extracellular? Mansour (1945) and Mansour-Bek (1945) describe ripe holocrine fragments originating from regions similar to those that others, e.g. Potts (1923), Millott (1937a) have described for the origin

of phagocytes. Barer (1951) has shown that the cell periphery of amoebocytes of *Helix* is extremely thin and, consequently, there is difficulty in recognizing amoebocytic outlines (particularly in areas where these cells are dense), which may explain why they are described by some as cell fragments, rather than true cells. Again the nucleus may be completely covered by granules or may itself give an intense staining reaction of the same nature as that of some of the granules.

The numbers of phagocytes that are present in sections under review have influenced the interpretation of their importance in absorption, assimilation, or transport. George (1952) found no phagocytes carrying fat to the tissues and relegated these cells to a very minor role in a system that was mainly extracellular. Yonge (1923–1946) and others have described at times very large numbers of phagocytes cooperating in digestion and passing through the tissues and the blood. These differences in numbers could be explained by a periodic activity of these cells and a greater difficulty in recognizing them when they are inactive thereby staining in a manner similar to the surrounding connective, or other tissues.

Mansour (1945) considers that the ripe holocrine fragments, which sometimes resemble phagocytes, are the direct source of the Lamellibranch's extracellular enzymes. Are these cytolyzed phagocytes? It is usually not disputed that phagocytes concerned with digestion are richly equipped with digestive enzymes, although Mansour-Bek (1945) found difficulty in obtaining from them rich supplies of enzymes by grinding and extraction. Takatsuki (1934) found the amoebocytes of *Ostrea edulis* contained sucroclastic, lipoclastic, and proteoclastic enzymes and possessed a complete oxidase system. Bourne (1935) found a similar oxidase system in amoebocytes of *Helix*. Zachs and Welsh (1953) found that one of the greatest centers of lipase activity was in the amoebocytes of *Venus*. Yonge (1926a) stated that amoebocytes ingested particles that were too large to enter the cells of the digestive diverticulum, but George (1952) did not agree that the wandering phagocytes were important agents in fat absorption in the gut. His experiments showed that neutral fat was reduced to fatty acids in the lumen of the gut and absorbed as independent particles through the ciliated epithelium of the gut and ducts of the digestive diverticulum. Little, if any, was carried by the phagocytes. Yet George agrees these phagocytes do absorb and hydrolyze considerable quantities of fat—for what purpose? The large wandering cells he found in the connective tissue had reserve material that was not provable as fat in the form of neutral fat, or fatty acid, but was probably a chromolipid, perhaps mixed with other substances. George found that his experiments still left as an open question whether the cells of the digestive gland were secretory

or phagocytic, but found that they left in no doubt the fact that phagocytes were insignificant in the nutrition of fat.

The Mollusca have been chosen for a detailed review of amoebocytes and few examples in other phyla have been examined. The system of freely moving cells in some invertebrates, e.g. Oligochaetes, is as highly differentiated as in the fishes. In the less highly evolved phyla amoebocytes serve as a very important transport system in almost all the principal functions of the body, particularly assisting digestion, excretion, growth, regeneration and repair, and occasionally respiration. When their activity is less vital in any process which is normally performed by a specialized organ, the amoebocytes may remain as a reserve force, or they may continue to perform small services within the whole organization. Again, whereas, in invertebrates, amoebocytes appear able to be released from many tissues, particularly epithelial and connective tissues, in vertebrates, freely moving cells are more strictly limited in their origin. One traces a further separation of the formation of blood cells from derivatives of the digestive tract (e.g. the spleen) to the bone marrow.

VIII. Summary

In the Molluscs amoebocytes originate mainly from connective and epithelial layers and from the blood. Their structure varies according to origin and species and their granular contents according to their function. Some pass freely through the tissues and have a wide range of operation, whereas others are reported as occurring only in limited areas. Many can mass together in very large numbers when their activity is at its height. Their individual structure and behavior has best been studied in tissue cultures, but recently tracer elements have allowed a new and valuable approach to their performance within the living animal. Increase in the number of cells is generally by amitosis and budding. The value of amoebocytes to the animal is differently interpreted; they are generally considered to form a primitive transport system that has been superseded to a greater or less extent in different phyla. Amoebocytes in Molluscs are claimed to assist to some degree in all stages of digestion, i.e. transportation, physical and chemical treatment, and absorption. They are known to be richly equipped with enzymes and to be capable of intracellular digestion. Those that engulf food particles are commonly called phagocytes. Amoebocytes have been classified, particularly in the invertebrates as lymphoidocytes, aiding digestion and trephocytes, aiding excretion. Growth, regeneration, and repair are other functions greatly assisted by amoebocytes and certain problems in invertebrates are more easily resolvable if one envisages the

possibility that this primitive amoebocytic transport system can move with speed to a certain area, act efficiently, and increase its numbers both from the reactivation of resting cells and by further budding or division of the appropriate tissues. Where emergency operations are required, as in the repair of damage endangering life, the animal may temporarily fall back on the use of amoebocytes, even though normally more specialized mechanisms are in force. In all phyla freely moving cells continue to protect the animal from the attack of small parasitic organisms. Considerable differentiation of blood cells occur in some phyla, e.g. Annelids, Echinoderms, and Chordates but there appears to be no real gap between the blood of various groups, the primordial blood cell originating as a cellular content in the coelomic fluid. In the Chordates wandering cells usually represent both numerically and functionally only a vestigial cell system.

Acknowledgments

I wish to express my thanks to Dr. G. N. C. Crawford for his generous assistance, particularly in the preparation of the photographs.

References

Baker, J. R. (1950) *Proc. Linnean Soc London Zool.* **162**, 67.
Barer, R. (1951) "Cytology and Cell Physiology." Oxford, New York.
Berrill, N. J. (1935) *Phil. Trans. Roy Soc.* **B225**, 327.
Bevelander, G. (1952) *Biol. Bull.* **102**, 9.
Bevelander, G., and Benser, P. (1948) *Biol. Bull.* **94**, 176.
Bevelander, G., and Krimsky, I. (1949) Bermuda Biol. Station Annual Report, p. 1.
Bevelander, G., and Martin, J. (1949) *Anat. Record* **105**, 614.
Bierdermann, W. (1903) *Z. allgem. Physiol.* **1**, 154.
Bohuslav, P. (1933a) *Arch. exptl. Zellforsch. Gewebezücht.* **13**, 673.
Bohuslav, P. (1933b) *Arch. exptl. Zellforsch. Gewebezücht.* **14**, 139.
Bourne, G. H. (1935) *Australian J. Exptl. Biol. Med. Sci.* **13**, 43-48.
Bourne, G. H. (1950) *J. Roy. Microscop. Soc.* **70**, 367.
Boutan, L. (1923) *Ann. Sci. nat. Zool.* [10] **6**, 1.
Bruyne, C. de (1896) *Arch. biol. (Paris)* **14**, 187.
Carriker, M. R. (1946) *Biol. Bull.* **91**, 88.
Coe, W. R., and Fox, D. L. (1944) *Biol. Bull.* **87**, 59.
Courtice, F. C., Harding, J., and Steinbeck, A. W. (1953) *Australian J. Exptl. Biol. Med. Sci.* **31**, 215.
Crawford, G. N. C. (1954) Unpublished.
Crawford, G. N. C., and Barer, R. (1951) *Quart. J. Microscop. Sci.* **92**, 403.
Crofts, D. R. (1929) Liverpool Marine Biology Committee, Memorandum No. 29. University Press, Liverpool.
Dakin, W. J. (1909) Liverpool Marine Biology Committee, Memorandum No. 17. Williams and Norgate, London.
Dakin, W. J. (1912) Liverpool Marine Biology Committee, Memorandum No. 20. Williams and Norgate, London.

Danielli, J. F. (1953) "Cytochemistry: A Critical Approach." Chapman and Hall, London.
Darboux, G. (1899) *Bull. soc. sci. nat. Nîmes* **27**, 53.
Darboux, G. (1900) *Bull. sci. France et Belg* **33**, 21.
Davis, J. R. A., and Fleure, H. J. (1903) Liverpool Marine Biology Committee, Memorandum No. 10. Williams and Norgate, London.
Drew, G. H. (1910) *Quart. J. Microscop. Sci.* **54**, 605
Dotterwech, H., and Elssner, E. (1935) *Biol. Zentr.* **55**, 138.
Enriques, P. (1902) *Mitt. zool. Sta Neapel.* **15**, 281.
Fauré-Fremiet, E. (1929) *Protoplasma* **6**, 521
Fisher, A., and Dolschansky, E. (1929) *Wilhelm Roux' Arch. Entwicklungsmech. Organ.* **116**, 123.
Fordham, M. G. C. (1925) Liverpool Marine Biology Committee, Memorandum No. 27. Hodder and Stoughton, London.
Fretter, V. (1937) *Trans. Roy. Soc. Edinburgh* **59**, 119.
Fretter, V. (1939) *Trans. Roy. Soc. Edinburgh* **59**, 599.
Fretter, V. (1952) *Quart. J. Microscop. Sci.* **93**, 133.
Galstoff, P. S., and Whipple, D. V. (1930) *U.S. Bur. Fisheries Bull.* **96**, 486.
Gatenby, J. B. (1932) *Nature* **130**, 628.
Gatenby, J B. (1933) *Arch exptl Zellforsch. Gewebezucht* **13**, 663
Gatenby, J B., and Duthie, E. S. (1932) *J. Roy. Microscop Soc* **52**, 395
Gatenby, J. B., and Hill, J. C. (1934) *Quart J. Microscop Sci* **76**, 331.
Gatenby, J. B., Hill, J. C., and MacDougald, T. J. (1934) *Quart J Microscop Sci.* **77**, 129.
George, W. C. (1939) *Quart. J. Microscop. Sci.* **81**, 391.
George, W. C. (1941) *Quart. Rev. Biol.* **16**, 426.
George, W. C. (1952) *Biol. Bull.* **102**, 118.
Graham, A. (1931) *Trans. Roy. Soc. Edinburgh* **56**, 725
Graham, A. (1932) *Trans. Roy. Soc. Edinburgh* **57**, 287
Gray, J. E. (1838) *London Med. Gaz* **21**, 10.
Goodrich, E. S. (1919) *Quart. J. Microscop. Sci* **64**, 19.
Gresson, R. A. R. (1937) *Quart. J. Microscop. Sci* **79**, 659.
Gutheil, F. (1912) *Z. wiss. Zool.* **99**, 444.
Haughton, I. (1934a) *J. Roy. Microscop. Soc.* **54**, 246.
Haughton, I. (1934b) *Quart J Microscop. Sci.* **77**, 157.
Hayasi, K. (1938) *Annotat. Zool. Japanese* **17**, 95
Hayasi, K. (1939) *Annotat Zool Japanese* **18**, 1.
Heaysman, J. E. M. (1951) Ph D. thesis, University of London.
Herdman, W. A., and Boyce, R. (1899) "Oysters and Disease." Lancashire Sea Fisheries Committee, Memorandum No. 1. London.
Heyde, H. C. van der, and Oomen, H. A. P. C (1924) *Arch. intern. physiol.* **24**, 41.
Hill, J. C. (1934) *J. Roy. Microscop. Soc.* **54**, 163.
Hirata, A. A. (1953) *Biol. Bull.* **104**, 394.
Horning, E. S. (1932) *Proc. Soc. Exptl Biol Med.* **29**, 809.
Horning, E. S., and Richardson, K. C. (1929) *Australian J. Exptl. Biol. Med. Sci.* **6**, 229.
Janssens, F. (1891) *Cellule* **9**, 15.
Jodrey, L. H. (1953) *Biol. Bull.* **104**, 398.
Jordan, H. E. (1933) *Quart. Rev. Biol.* **8**, 58

Jordan, H. E. (1938) *J. Morphol.* **63**, 143.
Kanajew, J. (1930) *Wilhelm Roux' Arch. Entwicklungsmech. Organ* **122**, 736.
Kessel, E (1933) *Z. Morphol. Ökol. Tiere.* **27**, 129.
Kindred, J. E. (1924) *Biol. Bull.* **46**, 228.
Kindred, J. E. (1929) *J. Morphol.* **47**, 435.
Kirchner, H. A (1934) *Z. Zellforsch. u. mikroscop. Anat.* **22**, 1.
Kollmann, M. (1908) *Ann. sci. nat. zool.* **9**, 8.
Koníček, H. (1933) *Arch. exptl. Zellforsch. Gewebezücht.* **13**, 709.
Lemberg, R. (1938) "Perspectives in Biochemistry." Cambridge Univ. Press, London.
Lewis, W. H. (1925) *Anat. Record* **31**, 43.
Lewis, W. H. (1937) *Science* **85**, 437.
Leydig, F. (1876) *Arch. Naturgeschichte* **42**, 209.
Liebman, E. (1927) *Zool. Jahrb. Abt* 3, **44**, 269.
Liebman, E. (1931) *Zool Jahrb. Abt.* 2, **54**, 417.
Liebman, E (1942a) *J Morphol.* **70**, 151.
Liebman, E. (1942b) *J Morphol* **71**, 221
Liebman, E. (1942c) *J. Exptl. Zool.* **91**, 373.
Liebman, E. (1945) *Am J. Anat.* **77**, 273.
Liebman, E. (1946) *Growth* **10**, 291.
Liebman, E. (1947) *Experientia* **3**, 442.
Liebman, E. (1950) *Biol. Bull.* **98**, 46.
List, T. (1902) *Fauna u. Flora Neapel* **27**, 1.
Longe, R, and Mer, E. (1880) *Compt. rend.* **90**, 882.
Luck, J. M. (1938) "Perspectives in Biochemistry." Cambridge Univ. Press, London.
MacGee-Russell, G (1954) Unpublished.
MacMunn, C. A (1900) *Phil. Trans. Roy Soc.* **B193**, 1.
Manigault, P. (1933) *Trav. sta. biol Roscoff.* **2**, 51.
Manigault, P. (1939) *Ann. inst. oceanog. (Paris)* **18**, 331.
Mansour, K. (1945) *Proc Egypt. Acad. Sci* **1**, 1.
Mansour, K (1946a) *Nature* **157**, 482.
Mansour, K. (1946b) *Nature* **158**, 378.
Mansour-Bek, J J (1945) *Proc Egypt Acad. Sci* **1**, 13.
Mansour-Bek, J. J. (1946) *Nature* **158**, 378.
Mathews, L. H. (1931) "South Georgia." Wright and Marshall, London.
Meckel, H. (1846) *Arch. Anat. Physiol.* Leipzig 1.
Mellanby, K. (1939) *Biol Revs.* **14**, 243.
Millott, N. (1937a) *J. Exptl. Biol.* **14**, 405.
Millott, N. (1937b) *Phil. Trans. Roy. Soc.* **B228**, 173
Moore, J. (1952a) *J. Exptl. Biol* **29**, 72
Moore, J. (1952b) *Quart. J. Microscop. Sci.* **93**, 269.
Morton, H. E. (1951) *Quart. J. Microscop. Sci.* **92**, 1.
Munro-Fox, H. (1954) *Nature* **173**, 849.
Nelson, T. C. (1925) *Biol. Bull.* **49**, 86.
Neumann, G. (1928) *Jenaische Z. Naturwiss.* **63**, 181.
Nicloux, M. (1923) *Compt. rend. soc. biol.* **89**, 1328.
Orton, J. H. (1923) *J. Marine Biol. Assoc. United Kingdom* **13**, 1.
Orton, J. H. (1928) *J. Marine Biol. Assoc. United Kingdom* **15**, 365.
Peczennik, O. (1925) *Z. vergleich. Physiol.* **2**, 215.
Pelseneer, P. (1906) "A Treatise on Zoology." Black, London.

Potts, F. A. (1923) *Biol. Revs.* **1**, 1.
Prenant, M. (1922) *Arch. Morphol. Gén. et Exptl.* **5**, 1.
Prenant, M. (1924) *Biol. Bull.* **58**, 331.
Prenant, M. (1927) *Biol. Revs.* **2**, 365.
Puckett, W. O. (1936) *Biol. Bull.* **70**, 392.
Rawitz, B. (1892) *Jenaische Z. Naturwiss.* **27**, 1.
Reaumur, R. (1709) *Hist. Acad. Sci. Paris* p. 364.
Regan, C. T. (1914) *Terra Nova Repts. Zool.* **1**, 1.
Reichert, W. (1928) *Z. Morphol. Ökol. Tiere* **2**, 624.
Rudd, J. T. (1954) *Nature* **173**, 848.
Sawano, E. (1929) *Sci. Repts. Tôhoku Univ. Fourth Ser.* **4**, 327.
Simmonds, W. J. (1953) *Australian J. Exptl. Biol. Med. Sci.* **31**, 77.
Sioli, H. (1934) *Zool. Jahrb. Abt.* 3, **54**, 507.
Stern, K. G. (1932) *Z. physiol. Chem.* **204**, 259.
Stock, A. (1949) *J. Roy. Microscop. Soc.* **69**, 20.
Stolte, H. A. (1936) *Biol. Revs.* **2**, 1.
Takatsuki, S. (1934) *Quart. J. Microscop. Sci.* **76**, 379.
Trueman, E. R. (1942) *J. Roy. Microscop. Soc.* **61**, 69.
Turek, P. (1933) *Arch. Naturgeschichte* **2**, 291.
Villepoix, M. R. de (1892) *J. Anat. (Paris)* **28**, 461.
Vonk, H. J. (1924) *Z. vergleich. Physiol.* **1**, 607.
Waele, A. de (1930) *Mem. Acad. Roy. Belgique Cl. Sci.* **10**, 1.
Wagge, L. E. (1951) *Quart. J. Microscop. Sci.* **92**, 307.
Wagge, L. E. (1952) *J. Exptl. Zool.* **120**, 311.
Wagge, L. E., and Mittler, T. (1953) *Nature* **171**, 528.
Willmer, E. N. (1935) "Tissue Culture." Methuen, London.
Willmer, E. N. (1945) *in* "Essays on Growth and Form" pp. 264-294. Oxford Univ Press, New York.
Wolff, E., and Dubois, F. (1948) *Rev. Suisse. Zool.* **55**, 218.
Yonge, C. M. (1923) *Brit. J. Exptl. Biol.* **1**, 15.
Yonge, C. M. (1925a) *Brit. J. Exptl. Biol.* **2**, 373
Yonge, C. M. (1925b) *J. Marine Biol. Assoc. United Kingdom* **13**, 938.
Yonge, C. M. (1926a) *Trans. Roy. Soc. Edinburgh* **54**, 703.
Yonge, C. M. (1926b) *J. Marine Biol. Assoc. United Kingdom* **14**, 295.
Yonge, C. M. (1928a) *J. Marine Biol. Assoc. United Kingdom* **15**, 643.
Yonge, C. M. (1928b) *Phil. Trans. Roy Soc.* **B216**, 221.
Yonge, C. M. (1931) *J. Conseil, Conseil Permanent intern. exploration Mer.* **6**, 175.
Yonge, C. M. (1932a) *Sci. Repts. Brit. Museum Nat. Hist.* **1**, 259.
Yonge, C. M. (1932b) *Science Progr.* **26**, 643.
Yonge, C. M. (1935) *J. Marine Biol. Assoc. United Kingdom* **20**, 341.
Yonge, C. M. (1936a) *Sci. Repts. Brit. Museum Nat. Hist.* **1**, 283.
Yonge, C. M. (1936b) *Mém. Museum hist. nat. Belgique* **31**, 77.
Yonge, C. M. (1937a) *Biol. Revs.* **12**, 87.
Yonge, C. M. (1937b) *J. Marine Biol. Assoc. United Kingdom* **21**, 687.
Yonge, C. M. (1938) *J. Marine Biol. Assoc. United Kingdom* **22**, 453.
Yonge, C. M. (1939a) *Phil. Trans. Roy. Soc.* **230**, 79.
Yonge, C. M. (1939b) *Quart. J. Microscop. Sci.* **81**, 367
Yonge, C. M. (1946) *Nature* **157**, 729.
Zacks, S. I., and Welsh, J. H. (1953) *Biol. Bull.* **105**, 200.
Zawansin, A. A. (1929) *Arch. Entwicklungsmech. Organ.* **115**, 1.

Problems of Fixation in Cytology, Histology, and Histochemistry

M. WOLMAN

Department of Pathology, Hebrew University, Hadassah Medical School, Jerusalem, Israel

		Page
I.	Introduction	79
II.	Chemical and Physical Effects of Fixatives on Tissue Constituents	80
	1. Formaldehyde	80
	2. Chromates	82
	3. Osmium Tetroxide	83
	4. Mercuric Chloride	85
	5. Picric Acid	85
	6. Acetic Acid	85
	7. Alcohol and Acetone	86
III.	Effect of Fixation on Living Protoplasm	87
	1. The Structure of Protoplasm	87
	2. Denaturation of Proteins	88
	3. Fixation of Protoplasmic Compounds	89
	4. Fixation of Protoplasmic Structures	91
	5. Good and Bad Fixation	94
IV.	Effects of Fixation on Some Characteristics of Tissue and Cell Constituents	96
	1. Hardness and Elasticity	96
	2. Diffusion	96
	3. Increased Availability of Reactive Groups	97
	4. Mordanting Effect	97
	5. Blocking Effect and Hydrolysis	97
	6. Oxidizing Action	98
	7. Effect on Enzymatic Activities	98
V.	Conclusions	99
VI.	References	99

I. Introduction

This review deals with the general principles and problems of fixation. It is not intended to cover the practical aspects of fixation which can be found in all the standard texts dealing with histological and cytological techniques.

Fixation is an empirical procedure which aims at the permanent preservation of cells and tissues in their native condition. It is obvious that the aim, as stated, is unattainable, as no appearance and composition of a dead structure can be identical with that of a living structure. Still, fixation is a necessity for most morphological studies, because of the many difficulties inherent in the examination of fresh living material.

Living cells of multicellular organisms are usually examined outside their native surroundings and under more or less unphysiological conditions which may cause changes of structure and composition. The scope of the methods of direct examination is limited. Visible light microscopy of living cells will show only differences in light absorption, which in most cases are rather small. Dark field examination will show only those particles which reflect the light. Ultraviolet microscopy, with which differences in absorption are more prominent than with light microscopy, may induce changes in the composition of living cells. Phase contrast microscopy is probably the best method of examining living tissue, as it shows differences in optical density. Electron microscopy cannot be applied to living tissues. None of these methods, when applied to living tissue, can demonstrate structures and compounds which do not differ from their surroundings either by their absorption of light or by their refractive index. Another disadvantage of examination of living tissue lies in the fact that no permanent preparations can be obtained.

Fixed tissues are usually further processed, i.e. embedded, sectioned, stained, and mounted, the procedure varying with the special requirements of the worker. Fixation aims, therefore, also at rendering the tissues and their constituents resistant to these manipulations. The aim of the investigator, whether it is to study special cellular structures, such as mitochondria, or to study the distribution of a chemical constituent, such as an enzyme, will affect the choice of the method of fixation. The choice of fixing procedure requires knowledge of the chemical and physical effects of the fixatives on tissue constituents.

II. Chemical and Physical Effects of Fixatives on Tissue Constituents

Knowledge regarding the chemical effects of fixatives on tissue is rather incomplete. In practice fixatives often contain a mixture of fixing agents which most probably act upon tissues independently and summatively. Only some of the most important simple fixatives will be considered here.

1. Formaldehyde

Formalin, a 38–40% solution of formaldehyde in water, is used as a fixative, usually as a 10% solution.

The effect of formaldehyde on proteins depends on a number of chemical reactions between formaldehyde and various reactive groups of the protein molecule. Blum (1926), who introduced formalin into the histological technique in 1890, suggested that formaldehyde reacts with amide and indole groups, with a resulting formation of methylenic bridges (—CH_2—)

between the protein molecules. Further studies have shown that formaldehyde forms primary additive compounds with amines or other groups containing an active hydrogen atom. The methylol (—CH_2OH) compounds formed may then react with amines or other groupings containing active hydrogen situated on the same or another protein molecule, thus forming a methylene bridge (—CH_2—). It is believed that the second reaction, involving the cross-linking, is responsible for the hardening and fixing effects of formaldehyde. The following chemical groups of protein molecules have been shown to react with formaldehyde: Amine, amide, peptide, imine, guanidine, indole, alcoholic hydroxyls, thiol, disulfide, and carboxyl groups (Meyer, 1929; Fraenkel-Conrat, Cooper, and Olcott, 1945; Nitschmann and Hadorn, 1944; Ratner and Clarke, 1937; Carpenter and Lovelace, 1944; Wormell and Kaye, 1944; Middlebrook and Phillips, 1942; Theis, 1944; Fraenkel-Conrat, and Olcott, 1948a, b; Gustavson, 1943. *Reviews:* Gustavson, 1949; French and Edsall, 1945; Walker, 1953).

Direct evidence showing that the protein molecules are cross-linked by formaldehyde has been adduced by Fraenkel-Conrat and Mecham (1949).

It is known (French and Edsall, 1945) that in the concentrations used for fixation (approximately 4% formaldehyde in water), formaldehyde exists mainly as a monomer, most of it in the hydrated form, as methylene glycol ($CH_2(OH)_2$). Concentrated formalin contains mostly the polymerized form, and the dilution, therefore, involves a depolymerization process which is not instantaneous. Although the final equilibrium does not seem to depend on the pH of the solution, the speed of the depolymerizations increases with higher pH values, being very rapid in the neutral and slightly alkaline ranges. It seems that histologists and cytologists have never paid any attention to this point (although the use of neutralized or buffered solutions has been advocated), but chemists (Gustavson, 1949) noticed some failures in the tanning process which might have been due to insufficient ageing of the diluted formaline solutions.

The possibility of a reaction between polymerized formaldehyde and tissue proteins has apparently never been studied. Such a possibility exists, as Stockinger (1951) has shown that formalin-fixed tissues fluoresce like formaldehyde polymers.

Formaldehyde is a fairly rapidly penetrating fixative (Medawar, 1941). The rapid penetration of formaldehyde and its lattice forming effect on proteins may explain the fact that it delays the diffusion of some macromolecular substances which it does not fix. Glycogen, for instance, is not fixed by formaldehyde, yet fixation in formalin, if not unduly prolonged, does not markedly reduce the amount of glycogen in tissues or disturb its visualization. Early warnings against the use of formaldehyde solutions

for fixing tissues in which glycogen is to be demonstrated, appear exaggerated in the light of modern contributions (Lillie, 1948; Vallance-Owen, 1948; Mowry and Bangle, 1951; Morrione and Mamelock, 1952; Lison, 1953; Wolman and Feingold, 1953).

Information regarding the effect of formaldehyde on lipids is fragmentary. Weil (1929), Mladenovic and Lieb (1929), Kimmelstiel (1929) and Brante (1949) found that solubility characteristics of various lipids changed after formalin fixation. Halliday (1939) found that the iodine number of unsaturated lipids was reduced as a result of fixation in formalin. Wolman and Greco (1952) adduced evidence for the binding of formalin with unsaturated lipids. That formalin fixation causes changes in the chemical composition of some lipids is evident also from the effects of formalin on some staining reactions of lipids. Thus Lorrain-Smith and Rettie (1921) found that some myelin stains were influenced by formalin fixation, whereas Ashbel and Seligman (1949), Hack (1952), Wolman and Greco (1952) and Fetzer (1953) found that formalin caused marked changes in the staining of lipids with carbonyl reagents.

To summarize: formaldehyde combines with proteins forming bridges between adjacent molecules; it probably does not change the solubility characteristics of polysaccharides, but their diffusion is slower because of the effect of formaldehyde on the proteins; it combines with some lipids changing their solubility and staining characteristics.

2. *Chromates*

Potassium dichromate is used as a fixative either in a neutral or in an acid solution. The types of compounds formed under these two conditions, and the morphological results differ (cf. Baker, 1945).

Berg (1926) suggested that fixation by chromates proceeds in two stages, a primary stage which can be reversed by washing, and a secondary stage of hardening in which the bound chromates are reduced to chromic (trivalent) compounds. The extent of the secondary process depends on the reducing capacity of the tissue constituents. Bowes and Kenten (1949) found that the initial reaction of the hexavalent chromium complexes involves carboxylic groups, and to a lesser extent also amino groups. The reaction with the carboxylic groups appears to be essential for stabilization (cf. Gustavson, 1949, for an extensive review). Green (1953) believed that a simple salt formation between chromium and carboxyl would not be sufficient to explain the stabilization of the structures and adduced evidence suggesting that the primary reaction between chromium complexes and carboxylic groups is followed by coordination with amino and hydroxyl groups. It may be summarized, therefore, that primary fixa-

tion by chromates is due to the formation of chromium bridges between protein molecules.

The different results obtained by fixation with chromate in decidedly acid medium on the one hand, and in neutral or slightly acid medium on the other hand, has not as yet been fully explained. It is known that an acid reaction accelerates fixation and tanning (Berg, 1926; Gustavson, 1949). The effect of acidity on chromate tanning has been explained by Gustavson in terms of an increase of size of the chromium complex (which facilitates multipoint attachment to the protein) and of increased availability of ionized carboxyls.

As fixation can be obtained also by chromic compounds, it is not quite clear whether the oxidation of proteins by chromates (i.e. the secondary stage) plays any part in fixation. In view of the known effects of chromates on reducible substances in the so-called chromaffin system, of their effects on lipids, and on polysaccharides (see below), it is logical to assume that oxidation of proteins plays a part in the process of fixation.

Bauer (1933) has shown that chromic acid converts glycogen into a compound insoluble in water. Many lipids are also converted into compounds insoluble in the usual fat solvents by chromate treatment (Lison, 1953). According to Kauffmann and Lehmann (1928), who worked with pure lipids and their mixtures, the insolubilization by chromates extends to all the unsaturated fats. The effect of chromate fixation on polysaccharides and lipids might be due to (a) their capacity to form compounds with aliphatic hydroxyl groups as shown by Green (1953), and (b) their possible combination with unsaturated fats (cf. Lorrain-Smith and Mair, 1911). There is evidence in the literature (cf. Markley, 1947) that the oxidation of lipidic double bonds by acid chromates proceeds via a peroxido or oxido compound to a dihydroxylic intermediate which is usually oxidized further into carboxylic acids.

3. Osmium Tetroxide

Although osmium tetroxide is generally considered to be one of the best or the best fixative for thin tissue slices, little is known of the way it affects tissue constituents. According to Michaelis (1926), Altmann and Starke have shown in the past century that OsO_4 is reduced by unsaturated fats and causes their blackening. The work of Paal and Amberger (1907), Hofmann (1912) and Hofmann, Ehrhart, and Schneider (1913) has demonstrated how OsO_4 and mixtures of OsO_4 with some other oxidizing agents affect many organic compounds. OsO_4 is a strong oxidizing agent, which, once reduced, can be slowly reoxidized by atmospheric oxygen, thus acting as an oxidative catalyst. This function can

be better performed by mixtures of OsO_4 with another oxidizing agent (such as chlorate) which reoxidizes the reduced osmium, keeping the OsO_4 at a constant concentration. OsO_4 and the mixtures of it with other oxidants oxidize both aliphatic and aromatic double bonds, alcoholic hydroxyl groups, and some amines, but usually not the carboxyl and carbonyl groups. Hofmann, Ehrhart, and Schneider (1913) mentioned also a special affinity of osmium towards nitrogenous groups. Oxidation by OsO_4 alone is usually accompanied by a deposition of black reduced osmium, whereas oxidation by an osmium-oxidant mixture does not usually involve blackening, as the reduced osmium is reoxidized. According to Berg (1927) fixation by OsO_4 depends mainly on its affinity towards nitrogenous groups and is not directly linked with its oxidative capacity. Criegee (1936) has shown how osmium tetroxide can form osmic acid diesters by oxidizing two ethylenic linkages of two adjacent molecules with a resulting osmium bridge between the molecules. A similar bridging may occur also between two diols (1,2 glycols). Recently, Porter and Kallman (1953) suggested that osmium fixation might be due to the formation of intermolecular linkages at points containing double bonds. It appears reasonable to assume that such osmium linkages are formed in OsO_4 fixed tissues, although the chances are that ethylenic bonds are not the only points where the links are formed. Alcoholic groups and especially 1-2 glycols, but also sulfhydryl (Wolman, unpublished data) and amino groups are probably also involved in the fixation reactions.

Osmium tetroxide renders most fats insoluble in fat solvents because of the relative insolubility of the esters and diesters formed. Some saturated fats dissolve OsO_4 (Michaelis, 1926), and when they are then transferred to alcohol the fat blackens, probably as a result of reduction of the dissolved OsO_4 by the alcohol. Thus these fats, although unreactive towards OsO_4 can be later visualized by ethanol treatment.

The chemical effect of OsO_4 on glycogen, if any, has not been investigated.

Recent work by Finean (1954) indicated that buffered OsO_4 is an excellent fixative for myelin and that the X-ray diffraction pattern of OsO_4-fixed myelin resembled that of unfixed myelin. Low (1954) has shown that short fixation of tissues in buffered OsO_4 is optimal for electron-microscopy, while longer periods produce distortion and disintegration. Low's data might indicate that the bridging effect occurs first, while further oxidation of the tissue by OsO_4 is detrimental.

4. *Mercuric Chloride*

Mercuric ion, being a bivalent heavy metal capable of combining with two different protein molecules and bridging them, is a powerful protein precipitant if used in concentrated solutions. Hughes (1950) has shown that SH groups of proteins bind mercuric ions. In some cases Hughes (1947) could demonstrate the formation of mercury bridges between two molecules of protein. Klotz, Faller, and Urquhart (1950) have shown that cupric compounds (whose behavior is probably similar to that of mercuric ions) are bound in acid pH ranges mainly by carboxylic groups. In neutral and alkaline solutions copper is bound mainly by the amino groups.

It may be concluded, therefore, that mercuric ions form intermolecular mercury links between SH groups, carboxyl groups, and amino groups, the sites of the links depending to a great degree on the pH of the solution.

5. *Picric Acid*

Baker (1945) stated that the fixing action of picric acid differs in its nature from that of other fixatives, and considers it probable that the anion combines with proteins, forming protein picrates. The same author quotes a personal communication from Holmes who found that 2,4-dinitrophenol, and *p*-nitrophenol behave differently from picric acid and do not precipitate proteins. It is obvious that the fixing (and precipitating) action of picric acid does not depend only on the hydroxyl group.

Picric acid is known to form additive, usually insoluble, compounds with many organic bases and also with relatively inert aromatic hydrocarbons. There is good evidence in the literature (Anderson and Hammick, 1950; Baddar and Mikhail, 1949) that the 2,4,6-nitro groups can act as inducing dipoles, and that a further increase of activity is due to the presence of several resonance states of the picric acid molecule. Thus, weak electrostatic forces may cause the bridging of protein molecules by picric acid, with part of the bonds being due to ionic forces (phenolic OH) and others being due to the induced polarity in the many polarizable moieties of the protein molecules. A partial explanation of the differences between the effects of picric acid and of the other nitrophenols, as observed by Holmes, can be found in the article of Teresi and Luck (1948).

6. *Acetic Acid*

Baker (1945) has shown that acetic acid precipitates nucleoproteins but not albumin. Von Tellyesniczky (1926) found that acetic acid coagulates the nuclear proteins but dissolves most of the cytoplasmic ones. Lassek (1950) reported that maximum precipitation of emulsions of liver

and brain by various fixatives are obtained at an acid pH. It seems obvious that acetic acid affects fixation of those proteins the isoelectric point of which lies near the pH value of the acid solution employed (i.e. mainly of nucleoproteins and mucoproteins). The mechanism of fixation would be similar in this case to the mechanism of precipitation. The bound water layer, which exists normally around the ionized groups of the protein molecules, disappears with the loss of the electric charge. The protein molecules can form, therefore, new cross-links as the reactive points get much nearer than before. Acetic acid does not seem to affect lipids and carbohydrates except for those which are soluble in it.

Acetic acid dissolves some metals. Thus, obviously, fixatives containing acetic acid are not suitable for material in which small precipitates of calcium are to be demonstrated.

7. *Alcohol and Acetone*

The old view (von Tellyesniczky, 1926) in which fixation of proteins by alcohol or acetone is due simply to replacement of water by the solvent (in which the proteins are insoluble) is untenable. Modern workers using the freeze-drying technique (cf. Simpson, 1941; Bell, 1952) found that treatment by ethanol was necessary for rendering the dried sections resistant to water. Seki (1937) remarked justly that the air-drying of blood films does not render the blood insoluble in water, whereas fixation in alcohol does. Seki explained the effect of these fixatives by (a) the reduction of the electric charge on the active hydrated groups, and (b) the lowering of the dielectric constant, which allows for a stronger reciprocal attraction between the protein molecules.

Although it is clear that Seki's explanation of the effects of these less polar fixatives explains most available data, there might be an additional way in which these substances fix proteins. Lipids constitute about one-third of the total dry mass of cells, most of them being firmly bound and making up part of lipoprotein molecules (Claude, 1949). Kendall (1941) found that crystalline serum albumin is associated with a free fatty acid which cannot be extracted without denaturing the albumin. Cohn, Hughes, and Weare (1947) found that serum albumins in the crystalline state contain fatty acids whose removal made crystallization difficult. The addition of sodium oleate or stearate improved crystallizability. Haurowitz (1950) mentions the fact that fatty acid anions prevent the coagulation of proteins denatured by heat or by other methods. It seems probable that the presence of some lipids bound to protein molecules by their hydrophilic group neutralizes some active, charged, molecular groups which are covered by relatively inert lipid moieties. The dissolution of the lipidic

groups with the subsequent unmasking of the charged groups might render the proteins more reactive and cause them to attract each other and form new cross links.

Recently Folch and Lees (1951) described a new type of proteins, the proteolipides which are soluble in fat solvents and are found in all tissues. The proteolipides might be expected to be extracted by some nonpolar fixatives or by subsequent treatment of the material by fat solvents. Thus, nonpolar fixatives can no longer be considered to be preservers of all proteins.

III. Effect of Fixation on Living Protoplasm

Fixation changes the chemical constitution, and often the localization of chemical constituents of cells and tissues and, therefore, also their morphology. In order to understand these changes, a short review of the structure of protoplasm is necessary.

1. The Structure of Protoplasm

The old controversy about the submicroscopic structure of protoplasm seems to have been settled, and most authorities agree that living protoplasm has an organized structure for which terms such as vacuolar, micellar are not quite adequate. The basis of this structure is chainlike protein molecules. These elongated protein molecules (fibrous or structural proteins) are held together at different points, giving the protoplasm a "structural continuity" (Seifriz, 1938) and elasticity. These molecules contain many different active groups at the surface and side chains. Some of the side groups serve as junctions between molecules, others are bound to lipids, carbohydrates, or other substances, while many are surrounded by bound water (Frey-Wyssling, 1953); Bensley (1943) expressed this concept as follows: "Protoplasm, however, does not possess either microscopic or submicroscopic homogeneity. Even when it seems optically structureless it may conceal behind the mask of simplicity those local differences of organization which express themselves in determinate cleavage." The relative rigidity of the protoplasm is not caused most probably by any fixed links, but rather by links which change constantly, with fission of some and reconstruction of others, in accordance with metabolic cell functions (Frey-Wyssling, 1953). Examples of changes in the amount of linking between molecules which occurred in sea urchin eggs as a result of fertilization were reported by Mirsky (1936) and by Moore and Miller (1937). These authors found that fertilization induced the protoplasmic proteins to form cross links and thereby a fibrous multimolecular network with birefringent properties.

The presence of an organized structure had to be surmised not only to explain the elasticity and plasticity of protoplasm, but also for most of its biological functions. Höber (1950) recognized the presence of chemical organization in areas where the protoplasm appears homogeneous. A spatial configuration is in reality a *sine qua non* condition for any biological activity in which a multi-enzyme system is doing any useful work. Without it, all secretion products would never be secreted, biochemical reactions would go uselessly back and forth, and the cell would behave similarly to a brei in which reactants and reaction products are all mixed up. A nice example of the necessity to assume a morphological structural arrangement of a series of different enzyme protein molecules, which have to be presumed to lie in a laminated fashion, layer upon layer, is given by Patterson and Stetten (1949) who attempted to explain gastric secretion of HCl.

Between the meshes of the loose structural network there exists a large number of other molecules. A large number of mobile or globoid proteins can be easily extracted from the cells (Bensley, 1943). These proteins probably represent chains which are more or less folded. There might exist multiple reversible changes of folding and unfolding of proteins (Seifriz, 1942).

The water of protoplasm is partly bound to the polar groups of the structural proteins, and, in part, is free or attached to mobile molecules. There is general agreement that the amount of water bound to proteins is about one-third of the weight of the protein mass (Sponsler, 1940; Edsall, 1949). Crystalloids, carbohydrates, and fats are found in the cytoplasm either bound to the structural proteins or to the globular ones, or else free in or out of solution in the unbound water. Figure 1 represents a scheme of the structure of protoplasm.

2. *Denaturation of Proteins*

Fixation is known to denature proteins. Apart from the etymological concept which indicates change of characteristics, denaturation was classically considered (Mirsky, 1938) as a change in the characteristics of solubility, viz. loss of solubility in water. Denaturation is usually associated also with changes in many other properties of the protein, such as crystallizability, serologic reactivity, enzymatic activity, chemical, and physical characteristics (Mirsky, 1938; Bull, 1938). It is generally assumed that denaturation is due to unfolding of the globular proteins through disruption of some of the forces which hold the chains closely folded, and subsequent formation of new links between the molecules with a resulting flocculation or coagulation (Mirsky, 1938; Moyer, 1942; Ferry *et al.*,

1947; Haurowitz, 1950). Active groups, which are unavailable for reactions in the folded molecule, become available for cross linkages, and also for reacting with other compounds when in the denatured state.

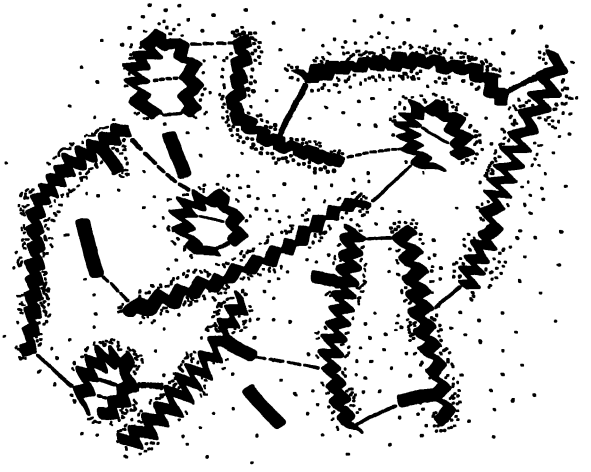


FIG. 1. A schematic representation of the structure of protoplasm. The thick chains represent protein molecules, the fibrous ones being linear, the curved ones representing globoid proteins. The thick straight lines represent fats and fatty radicals. The dots represent water and crystalloids The thin lines represent intra- and intermolecular bonds.

It should be emphasized that denaturation is not a single, uniform process. A protein may be denatured in many different ways by using different denaturing agents. Furthermore, not all of the changes which are considered to be typical for denaturation must proceed to the same extent. It would be probably better not to speak of denaturation generally, but rather of denaturation of certain specific properties of the proteins. A short fixation with alcohol, for example, will denature the solubility properties of some proteins, but it will not destroy a great part of the activity of alkaline phosphatase. To the writer's mind, references in the literature to fixing agents which do not denature the proteins which are being fixed (statements which are based on the preservation of a single characteristic in which the worker was interested, as for example enzymatic activity), are misleading.

3. *Fixation of Protoplasmic Compounds*

Fixation of any molecular constituent of the cell involves its linking to other molecules so that, unless its bonds are broken, it cannot move or

be dissolved away from its place. Fixation, therefore, invariably involves chemical and physico-chemical changes of the fixed compounds.

Fixation of tissue may or may not be attended by fixation (i.e. formation of cross links) of the molecules chosen for study. In the first case it is necessary that the fixed molecules should still contain some characteristic chemical groups which may serve for their visualization. Fixed enzyme molecules should therefore still exhibit the specific activity, if it is intended to visualize them. In the second case, when the studied molecules are not directly affected by the fixative, a good fixation of the other tissue constituents often helps in the preservation of the studied compound. Thus, neutral triglycerides may be demonstrated in unfixed tissue or in tissue fixed by watery fixatives. This seems to be the case also in the demonstration of glycogen after fixation by agents which do not directly affect the compound (Lison, 1953). The same principle, i.e. insolubilization of surrounding constituents applies also to the fixation of small molecules in the cells by rapid freezing or to the fixation of glycogen by the same method (Mancini, 1949). In these cases the only really fixed phase is water.

For the demonstration of most substances fixation is a necessity, and, provided the worker understands the mechanism of his fixation and staining procedures, it should not be considered as an evil to which one must submit unwillingly. All the known methods of visualizing compounds depend on the presence of some specific molecular groups which can be recognized by their chemical affinities, absorption spectra, or other characteristics. This general statement applies even to molecules characterized by their antigenic features, as for example in Coons' experiments (cf. Coons, Leduc, and Kaplan, 1951) and to a large degree also to solubility characteristics. The attitude, often adopted by histochemists and histologists, who prefer to use fixatives which are not very reactive from the chemical point of view, such as alcohol for example, is usually based on a fallacy. The chemical change caused by the fixative, whether it is due to a combination with the fixative molecule (as with $HgCl_2$), or is caused by the fixative without combination of the fixing agent with the studied molecules (such as alcohol and even more obviously in fixation by heat), results in the bonding together of different molecular groups. If it is intended to study any given reactive groups of a compound, one should first find out what effect the different fixing agents have on these groups. If it is the intention to study the distribution of reactive SH groups in tissue, it should be realized that most, if not all fixatives, through their denaturing effect, increase the availability of these groups, and furthermore that fixatives which might change these compounds (such as $HgCl_2$, OsO_4, chromates) should be avoided. It is obvious that in such a study attention

should also be paid to the solubility characteristics of the different compounds containing the reactive group.

It may be concluded that there are no ideal fixatives for histochemistry or chemically inert fixatives. There are better and worse fixatives for the demonstration of any given molecular group. The consideration of alcohol as an "ideal fixative" for chemical studies (Schiller, 1930), or as a chemically indifferent agent (Zeiger, 1930; Lison, 1953), the notion that formaldehyde, OsO_4 and chromates hardly affect the valence and molecular interrelationship of tissue constituents (Bretschneider, 1952) and even the notion that "freeze-drying is the best fixation method for use in cytochemistry" (Danielli, 1950), cannot be regarded as accurate. All these agents cause chemical changes in protein, and the choice of any one of them should depend on the specific aim of the investigator.

The process of fixation is complicated by the fact that fixing agents need time to reach the different tissue structures. The rate of penetration of chemical fixatives has been studied extensively by Mann (1902), von Tellyesniczky (1926), Medawar (1941), Baker (1945) and others. Most workers concluded from their results that fixation of the external layers interfered with the penetration of fixatives (membranogenous activity, Tellyesniczky). Although theoretical considerations render the possibility of a barrier created by the fixed external layer plausible, as penetration through a finely interlinked mesh would be expected to be slower than through a looser mixture, Medawar (1941) found that the penetration of liquid fixatives into a plasma clot obeys the physical laws of diffusion through a homogeneous system.

The speed of penetration and of action of the fixing agent is of primary importance. The cells or tissues which are subjected to the action of a fixative are changing continuously in composition before having actually been fixed. Furthermore, there are some indications that before fixation is complete cells undergo violent changes (Buchsbaum, 1948; Crawford and Barer, 1951). One of the great advantages of freeze-drying, as used today, is the speed with which the freezing of the tissue takes place.

4. Fixation of Protoplasmic Structures

Cytologists who examine fixed specimens microscopically, debated a great deal whether the structures seen "really existed" before fixation. The usual criterion for "real existence," which has apparently been accepted by almost all the workers in the field, was the possibility of recognizing the same structures in the unfixed state. A good example of such a controversial issue in the literature for the past 50 years is given by the problem of structure of the interphase nucleus. Von Tellyesniczky (1926)

who observed cell nuclei mainly with the light microscope, maintained that the long use of 1% acetic acid as a fixative caused errors in the understanding of the nuclear structure. In his opinion, acetic acid caused precipitation of the nucleoproteins which in the fresh state were simply in solution. Von Tellyesniczky stated that "the importance of the examination of living cells and tissues cannot be stressed enough." Zeiger (1935), who found that the position of nucleoli within nuclei did not change during many hours of exposure to fixatives, believed that the optical homogeneity of the nucleus does not necessarily mean that the resting nucleus is a homogeneous solution. The possible existence of an invisible skeleton which would cause the appearance of a "Vitalartefact" in the sense of von Herwerden (an artifact caused by a pre-existing molecular arrangement) was proposed. Pischinger (1937) found that interphase nuclei were empty when examined both with the light and the dark-field microscopes and confirmed the observation of von Tellyesniczky regarding the homogeneous appearance of nuclei fixed by OsO_4. He therefore postulated the existence of completely (not only optically) homogeneous nuclei. By the use of phase microscopy Buchsbaum (1948) and Zollinger (1948) found that a chromatic network was apparent in unfixed interphase nuclei. Similar results were obtained by Ludford and Smiles (1954) with ultraviolet-microscopy of living cells. Ris and Mirsky (1949) found that chromosomes exist in the interphase nuclei, that they can be isolated from them, and that their invisibility under the light microscope is probably due to their swollen (hydrated?) state.

It is apparent from the history of this debate that the criteria of "real existence" were different at different times and depended very much on the techniques available. The limitations of the examination of cells and tissues in the fresh state have been discussed in the first paragraph of this paper. A structure which is colorless and does not differ from its surroundings in its refractive index and density cannot be recognized in the fresh state. Furthermore, a structure may have many different forms in accordance with the criteria used for its identification. Fig. 2 represents a case in point. Supposing a certain well delimited structure as represented in Fig. 2A, which is made of hydrated molecules containing certain reactive groups spread at random, and other reactive groups (●) at one of the ends of the molecules. While the molecules are arranged in a haphazard manner, the '●' groups are arranged in a line. That structure might, or might not, be visible under the light and the phase microscopes, according to its physical properties. Assuming the possibility that fixation did not affect markedly the structural pattern and the studied reactive groups of these molecules, we may now demonstrate the structure by two means.

The molecules may be demonstrated by visualizing the first group, in which case the structure will have a certain irregular form, Fig. 2C. If, on the other hand, the "•" groups were visualized, the structure will appear as in Fig. 2B, rectilinear. This imaginary structure (which in fact

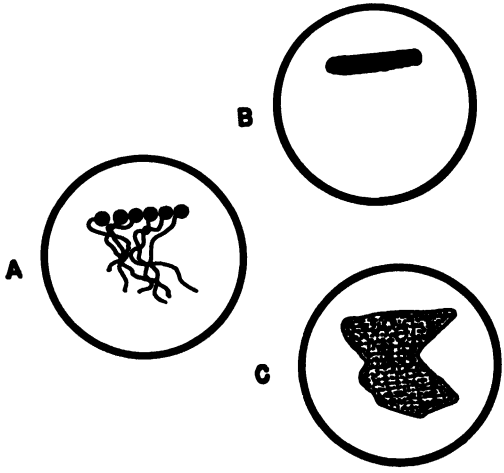


FIG. 2. A schematic representation of a hypothetical cellular structure. A: The arrangement of the molecules constituting the structure. B: Visualization of the structure by reactive groups (•) found at one end of the molecules. C: Visualization of the structure by reactive groups spread at random in the molecules.

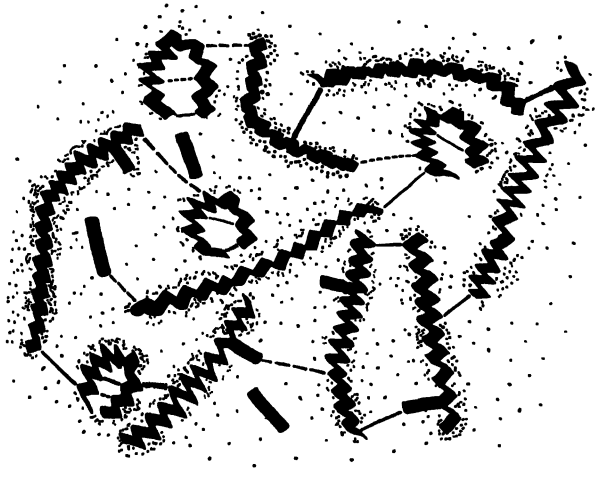
might not be too far off the truth in the case of membranes and cellular organelles) might appear invisible under the light and the phase microscopes, have a polygonal appearance after one fixation and staining procedure, and appear linear in another. One has to admit that many scientists would have called both appearances "artifacts". This scheme can be applied with many variations (such as differences between appearances due to different refractive indices without differences in density, or vice versa, differences in density which are masked by marked hydration and which can be unmasked by dehydration, etc.) to a great number of organelles the real structure of which is debated in histology. The answer seems to be that a "really existing" form may have at the same time different forms.

The foregoing discussion should not be considered to indicate that all methods of fixation are good and that all artifacts represent the true structure of protoplasm. Many proteins in solution can be precipitated by fixatives to form granules and fibrils which represent only the forms in which these substances are precipitated or crystallized.

5. *Good and Bad Fixation*

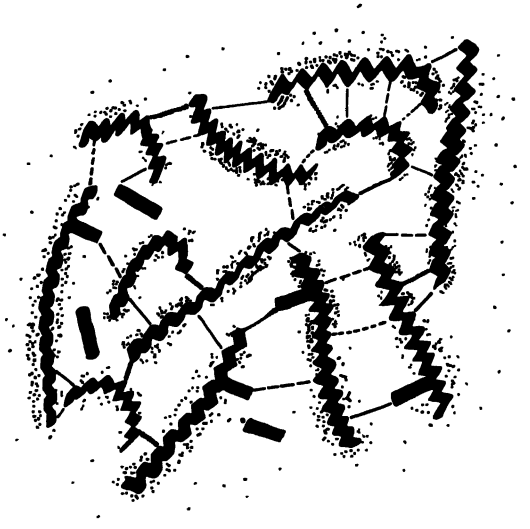
The criteria for good fixation are somewhat different for cytology and histology on the one hand and histochemistry on the other. It should be emphasized, however, that a *bad* histological fixative is never good for histochemistry as gross distortions of cellular structures will result in displacement of the studied chemical compounds. Thus a moderately good histological fixation is a *sine qua non* also for histochemistry.

From the morphological standpoint a good fixative is one which causes the least distortion of the tissues, cells, and cellular constituents. Although fixation invariably involves formation of new cross links between the tissue molecules, and therefore the operation of new forces of attraction between these particles, the energy of these forces is probably different in the various cases. If new links are formed, which cause the clumping together of the particles, the result will be a bad fixation. (An example of bad fixation is fixation by boiling water or by strong precipitating agents). This strong bonding of particles will result in the formation of irregular coacervates and between them empty vacuoles (Fig. 3c). It seems probable that morphologically good fixation is the result of action of agents which cause the formation of many links between the molecules, provided that the attractive forces which operate in the linking are not too strong

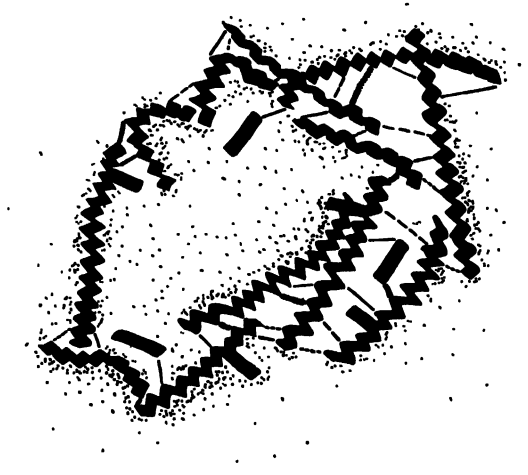


A

FIG. 3. A schematic representation of good and bad fixation. A: The arrangement of the molecules in the native state. B: Good fixation—formation of cross linkages without extreme displacement of the molecules. C: Bad fixation—formation of cross linkages with separation of a liquid phase from a coarse coacervate.



B



C

(Fig. 3b). There seem to be some exceptions to this statement. A good fixation of some highly hydrated structures (such as chromosomes), which are probably already linked together in relatively immobile units, involves usually coarse precipitation with shrinkage and dehydration of the structures. It is obvious, therefore, that fixatives can be good for certain structures and bad for others.

If microscopy is taken to include all the possibilities from the lowest power to electron microscopy, it is clear that all fixatives cause artifacts. Fixation is always attended by some displacement of structures, although one can try to keep these displacements beyond the resolving power of the optics used (Danielli, 1953). Another approach which seems to be sound, is that of Nissl (1910), who considered fixation as an artifact and proposed to standardize it. By using organs of animals killed in a definite way and processed in an exactly similar manner Nissl obtained comparable "Aequivalentbilder."

It may be safely concluded that fixation always creates structural artifacts, that a fixation is good when these artifacts occur mainly beyond the resolving power of the optical system, and that the scientist is good if he realizes that he works with "Aequivalentbilder."

IV. Effects of Fixation on Some Characteristics of Tissue and Cell Constituents

Many of the physical and chemical characteristics of tissues are changed as a result of fixation.

1. Hardness and Elasticity

The increased hardness and the often encountered decrease of elasticity are due to the formation of cross links between the molecules and to the unfolding and subsequent linking of the globular proteins (cf. Bjorksten, 1951). It is interesting to note that Petry (1951) found that the elasticity of elastic fibers is not markedly affected by formalin fixation. This fact might be due to an inability of the elastic molecules which does not allow formation of interfibrillar cross links. The phenomena of cross-linking and unfolding are also responsible for the insolubilization of many tissue constituents.

2. Diffusion

Fixation is always attended by diffusion and loss of some chemical constituents. In the freeze-drying procedure this is minimal and occurs (providing the eutectic point of the cytoplasm is not reached during drying) for a very short time until the tissue is frozen. In the other procedures diffusion and dissolution occur both before the fixative reaches the studied area and afterwards. The late diffusion might be due to the fixative itself (e.g. dissolution of fats in alcohol), or to the lack of fixation of the compound. This fact seems not to have been appreciated to its full extent by many research workers. Not all the proteins are insolubilized by fixatives, not even by those which are known as protein precipitants. Many low molecular weight proteins might remain soluble in water when

the tissue is treated by the usual fixatives. Alcohol might actually solubilize the proteolipides. It is not surprising, therefore, that Bell (1952) found that freeze-drying preserved certain cellular structures which were not preserved by fixation in Carnoy's solution.

3. *Increased Availability of Reactive Groups*

Fixation *per se*, involving the unfolding of globular proteins, results in an increased availability of reactive groups of the protein molecules. This causes an increased stainability by acid and basic dyes, as well as increased reducing activity which can be visualized by the metallic impregnation methods. The various fixatives affect differently the binding capacity of acid and basic dyes. Fixatives which affect the basic groups of protein molecules will shift the isoelectric point to the acid range, thus decreasing acidophilia (e.g. formalin). On the other hand, fixatives which bind mainly the acid groups will render the tissues less basophilic (e.g. bichromate, picric acid). The relationship between staining characteristics and the isoelectric point has been studied by many workers (Seki, 1933; Singer and Morrison, 1948, and others) and thoroughly reviewed by Singer (1952). Seki also suggested that fixatives might affect the staining capacity of tissue elements by changing their density and porosity. He considered the possibility of a disturbed penetration of stains of high molecular weight into denser structures. It should be added that a change in density, for example a concentration of a structure by loss of bound water, will cause a stronger and more distinct staining even when the total number of the dye binding groups remains unchanged.

4. *Mordanting Effect*

Fixatives which cause the formation of cross-linkages by the binding of adjacent tissue molecules to the molecules of the fixative itself, often exert a mordanting effect. This effect is due to the possible binding of dye molecules to additional valencies of the fixative (e.g. chromates).

5. *Blocking Effect and Hydrolysis*

Besides the effect on the acid and basic groups of proteins, fixatives often exert a blocking effect on other reactive groups of tissue molecules. $HgCl_2$ for example will block SH groups. On the other hand, this same reagent, as well as most acid fixatives, hydrolyzes some tissue components with the liberation of new active radicals, as for example in the hydrolysis of plasmalogen to plasmal (Feulgen and Bersin, 1939), or in the effect of formalin-ammonium-bromide on glial elements (Wolman, unpublished data).

6. *Oxidizing Action*

Some fixatives exert an oxidizing action on tissue constituents. OsO_4 and chromates are reduced during fixation, and the oxidizing effect may go on after primary fixation has been achieved. Many histological procedures are based on the formation of new radicals as a result of oxidative fixation. For example, the Golgi procedure for elements of the central nervous system is based on the formation of reducing groups in tissues, probably as a result of oxidative cleavage. Some oxidative changes occur in nonoxidizing fixatives because of the oxygen dissolved in the fixing solutions. Unsaturated lipids fixed in aqueous solutions of formaldehyde may be oxidized to aldehydes and give the so-called "pseudo-plasmal" reaction (Verne, 1929; Gomori, 1942; Cain, 1949).

7. *Effect on Enzymatic Activities*

The effect of fixatives on enzymatic activities is of a great theoretical and practical importance. From a theoretical standpoint the main problem in most cases is that of finding the conditions of optimal cross linkages with consequent insolubilization of the enzyme molecules, without affecting markedly the active groups. In the special case of structural enzymes the fixation is directed less to the enzyme molecules themselves (as their positions are fixed anyway) but rather to the other tissue constituents which have to be processed without undue shifting, and made microscopically recognizable. In both cases, unless the necessary cofactors are also fixed near the enzyme molecules, they have to be supplied from the outside during the development of enzymatic activities. It is tempting to speculate that in the histochemical demonstration of succinic dehydrogenase, the often observed failing of thin tissue sections and the lack of staining of the outer layers of tissue blocks (Seligman and Rutenburg, 1951; Shelton and Schneider, 1952; Foraker, Denham, and Johnston, 1953) is due to diffusion out of the tissue of a cofactor which has not yet been identified. The problem of fixation in well organized multi-enzyme systems is further complicated by the fact that the inhibition of one member of the series might cause the loss of activity of the whole system. The same applies to structural disruption of the continuity between the different enzymes, which might cause loss or decrease of activity. In the case of the oxidative enzymes of mitochondria, fixation presents a major difficulty. None of the known fixatives will preserve dehydrogenase activity. Even freezing, and especially rapid freezing (which is best for the preservation of cellular details) destroys a great part of activity, probably through mechanical disruption (Porter *et al.*, 1953).

The dilemma of a choice of fixative which would both fix the tissues

reasonably well for microscopical examination, and yet preserve a great part of the enzymatic activity has not yet been solved satisfactorily for the hydrolytic enzymes either. Relatively good results for some enzymes have been obtained by Wolman and Behar (1952) who used acetic acid-alcohol mixtures at temperatures somewhat below zero.

Emmel (1946), Wang and Grossman (1949) and Berenbom, Yokoyama, and Stowell (1952) have shown that a much greater percentage of some enzymes was destroyed by chemical fixation than by freeze-drying. Many authors believe, therefore, that freeze-drying is the ideal method of preparing material for the histochemical study of enzymes. It should be noted, however, that the method is far from being ideal for the two following reasons: (a) it does not give satisfactory results as far as morphology is concerned in a number of organs (brain, adrenals), and (b) in the unfixed tissue the chances of diffusion of enzyme molecules are considerable. For practical reasons most workers still prefer to use chemical fixation in the study of enzymatic activities.

A major objection to the use of chemical fixation in histochemical enzyme studies stems from the work of Maengwyn-Davies and Friedenwald (1950). The results of this interesting work seem to indicate that the specificity of phosphatases is lost as a result of fixation and preparation of paraffin sections.

V. Conclusions

Fixation involves the linking together of molecules of the fixed tissue compounds and is, therefore, always accompanied by morphological and chemical change. A good morphological fixation is one in which the changes occurring in the structures studied occur beyond the resolving power of the optics used. Different appearances of the same structures studied by different methods do not necessarily mean that one of the methods elicits the formation of artifacts. A good histochemical fixation is a good morphological fixation in which the chemical groups studied can still be recognized by the appropriate reagents.

There are no good universal fixing agents any more than there are universally good stains. The fixing agent must be chosen according to the use which is to be made of the fixed specimen.

VI. References

Anderson, H. D., and Hammick, D. L. (1950) *J. Chem. Soc.* **2**, 1089.
Ashbel, R., and Seligman, A. M. (1949) *Endocrinology* **44**, 569.
Baddar, F. G., and Mikhail, H. (1949) *J. Chem. Soc.* **4**, 2927.
Baker, J. R. (1945) "Cytological Technique." Methuen, London.
Bauer, H. (1933) *Z. mikroskop. anat. Forsch.* **33**, 143.
Bell, L. G. E. (1952) *Intern. Rev. Cytol.* **1**, 35.

Bensley, R. R. (1943) *in* "Frontiers in Cytochemistry." Cattell Press, Lancaster.
Berenbom, M., Yokoyama, H. O., and Stowell, R. E. (1952) *Proc. Soc. Exptl. Biol. Med.* **81**, 125.
Berg, W. (1926) *in* "Enzyklopaedie der Mikroskopischen Technik" (Krause, ed.), Vol. 1, p. 362. Urban and Schwarzenberg, Berlin.
Berg, W. (1927) *in* "Enzyklopaedie der Mikroskopischen Technik" (Krause, ed.), Vol. 3, p. 1742. Urban and Schwarzenberg, Berlin.
Bjorksten, J. (1951) *Advances in Protein Chem.* **6**, 343.
Blum, F. (1926) *in* "Enzyklopaedie der Mikroskopischen Technik" (Krause, ed.), Vol. 2, p. 791. Urban and Schwarzenberg, Berlin.
Bowes, J. H., and Kenten, R. H. (1949) *Biochem. J.* **44**, 142.
Brante, G. (1949) *Acta Physiol. Scand.* **18**, Suppl. 63.
Bretschneider, L. H. (1952) *Intern Rev. Cytol.* **1**, 305
Buchsbaum, R. (1948) *Anat. Record* **102**, 19.
Bull, H. B. (1938) *Cold Spring Harbor Symposia Quant. Biol.* **6**, 140.
Cain, A. J. (1949) *Quart. J. Microscop. Sci.* **90**, 75
Carpenter, D. C., and Lovelace, F. E. (1944) *Ind. Eng. Chem.* **36**, 680.
Claude, A. (1949) *Advances in Protein Chem.* **5**, 423.
Cohn, E. J., Hughes, W. L., Jr., and Weare, J. H. (1947) *J. Am. Chem Soc.* **69**, 1753.
Coons, A. H., Leduc, E. H., and Kaplan, M. H. (1951) *J. Exptl. Med.* **93**, 173.
Crawford, G. N. C., and Barer, R. (1951) *Quart J. Microscop. Sci.* **92**, 403.
Criegee, R. (1936) Ann. **522**, 75.
Danielli, J. F. (1950) *Cold Spring Harbor Symposia Quant. Biol.* **14**, 32.
Danielli, J. F. (1953) "Cytochemistry: A Critical Approach" Wiley, New York.
Edsall, J. T. (1949) *Fortschr. chem. Forsch.* **1**, 119.
Emmel, V. M. (1946) *Anat Record* **95**, 159.
Ferry, J. D., Singer, M., Morrison, P. R., Porsche, J. D., and Kutz, R. L. (1947) *J. Am. Chem. Soc.* **69**, 409.
Fetzer, S. (1953) *Mikroskopie* **8**, 189.
Feulgen, R., and Bersin, T. (1939) *Z. physiol. Chem.* **260**, 217
Finean, J. B. (1954) *Exptl. Cell Research* **6**, 283.
Folch, J., and Lees, M. (1951) *J. Biol. Chem.* **191**, 807.
Foraker, A. G., Denham, S. W., and Johnston, M. H. (1953) *Arch. Pathol.* **55**, 147.
Fraenkel-Conrat, H., and Mecham, D. K. (1949) *J. Biol. Chem.* **177**, 477.
Fraenkel-Conrat, H., and Olcott, H. S. (1948a) *J. Am. Chem. Soc* **70**, 2673.
Fraenkel-Conrat, H., and Olcott, H. S. (1948b) *J. Biol. Chem.* **174**, 827.
Fraenkel-Conrat, H., Cooper, M., and Olcott, H. S. (1945) *J. Am Chem. Soc.* **67**, 950.
French, D., and Edsall, J. T. (1945) *Advances in Protein Chem.* **2**, 277.
Frey-Wyssling, A. (1953) "Submicroscopic Morphology of Protoplasm." Elsevier, Houston.
Gomori, G. (1942) *Proc. Soc. Exptl. Biol. Med.* **51**, 133.
Green, R. W. (1953) *Biochem. J.* **54**, 187.
Gustavson, K. H. (1943) *Kolloid-Z.* **103**, 43.
Gustavson, K. H. (1949) *Advances in Protein Chem.* **5**, 353.
Hack, M. H. (1952) *Anat. Record* **112**, 275.
Halliday, N. (1939) *J. Biol. Chem.* **129**, 65.
Haurowitz, F. (1950) "Chemistry and Biology of Proteins." Academic Press, New York.

Höber, R. (1950) "Physical Chemistry of Cells and Tissues." Blakiston, Philadelphia.
Hofmann, K. A. (1912) *Ber.* **45**, 3329.
Hofmann, K. A., Ehrhart, O., and Schneider, O. (1913) *Ber.* **46**, 1657.
Hughes, W. L., Jr. (1947) *J. Am. Chem. Soc.* **69**, 1836.
Hughes, W. L., Jr. (1950) *Cold Spring Harbor Symposia Quant. Biol.* **14**, 79.
Kauffmann, C., and Lehmann, F. E. (1928) *Virchow's Arch.* **270**, 360.
Kendall, F. E. (1941) *J. Biol. Chem.* **138**, 97.
Kimmelstiel, P. (1929) *Z. physiol. Chem.* **184**, 143.
Klotz, I. M., Faller, J. L., and Urquhart, J. M. (1950) *J. Phys. and Colloid Chem.* **54**, 18.
Lassek, A. M. (1950) *Anat. Record* **107**, 409.
Lillie, R. D. (1948) "Histopathologic Technic." Blakiston, Philadelphia.
Lison, L. (1953) "Histochimie et Cytochimie Animales." Gauthier-Villars, Paris.
Lorrain-Smith, J., and Mair, W. (1911) *J. Pathol. Bacteriol.* **15**, 179.
Lorrain-Smith, J., and Rettie, T. (1921) *J. Pathol. Bacteriol.* **24**, 367.
Low, F. N. (1954) *Anat. Record* **120**, 827.
Ludford, R. J., and Smiles, J. (1954) *J. Roy. Microscop. Soc.* **73**, 173.
Maengwyn-Davies, G D, and Friedenwald, J. S. (1950) *J. Cellular Comp. Physiol.* **36**, 421.
Mancini, R. E. (1949) *Rev. assoc. méd. Argentina* **63**, 653.
Mann, G. (1902) "Physiological Histology." Clarendon Press, London.
Markley, K. S. (1947) "Fatty Acids, Their Chemistry and Physical Properties." Interscience, New York.
Medawar, P. B. (1941) *J. Roy. Microscop. Soc.* **61**, 46.
Meyer, K. H. (1929) *Biochem. Z.* **208**, 1.
Michaelis, L. (1926) *in* "Enzyklopaedie der Mikroskopischen Technik" (Krause, ed.), Vol. 1, p. 723. Urban and Schwarzenberg, Berlin.
Middlebrook, W. R., and Phillips, H. (1942) *Biochem. J.* **36**, 294.
Mirsky, A. E. (1936) *Science* **84**, 333.
Mirsky, A. E. (1938) *Cold Spring Harbor Symposia Quant Biol.* **6**, 150.
Mladenovic, M., and Lieb, H. (1929) *Z. physiol Chem.* **181**, 221.
Moore, A. R., and Miller, W. A. (1937) *Proc. Soc. Exptl Biol. Med.* **36**, 835
Morrione, T. G., and Mamelock, H. L. (1952) *Am. J. Pathol.* **28**, 497.
Mowry, R. W., and Bangle, R., Jr. (1951) *Am. J. Pathol.* **27**, 611.
Moyer, L. S. (1942) *in* "A Symposium on the Structure of Protoplasm." Iowa State College Press, Ames.
Nissl, F. (1910) *in* "Enzyklopaedie der Mikroskopischen Technik" (Erlich *et al.*, eds.), Vol. 2, 2nd Ed., p. 268. Urban and Schwarzenberg, Berlin.
Nitschmann, H., and Hadorn, H. (1944) *Helv. Chim. Acta* **27**, 299.
Paal, C., and Amberger, C. (1907) *Ber.* **40**, 1907.
Patterson, W. B., and Stetten, D., Jr. (1949) *Science* **109**, 256.
Petry, G. (1951) *Z. Zellforsch. mikroskop. Anat.* **36**, 338.
Pischinger, A. (1937) *Z. Zellforsch. mikroskop. Anat.* **26**, 249.
Porter, K. R., and Kallman, F. (1953) *Exptl. Cell Research* **4**, 127.
Porter, V. S., Denning, N. P., Wright, R. C., and Scott, E. M. (1953) *J. Biol. Chem.* **205**, 883.
Ratner, S., and Clarke, H. T. (1937) *J. Am. Chem. Soc.* **59**, 200.
Ris, H., and Mirsky, A. E. (1949) *J. Gen. Physiol.* **32**, 489.

Schiller, W. (1930) *Z. Zellforsch. mikroskop. Anat.* **11**, 63.
Seifriz, W. (1938) *Science* **88**, 21.
Seifriz, W. (1942) *in* "A Symposium on the Structure of Protoplasm." Iowa State College Press, Ames.
Seki, M. (1933) *Z. Zellforsch. mikroskop. Anat.* **18**, 21.
Seki, M. (1937) *Z. Zellforsch. mikroskop. Anat.* **26**, 305.
Seligman, A. M., and Rutenburg, A. M. (1951) *Science* **113**, 317.
Shelton, E., and Schneider, W. C. (1952) *Anat. Record* **112**, 61.
Simpson, W. L. (1941) *Anat. Record* **80**, 173.
Singer, M. (1952) *Intern. Rev. Cytol.* **1**, 211.
Singer, M., and Morrison, P. R. (1948) *J. Biol. Chem.* **175**, 133.
Sponsler, O. L. (1940) *in* "The Cell and Protoplasm." American Association for the Advancement of Science, Washington.
Stockinger, L. (1951) *Anat. Anz.* **98**, 82.
Tellyesniczky, K., von (1926) *in* "Enzyklopaedie der Mikroskopischen Technik" (Krause, ed.), Vol. 2, p. 750. Urban and Schwarzenberg, Berlin.
Teresi, J. D., and Luck, J. M. (1948) *J. Biol. Chem.* **174**, 653.
Theis, E. R. (1944) *J. Biol. Chem.* **154**, 87.
Vallance-Owen, J. (1948) *J. Pathol. Bacteriol.* **60**, 325.
Verne, J. (1929) *Ann. physiol. physicochim. biol.* **5**, 245.
Walker, J. F. (1953) "Formaldehyde." Reinhold, New York.
Wang, K. J., and Grossman, M. I. (1949) *Anat. Record* **104**, 79.
Weil, A. (1929) *J. Biol. Chem.* **83**, 601.
Wolman, M., and Behar, A. (1952) *Exptl. Cell Research* **3**, 619.
Wolman, M., and Feingold, D. (1953) *Acta Med. Orient.* **12**, 218.
Wolman, M., and Greco, J. (1952) *Stain Technol.* **27**, 317.
Wormell, R. L., and Kaye, M. A. G. (1944) *Nature* **153**, 525.
Zeiger, K. (1930) *Z. Zellforsch. mikroskop. Anat.* **10**, 481.
Zeiger, K. (1935) *Z. Zellforsch. mikroskop. Anat.* **22**, 607.
Zollinger, H. U. (1948) *Am. J. Pathol.* **24**, 797.

Bacterial Cytology

ALFRED MARSHAK

Marine Biological Laboratory, Woods Hole, Massachusetts

		Page
I.	Introduction	103
II.	Nuclei	104
III.	Centrioles	105
IV.	Chromosomes	106
V.	Mitochondria	107
VI.	Vacuoles	107
VII.	Membranes	108
VIII.	Transverse Wall: Flagella	108
IX.	Nucleus of Bacterial Spores	109
X.	Formation of Giant and Dwarf Forms	110
XI.	Comparison with Cells of Higher Forms	110
XII.	Conclusions	112
XIII.	References	113

I. Introduction

In the study of the fine details of the structure of bacteria, as in other human activities, the turning of the wheels of progress appears in retrospect to have been amazingly slow while in the turmoil and excitement of present activity much seems to be happening, although it is not easy to see how much of this is pure spin and how much represents traction onto new ground. The whims of fashion have again brought the bacterial nucleus into prominence, not only as judged by the volume of words about it appearing in the literature, but apparently also in the thinking of bacteriologists not formerly accustomed to giving it much consideration. The magic of the word chromosome, reintroduced into bacterial cytology in 1945, may have had a good deal to do with the increase in interest in the bacterial nucleus, but more likely the development of microbial genetics, with its concentration on genetic factors involved in the control of familiar biochemical systems, has prepared more minds for thinking in terms of nuclear phenomena in bacteria. Certainly the development of fundamentally new techniques has had little to do with it. We find the same words (chromosomes, centrioles, nucleolus, mitotic process) being applied now, as in the past, to images obtained by staining procedures not very different from those used 20–30 years ago. There are in the current literature indications of a very natural tendency to seek in bacteria morphological counterparts of intracellular structures seen in higher forms. It seems apropos to quote some words of caution given some 20 years ago " . . . if taking the point of view of the uniformity of the living world, we

have all reason to expect the presence of a nucleus in bacteria as well as in any other form, we have not any right to demand that in the morphological respect their nuclear apparatus should entirely coincide with nuclei of different organisms. It is the duty of the biologist to apprehend its actual structure and not lay it forcibly on the Procrustes bed of preconceived notions, and, by the way, many shrink from it only because they cannot seem to make it fit this bed." (Epstein *et al.*, 1936.)

II. Nuclei

Although Feulgen and Rossenbeck (1924) applied their new procedure to bacteria they obtained negative results, but in the following year Voit (1925) presented evidence for a positive nucleal reaction in bacteria, although localization of the thymonucleic acid in nuclei was not proven. However, by 1936 the technique was sufficiently well advanced for a clear demonstration of nuclei in particularly difficult material, *M. tuberculosis*, to be presented by Epstein *et al.* (1936). They found that, after lipid extraction with carbon disulfide as well as alcohol, Feulgen-positive nuclei could be seen and readily distinguished from volutin granules. Piekarski (1937), with a fortunate choice of bacteria which did not require such extraction, demonstrated nuclei by the Feulgen procedure and also with the Giemsa stain, as modified by Badian, using aqueous eosin for differentiation and counterstaining. The latter procedure in various modified forms has become popular in recent years although it lacks the specificity of the Feulgen method, and the necessity for thorough lipid extraction prior to staining by either the Feulgen or modified Giemsa methods is still usually overlooked. I have applied the Feulgen procedure after thorough lipid extraction to a large variety of bacteria and with a single exception found very obviously identifiable nuclei (Marshak, unpublished data). The exception was an unidentified mycobacterium isolated from soil in which no nucleus could be detected by either the Feulgen or acid-Giemsa method, but which on chemical analysis was found to contain large quantities of thymine, the nitrogenous base considered to be peculiar to deoxyribonucleic acid (Marshak and Vogel, 1951). The significance of this observation remains undetermined, but it is mentioned here to emphasize the desirability of exploring not only phenomena related to the bacterial nucleus which conform to an expected pattern, but also those apparent exceptions which may ultimately throw some light on the general problem of nuclear organization.

III. Centrioles

Having become aware of the existence of bacterial nuclei, it was to be expected that the appurtenances of the nuclear apparatus would be sought and, as in other instances in cytology, where observations are made on structures near the limit of resolution of the light microscope, the inevitable controversy over interpretation has again arisen. DeLamater (1953) has described granules which he considers to be centrioles. They are stained purple by his double staining procedure, which is a combination of the thionine-SO_2 procedure he described previously, and a modification of the Cassel procedure (1951) in which the cells are mordanted with tannic acid before staining with basic fuchsin. The spindle and chromosomes are also purple, the cell wall red and "mitochondria" a "red-violet". Bisset (1952, 1953a, b), using the combination stain, Giemsa and tannic acid-crystal violet, but avoiding dehydration by making examinations in water mounts, concludes that the structures called centrioles and mitochondria by DeLamater, Mudd *et al.* (1951), and Mudd (1953) are nothing more than areas of greater basophilia at the growing tips and points of division of the cells. The picture is complicated by the designation of some granules (which also appear in association with the spindle but not at its poles) as "mitochondria," i.e. those granules that stain red-violet rather than purple, which seems to be a rather fine distinction (DeLamater, 1953). By treatment with drugs (colchicine, formaldehyde, and antibiotics) DeLamater finds arrests or deviations from the normal behavior which support his contention that the granules associated with the spindle are centrioles. According to his interpretation the centriole is at first associated with the nuclear membrane, then migrates away from it as the spindle develops and elongates. In prometaphase there is a single centriole which then divides, each half going to the spindle poles as the cell proceeds into metaphase and anaphase. It is claimed that the drug action enlarges the centriole. Arrests at various phases of the centriole division cycle may be produced by the actinomycete-derived antibiotics which also inhibit mitosis in *B. megatherium*. Para-aminosalicylic acid differs from the above in inhibiting centriolar division resulting in unipolar spindles and increase in size of the nuclear mass suggestive of polyploidy. In the recovery from antibiotic inhibition there is segmentalization of the elongated nuclear mass into portions, each of which eventually contain the three chromosomes of the haploid complement. Colchicine acts differently in these organisms in producing directly a doubling of the chromosomes. Work by Szybalski and Hunter is cited by DeLamater and illustrations are given of stages purported to be zygotene and pachytene.

Hunter (cited by DeLamater) has described a complete meiotic cycle for *Micrococcus cryophilus* from zygotene through the two meiotic divisions, but this reviewer has not yet seen the published descriptions.

IV. Chromosomes

A fine series of parallel observations made with the electron microscope and with the light microscope after acid-Giemsa staining on strains of *E. coli* has been given by Kellenberger (1953). During the exponential phase of growth all of the nucleoid bodies have the form of chromosomes, as described by Robinow (1944, 1945). At the end of the growth phase the chromosome-like appearance is no longer found but instead there are larger masses of ellipsoidal, dumbbell-shaped, and rod-like bodies. Strain C of *E. coli* shows a characteristic peripheral disposition of the "chromosomes" instead of the paracentral one found in strains S and K12. In the presence of low concentrations of aureomycin or chloromycetin, the nucleoid body assumes a globular shape with a denser portion usually more or less centrally located in the less dense mass of the nucleoid. In strain C the formation of a similar centrally located body is induced by action of the antibiotic. Upon returning the bacteria to medium free of antibiotic, the "chromosomes" again appear at first in numbers much greater than normal (polychromosome phase), after which they regroup to form the normal nucleoids following which cell division takes place. The polychromosomal condition is also induced by exposure to ultraviolet light or to methyl-bis-chloroethylamine, but the polychromosome stage in these cases is followed by one suggesting fragmentation of the chromosomes to such an extent that they appear to fill the cell. Subsequently in those cells which do not lyse there is a regrouping to form the usual nucleoids. The amount of fragmentation appears to be dependent upon dose. Kellenberger makes the interesting observation that the agents which are effective in the conversion of prophage to phage, all induce the "fragmented chromosome" stage in *E. coli*. He suggests that the mechanical rupturing of the chromosomes liberates the prophage from inhibiting influences so that it may then proceed to develop at the expense of the bacterial cell. Using staining procedures on *B. megatherium*, Bergersen (1953a) reports apparent fusion of paired nuclear bodies, when the culture proceeds beyond the logarithmic phase, to result in the formation of elongated chromatinic bars which then become converted to "zigzags" of short filaments. Subsequently on division of the cells typical bacterial rods are formed with a single, central, elliptical nuclear body. He finds that chloroamphenicol appears to inhibit cell division while cell growth and increase in the chromatinic material goes on (Bergersen, 1953b).

V. Mitochondria

Mudd (1953) and his co-workers (Mudd *et al.* 1951; Winterschied and Mudd, 1953) have described localization of Janus green, the indophenol blue of the Nadi reagent, and the formazan of the tetrazoliums in definite regions or granules. This localization has been taken as an index of oxidation-reduction activity which is assumed to be characteristic of mitochondria, and hence that the regions defined by accumulation of these materials are mitochondria. Bisset (1952, 1953a, b) has pointed out that the granules designated as mitochondria are in regions of active growth, and hence of synthesis and intense oxidation-reduction activity, so that the agents used as indicators would be expected to accumulate there even if they contained no specialized organelles analogous to the mitochondria of higher forms. Chapman and Hillier (1953) have published some very fine electron micrographs of thin sections of *B. cereus* which show "peripheral bodies" or vacuoles which appear empty except for small dense granules. These are located near the site of formation of new transverse septa corresponding to the regions of the mitochondrial bodies of Mudd *et al.* However, there is no indication of any of the typical mitochondrial structure (Palade, 1952). It should be mentioned that granules of formazan from tetrazolium do not necessarily accumulate in mitochondria. The writer has observed formazan granules in psoas muscle of the rat, which has no mitochondria (unpublished data). In *B. megatherium* fixed in OsO_4 vapor and stained with acid-Giemsa, Bergersen (1953a) has found stained granules similar to those described by Bisset at the terminal growing points and at the lateral septa. These regions also stained with Janus green and the Nadi reagent. Weibull (1953a) has found by continuous observation in phase contrast of *B. megatherium* in triphenyltetrazolium solutions that the formazan first appears as minute granules which then coalesce to form the large secondary granules described as mitochondria by Mudd (1953). He pointed out that the primary granules may indicate sites of oxidation-reduction activity but the larger secondary ones certainly do not. In mammalian tissue where succinic dehydrogenase is associated exclusively with mitochondria it has been impossible to obtain a clear indication of staining within the mitochondria, although formazan is abundantly produced at their surfaces. It has also been found that intracellular fat droplets will be colored by the tetrazolium salts (Shelton and Schneider, 1952).

VI. Vacuoles

Schuster (1952) has described vacuoles, which are probably liquid-filled, in *Malleomyces mallei;* these were observed using both light and electron microscopes. On further growth in high phosphate media,

granules appear at the vacuolar sites which consist primarily of metaphosphate as determined by toluidine blue staining and extraction techniques.

VII. Membranes

Weibull (1953b) has been able to remove the cell walls of *B. megatherium* with lysozyme and preserve the protoplast by keeping the cells in sucrose solution. When they are transferred to buffer or saline solutions they lyse. By centrifugation of the lysed cells, ghosts were isolated which were considered to be the cytoplasmic membranes. However, since the ghosts are pigmented and contain enzymes it seems to the writer more likely that the ghosts are cytoplasmic residua rather than membranes. The absence of any true cytoplasmic membrane in electron micrographs of thin sections of bacteria (Chapman and Hillier, 1953) also indicates the necessity for caution in the use of the term membrane in such cases as these. Weibull was unable to recover nuclei from his protoplasts but did obtain a rapidly sedimenting gel which appeared to be made up primarily of deoxyribonucleic acid. Robinow and Murray (1953) have described specific staining of the cytoplasmic surface of some bacteria. They find a thin cytoplasmic membrane in electron micrographs of cells in which retraction of the protoplast from the cell wall is induced by fixation in formalin. However, judging from the micrographs published, the "membrane" may be the residual cytoplasm not clumped with the main mass. This interpretation seems all the more plausible since inspection of the micrograph reveals at least two lines of density demarcation instead of the one expected on the membrane hypothesis. The protoplast surface does appear to provide a limiting surface which precipitates or accumulates the dye, but these observations raise again rather than settle the old question of whether there is a membrane in the structural sense, or a surface with differentiated physiological properties. Chapman and Hillier (1953) deduce from their studies of thin sections of *E. coli* that if a structural cytoplasmic membrane does exist, it must be less than 40 Å wide.

VIII. Transverse Wall: Flagella

Analyzing the behavior of lysozyme-treated *B. megatherium* towards stains, Welshimer (1953) finds evidence for different actions of the enzyme on the wall and on the capsular substances, an observation which is correlated with previous reports of polypeptides found in the capsule but not in the wall (see also Tomcsik and Guex-Holtzer, 1951).

The formation of the transverse wall by growth through invagination of the peripheral wall is clearly shown in the electron micrographs of Chapman and Hillier. They find the wall to be 200 Å thick, with an inner

portion which is denser and distinct from the outer portion. The transverse wall material is laid down as an annulus growing inward until a solid disc is formed, after which it thickens and divides into two layers. The cytoplasmic surface is always in contact with the forming wall. Van Iterson (1953) has described flagella in *Spirillum, Proteus,* and *Vibrio,* with definite basal granules which appear to be located in the cytoplasm beneath the cell wall. Piper *et al.* (1953) believe that the flagellum of *Spirillum serpens* is a continuation of the cell wall to which it is attached by a narrow stem. They find marked differences in the structure of flagella of *Vibrio, Salmonella,* and *Spirillum.* Van Iterson (1953) has shown that the flagellum of *Vibrio* has a definite sheath of material differing from that of the core. She concludes that motion of the flagellum cannot be as simple as the monomolecular mechanism proposed by Astbury (1949).

IX. Nucleus of Bacterial Spores

Robinow (1953) now agrees with Bisset's interpretation of the nuclear apparatus of bacterial spores. The spore contains a more or less central, vesicular, Feulgen-positive nucleus. Any of the procedures which involve acid hydrolysis result in the ejection of the nuclear material through a weak point in the spore wall to produce a diffusely Feulgen-positive mass on the surface of the spore. Chance (1953a, b) has described observations on stained preparations which suggest that in some species the transverse wall is laid down as a plate beginning at, or in the dividing nucleus in a manner resembling that found in higher plants. Clark and Webb (1953) have described instances where nuclei of the vesicular type, apparently in a state of division, are associated with a developing cell plate, suggesting to them that this type of nucleus is not limited to the resting stages. However, in *Nocardia corallina,* they found vesicular nuclei only in the coccal forms of old cultures and not in the rapidly growing filamentous forms which had only the chomosomal type of nucleus. Lack and Tanner (1953) have examined the changes in morphology of *M. tuberculosis* under conditions of nitrogen starvation which lead to lysis (Schaefer *et al.* 1949, Marshak & Schaefer 1952). They find that the nuclei swell to such an extent that the isolated cells take on a fusiform appearance. In those cells which are in cords or rafts, nuclear enlargement is accompanied by fusion of the cytoplasm of adjacent cells after which globules are formed which have the appearance of clusters of cocci. Similar coccoid forms are found in tissues of tuberculous organisms where the bacteria are growing under adverse conditions. Living forms may be distinguished from the dead ones by staining with malachite green and safranin, the viable cells staining green.

X. Formation of Giant and Dwarf Forms

In a series of investigations, employing continuous observation with phase contrast, staining reactions, and electron microscopy, Tulasne (1953) has elucidated the sequence of events in the L cycle in *Proteus* and *Salmonella*. Under the appropriate conditions, the normal bacterium is converted to a giant form which may then become divided to reproduce the normal type. Alternatively, the giant cell may by rupture or other means give rise to minute bodies which are designated as dwarfs that have the same morphology as the pleuro-pneumonia-like organisms with dimensions in the range 0.1–0.3 μ. The dwarfs may reproduce themselves, or in some circumstances give rise to giants. The formation of giants from the normal type begins with repeated chromosome division resulting in a filament containing as many as a hundred or more chromosomes. By swelling, the filament is then converted to a spherical body about 25 μ in diameter which has no detectable cytoplasmic membrane. The dwarf has a Feulgen-positive nucleus and a very small amount of peripheral material whose composition is not defined but which appears to contain appreciable amounts of lipid and very little ribonucleic acid. That this peripheral substance plays the role of a kind of cytoplasm is deduced from the fact that the dwarf is metabolically active. Electron micrographs of tungsten-shadowed dwarfs show crenulation of the surfaces parallel to the long axis of the particles, which Tulasne believes indicates a kind of granulation of the nuclear substance.

XI. Comparison with Cells of Higher Forms

One type of evidence needed to establish the homology of the chromatinic bodies of bacteria with nuclei and chromosomes of higher forms has, until recently, been lacking. Electron micrographs of whole bacteria have failed to reveal any internal chromosomal structure. Indeed, the bacterial nucleus rather surprisingly proved to be electron-optically less dense than the surrounding cytoplasm (Hillier *et al.*, 1949). Even the electron micrographs of thin sections of bacteria, which show better resolution than those previously published, have not revealed any structures that might obviously be taken to be chromonemata or chromomeres. These sections showed the same less dense areas at the nuclear sites, seen in the whole bacteria, which appeared to have a fibrous structure and have contained within them bodies of a greater density, comparable to that of the cytoplasm (Chapman and Hillier, 1953). Unfortunately, criteria for clearly identifying chromosomal structures in electron micrographs of osmic acid-fixed preparations such as these are lacking even for the cells of higher plants and animals. Osmic acid has become the fixative of choice for electron microscopy, but it is not

necessarily the fixative best suited for revealing the relationship of objects visualized by this means to the familiar chromosome components of the cells of higher organisms as seen with the light microscope.

Evidence for a chromonema-like internal structure in bacterial chromosomes has been obtained by procedures utilizing fixatives other than osmic acid (Marshak, 1951a, b). By differential centrifugation of fragments obtained by subjecting *E. coli* (in the early log phase of growth) to sonic vibration in either acetic or citric acid solutions, a heavy particle fraction was isolated in which the particles had dimensions similar to those of the Feulgen and Giemsa-positive bodies of these bacteria. Electron micrographs of acetic acid-treated bacteria showed depressed empty areas in many of the cells which suggested loss by ejection of the nuclear mass. This phenomenon and the comparable one described by Bisset and by Robinow in spores indicates that the proteins of the bacterial nucleus are quite different from those of the cytoplasm. The heavy particle fraction, after being subjected to freeze-drying and chromium shadowing, was examined with the electron microscope. Almost all of the particles of this fraction showed a regular crenulation of the surfaces along the long axis. In those particles which were not too dense, it could be seen that the crenulation was due to the presence of a helical structure, in which the coils of each helix were made up of bipartite strands each helically coiled. Fragments of about one-fourth the width of the usual heavy particle were found to contain a single helix composed of bipartite strands. The usual particle had four such helices. The structure seemed to be entirely comparable to the primary and secondary helices (major and minor coils) of the chromosomes of higher forms (Marshak, 1936). Although many types of structures examined in the electron microscope had been found to be helically coiled, this kind of system of helices had not been found elsewhere than in chromosomes. However, chemical analyses did not show any greater concentration of thymine in the heavy fraction than in either the light fraction or the whole bacteria. The significance of the chemical findings was not at all clear since loss of deoxyribonucleic acid to the small particle fraction during the vibration could not be excluded as a possibility. Most of the large particles could not be stained because they were coated with a dense black pigment, although occasionally such particles were found embedded in an amorphous matrix and these gave a positive Feulgen reaction.

The morphology of the chromosome structures of *E. coli* described by Marshak suggests comparison with the electron micrographs of the dwarf forms described by Tulasne (1953). Assuming that the surface lobulations in the electron micrographs of the dwarfs represent incompletely

resolved helices, the following rough approximations may be made from measurements taken from Tulasne's illustration (Tulasne, 1953, Fig. 12). The width of the dwarf, i.e. the diameter of the helix, is 0.32μ, the pitch is 0.08μ and the thickness of the chromonema, i.e. the diameter of the minor coil, is 0.42μ. In *E. coli* chromosomes, the comparable measurements are 0.60μ for the width of the chromosome pair or 0.30μ for the chromosome or chromatid width, depending upon whether the system of four helices or two helices is taken as the basic chromosome. The pitch of the primary helix (major coil) is 0.17μ or exactly twice that of the dwarf, while the diameter of the secondary helix is 0.05μ and its pitch 0.08μ. In the bacterial viruses also, particularly in the immature forms, one finds an apparent helical structure. The electron micrographs by Wyckoff of immature *E. coli* bacteriophage T4 (Wyckoff, 1949; Fig. VIII, 32) and of immature *Staphylococcus* K bacteriophage (Fig. VIII, 30) as well as those by Levinthal and Fisher (1952, Figs. 4 and 6) of *E. coli* bacteriophage T2 show that the principal structure of the phage at this stage is a helix with about one and one quarter turns. The dimensions of the diameter and the pitch, respectively, taken from the *E. coli* phage illustrations mentioned are approximately 0.7 and 0.5μ which may be compared with the values 0.5 and 0.8μ for the *E. coli* chromosome. Although it must be recognized that these measurements are rough and limited, the similarity in the dimensions as well as the general morphology suggests the possibility of there being a basic structural unit common to the chromosomes of normal bacteria, the dwarf or pleuro-pneumonia-like bacteria, and the bacterial viruses. It is of course quite possible that the correspondence in the dimensions found is accidental, but it would seem well worthwhile to make an extended series of accurate measurements in these superficially diverse organisms, to determine whether these indications are more than just coincidence.

XII. Conclusions

Perhaps our measures are still Procrustean. We may still be a long way from understanding the structural organization of the nucleus of the living bacterium as the disparity in the images observed with the electron microscope after osmic acid as compared with other fixatives suggests. The coordinated flocks of agglomerated flagella, such as those described by Van Iterson (1953), may serve to remind us again of the possibility of obtaining regularities in fixed materials that are imposed by the procedures used. Nevertheless, since a uniform pattern seems to be emerging from diverse materials treated by various methods, we appear to be making a closer approach to reality. We do find sufficient points of resemblance between the chromosomes of higher organisms, the chromatinic bodies of

ordinary bacteria, the principal components of the pleuro-pneumonia-like organisms, and the bacterial viruses, to conclude with reasonable assurance that they are analogous in structure and probably in function.

XIII. References

Astbury, W. T., and Weibull, C. (1949) *Nature* **163**, 280.

Bergersen, F. J. (1953a) *J. Gen. Microbiol.* **9**, 26.

Bergersen, F. J. (1953b) *J. Gen. Microbiol.* **9**, 353.

Bisset, K. A. (1952) *Exptl. Cell Research* **3**, 681.

Bisset, K. A. (1953a) *J. Gen. Microbiol.* **8**, 50.

Bisset, K. A. (1953b) Symposia on Bacterial Cytology. Fondazione Paterno, Rome, p. 9.

Cassel, W. A. (1951) *J. Bacteriol.* **63**, 239.

Chance, H. L. (1953a) *J. Bacteriol.* **65**, 593.

Chance, H. L. (1953b) *J. Bacteriol.* **66**, 239.

Chapman, G. B., and Hillier, J. (1953) *J. Bacteriol.* **66**, 362.

Clark, J. B., and Webb, R. B. (1953) *J. Bacteriol.* **66**, 498.

DeLamater, E. D. (1953) Symposia on Bacterial Cytology, Fondazione Paterno, Rome, p. 108

Epstein, G. W., Ravich-Birger, E. D., and Svinkina, A. A. (1936) *Giorn. batteriol. immunol.* **16**, 1.

Feulgen, R., and Rossenbeck, H. (1924) *Z. physiol. Chem.* **135**, 203.

Hillier, J., Mudd, S., and Smith, A. G. (1949) *J. Bacteriol.* **59**, 319.

Kellenberger, E. (1953) Symposia on Bacterial Cytology. Fondazione Paterno, Rome, p. 45.

Lack, C. H., and Tanner, F. (1953) *J. Gen. Microbiol.* **8**, 18.

Levinthal, C., and Fisher, H. (1952) *Biochim. et Biophysl Acta* **9**, 419.

Marshak, A. (1936) *J. Heredity* **27**, 459.

Marshak, A. (1951a) *Proc. Natl Acad. Sci. U.S.* **37**, 38.

Marshak, A. (1951b) *Exptl. Cell Research* **2**, 243.

Marshak, A., and Schaefer, W. B. (1952) *Am. Rev. Tuberc* **65**, 75.

Marshak, A., and Vogel, H. J. (1951) *J. Biol. Chem.* **189**, 605.

Mudd, S. (1953) Symposia on Bacterial Cytology. Fondazione Paterno, Rome, p. 67.

Mudd, S., Winterschied, L. C., DeLamater, E. D., and Henderson, H. J. (1951) *J. Bacteriol.* **62**, 459.

Palade, G. E. (1952) *Anat. Record* **114**, 427.

Piekarski, G. (1937) *Arch. Mikrobiol.* **8**, 428.

Piper, A., Crocker, C. G., Van der Walt, J. P., and Savage, N. (1953) *J. Bacteriol.* **65**, 628.

Robinow, C. F. (1944) *J. Hyg.* **43**, 413.

Robinow, C. F. (1945) *in* "The Bacterial Cell" (Dubos, ed.). Harvard Univ. Press, Cambridge.

Robinow, C. F. (1953) *J. Bacteriol.* **66**, 300.

Robinow, C. F., and Murray, R. G. E. (1953) *Exptl. Cell Research* **4**, 390.

Schaefer, W. B., Marshak, A., and Burkhardt, B. (1949) *J. Bacteriol.* **58**, 549.

Schuster, G. P. K. (1952) *Z. wiss. Mikroskop.* **61**, 101.

Shelton, E., and Schneider, W. C. (1952) *Anat. Record* **112**, 61.

Tomcsik, J., and Guex-Holtzer, S. (1951) *Schweiz Z. allgem. Pathol. u. Bakteriol.* **14**, 515.

Tulasne, R. (1953) Symposia on Bacterial Cytology. Fondazione Paterno, Rome, p. 144.
van Iterson, W. (1953) Symposia on Bacterial Cytology. Fondazione Paterno, Rome, p. 24.
Voit, K. (1925) *Z. ges. exptl. Med.* **47**, 183.
Weibull, C. (1953a) *J. Bacteriol.* **66**, 137.
Weibull, C. (1953b) *J. Bacteriol.* **66**, 696.
Welshimer, H. J. (1953) *J. Bacteriol.* **66**, 112.
Winterscheid, L. C., and Mudd, S. (1953) *Am. Rev. Tuberc.* **67**, 59.
Wyckoff, R. W. G. (1949) "Electron Microscopy." Interscience, N. Y.

Histochemistry of Bacteria

R. VENDRELY

Centre de Recherches sur les Macromolécules, Strasbourg, France

		Page
I.	Introduction	115
II.	The Envelopes of the Bacteria	116
	1. Capsule, Cell Wall, and Cell Membrane	116
	2. Cytochemical Studies	117
III.	The Bacterial Nucleus	120
	1. Chemical Studies of the Nucleic Acids of Bacteria	121
	2. Cytochemical Demonstration of the Bacterial Nucleus	122
IV.	The Cytoplasmic Bodies	130
	1. The Metachromatic Granules	130
	2. The Equivalents of Mitochondria	131
V.	The Isolation of Structural Elements of Bacteria	134
	1. Isolation and Chemical Study of the Cell Wall	135
	2. Isolation of Nuclear Structures	136
	3. Isolation of Cytoplasmic Elements	136
VI.	Conclusion	138
VII.	References	138

I. Introduction

Most of the techniques of general histochemistry available at present cannot be applied to bacteria. The small size of the cells and their thick capsules could perhaps explain the difficulty of their staining with the usual reagents of histochemistry. Another point confusing the question is that the internal morphology of bacteria is still a subject for controversy. In the cells of higher organisms, a histochemical test is used for the detection of some particular substance in one or the other of well-known organelles. But in bacteria, the staining is applied to structures, the real significance of which is not clearly seen and, in these cases, the results of histochemistry are essential for an understanding of the nature and functional significances of the organelles thus studied. For these reasons, the problems concerning histochemistry in bacteria are particularly complex and important. First, we must find techniques of histochemistry which will fit the bacteria and give a precise staining of morphological features without distortion and diffusion and, since the specificity of the techniques giving good morphological results in bacteria is often questionable, the results must be checked in other ways: gross chemical analysis (for the chemical specificity), phase contrast *in vivo* observations and electron microscopy (for the morphology), and eventually, physiological studies (for the interpretation of the biological functions of the observed features).

So that we can say that the study of the whole internal anatomy of bacteria is chiefly a matter of histochemistry.

II. The Envelopes of the Bacteria

1. Capsule, Cell Wall, and Cell Membrane

The very first data on the histochemistry of bacteria were derived from indirect methods. Immunochemists studying the reactions of antigens and antibodies were able to determine the morphological partition of certain chemical substances acting as antigens around the bacteria. In this way the capsule of *Pneumococcus* was found to contain an important polysaccharide moiety. In motile bacteria, the flagella bear protein antigen H and, in a great number of gram-negative bacteria, antigen complexes O (lipids, polysaccharides, proteins) are situated in the periphery. On the other hand, when two sorts of antigens are present simultaneously in a bacteria, it is possible to determine by immunochemical method which of them constitutes the outside layer and which the inside.

Subsequently, the surfaces of the bacteria were studied and stained cytochemically. The outer layer, which is a capsule or a slime layer, was shown to be constituted of polysaccharides or polypeptide mucilages. This layer has great importance in immunology. But it does not have much influence on the behavior of the cell, as Bisset (1950) pointed out, and the rigidity of the bacteria and the persistence of its shape is due to the existence of a cell wall all around the bacterium. This cell wall, like other cell walls, is a dead envelope secreted by the bacteria. On the other hand, the true surface of the cytoplasm in every cell bears a thin membrane regulating exchanges with the external medium. The existence of such a membrane in bacteria is very probable but still under discussion.

Cell walls and cytoplasmic membranes are not readily demonstrated. The basic dyes stain the whole body of the bacteria intensively and the cell surfaces cannot be distinguished. The method of Eisenberg (1910), used extensively by a great number of authors, consists in a treatment of the preparation with tannic acid and a subsequent staining with crystal violet; the inside of the bacteria is unstained and a cell wall is demonstrated. The tannic acid acts as a mordant and renders the cell wall more easily stainable, and on the other hand may destroy cytoplasmic proteins inside the bacteria. In fact, this technique stains the contours of bacteria and does not allow a distinction of any structure i.e. cell wall or cytoplasmic membrane. Robinow and Murray (1953) think that under these conditions the cell wall and the cytoplasmic membrane stain as a single structure, but Bisset (1953b) estimates that the cytoplasmic membrane is destroyed and the basic nucleoproteins are precipitated upon the cell wall and stain with

crystal violet. Hale (1953) and Bisset and Hale (1953) use phosphomolybic acid instead of tannic acid and stain with methyl green. They obtain better results, especially for the demonstration of septa. Chance (1953) stains the bacteria with fuchsin or crystal violet and then decolorizes the cell content with an acid dye, Congo red. Webb (1954) combines tannic acid as a mordant and Congo red as a decolorizing agent after staining with crystal violet. This staining gave uniformly good results in several genera of bacteria. Robinow and Murray (1953) found that Victoria blue, in gram-positive bacteria, selectively stains the cytoplasmic contours, but the dye diffuses after a short time into the bacterial cytoplasm; pre-treatment with a saturated solution of mercuric chloride retards this diffusion. The mechanism of this selective staining is not well known. Schumacher (1925) has shown that Victoria blue shaken up with lecithin or fatty acids in ether was almost entirely extracted from water by this lipoid solution, while thirty other basic dyes and acid dyes were little, if at all, soluble in the lipoid phase. This affinity of Victoria blue for fatty substances could be the explanation of its selective staining action on the membrane (which probably contains a certain amount of lipids) but Robinow and Murray, however, think that the action of the Victoria blue is due to its great surface activity. Victoria blue would stain the cell membrane rather than the cell wall and this staining reflects chiefly some property of the cytoplasmic membrane. This membrane appears separated from the cell wall, whenever the protoplast retracts from the cell wall, which can be realized by plasmolysis, air drying of the preparation, or exposure to ether vapors (Robinow and Murray, 1953). The cell wall can also be clearly seen, together with a remnant of the cytoplasmic membrane, by electron microscopy of spontaneously lysed bacteria (Knaysi, 1946).

The cytochemical techniques for polysaccharides and for lipids were applied and often gave positive results on the contours of bacteria. The cell wall is destroyed by hydrolysis with dilute hydrochloric acid, which Bisset (1950) considered as a sign of its polysaccharide structure. These results indicate that the cell wall contains polysaccharides and the cytoplasmic membrane is certainly rich in lipids.

2. *Cytochemical Studies*

a. Polysaccharides. Many polysaccharides are stained specifically by the method of Hotchkiss (1948) and McManus (1946) (Periodic acid, Schiff, i.e. PAS reaction), which is now extensively used in general histochemistry. It has been applied to bacteria (Pennington, 1949; Norris *et al.*, 1954). This technique consists of an oxidation of carbohydrates con-

taining the 1,2-glycol grouping, with a conversion of these groups to aldehydes, which are then able to recolorize the Schiff reagent. The cell wall is stained and also the cytoplasm, either uniformly or, in other cases, only in certain parts of the bacteria. Norris *et al.* (1954) performed the PAS reaction on variants of *Lactobacillus bifidus*. In the strain Jackson SC8 large globules were stained intensely and appeared distributed unevenly in the organisms; it cannot be decided whether they are in the cytoplasm or in the cell wall. In the case of Jackson mucoid, the polysaccharide substance is distributed more often as cross striae or bands on the bacteria. In the case of the unbranched mutant, Jackson B6 *parabifidus*, there is no staining. The hydrolysis of the polysaccharide substance yields monosaccharides including glucose, galactose, fucose, and a pentose.

Welshimer (1953), studying the action of lysozyme on cells of *Bacillus megatherium* killed with 15% formaldehyde, showed that the capsule and the cell walls are attacked by the enzyme, which indicates the presence of an acetylaminopolysaccharide component in these structures.

The polysaccharides of bacteria have also been demonstrated by oxidation with periodic acid followed by reduction of silver nitrate by the soformed aldehydes (Jarvi and Levanto, 1950). Alcian blue 8GN150 has also been used as a stain for polysaccharides in bacteria; it stains the capsule but its specificity is not absolute (McKinney, 1953).

b. Lipids. The lipids are other important components of the cell envelopes. Burdon (1946) used Sudan black for their cytochemical demonstration. He found lipids on the cell surface and also in some cytoplasmic inclusions. He used an alcoholic solution of Sudan and counterstained with safranin. He was thus able to classify the bacteria into three classes:

(1) intracellular lipids were regularly present in considerable amounts in *Clostridium, Corynebacterium* and *Mycobacterium.*

(2) the lipids are not regularly present and do not appear when the bacteria are cultivated on certain media in *Staphylococcus, Streptococcus,* and *Pneumococcus.*

(3) in other bacteria no lipids are demonstratable.

He noted that there are more lipids present in gram-positive than in gram-negative bacteria. Knaysi (1946) stained the cytoplasmic surface with Sudan black B, but Robinow and Murray (1953) did not believe that this dye is able to stain the cell wall and the cytoplasmic membrane; the time necessary for the staining is too long and the reaction cannot be significant. On the other hand, we saw that Victoria blue which stains the cell wall and the cytoplasmic membrane very selectively has a strong affinity for lipoid substances, which would indicate the existence of a certain amount of lipids on the cell surfaces. Bisset (1953b) noted that

the lipid component of the cell wall is an important factor of rigidity of this structure. Hurst (1952) showed that, when the lipids are removed, the strength of the cell wall is reduced.

Lastly, we saw that the nucleoproteins are certainly an important component of the cytoplasmic membrane of bacteria as shown by its strong staining with basic dyes. The Gram stain is probably due, at least in part, to differences in this nucleoprotein layer.

c. The Gram Stain. The Gram stain is universally employed by bacteriologists and allows a general distinction between gram-positive and gram-negative bacteria. It is a valuable means of identification, but for a long time it was purely empirical. Gentian violet is first fixed on the cells and forms with the iodine reagent a complex which is soluble in alcohol-acetone in gram-negative bacteria and insoluble in gram-positive bacteria. This fact had no explanation from the point of view of histochemistry, but the important works of Henry and Stacey (1943) throw some light upon this problem. These authors have shown that the Gram reaction is characteristic of a complex of magnesium ribonucleate and protein concentrated at the surface of the cell. The character, gram-positive, disappears after action of ribonuclease, and, if then some ribonucleate is added to the bacteria, they become gram-positive once again. Confirming this view, it can be pointed out that a number of factors which remove RNA from bacteria cause the character, gram-positive, to disappear; treatment by hot hydrochloric acid (which extracts the RNA from the cell), ultraviolet irradiation (which disturbs the synthesis of RNA; Bartholomew and Mittwer, 1952b), culture on a medium without nitrogen (De Ley, 1951), and lastly the action of penicillin (Dufrenoy and Pratt, 1947) which inhibits the formation of RNA (Vendrely, Tulasne, and Minck, 1948). All these factors change the Gram character from positive to negative.

Notwithstanding these data, a number of authors criticized the interpretation of Henry and Stacey (1943) and obtained results which are not in total agreement with it. For instance, Dianzani and Maccari (1950) and before them Dreyer, Scott, and Walker (1911), stressed the influence of lipid components upon the Gram reaction. More recently, Salton (1952) developed this point: the amount of lipids is higher in gram-negative bacteria than in gram-positive. He also pointed out the possible importance of the protein components of the bacterial membrane. Webb (1948), studying the action of lysozyme on bacteria, draws attention to the part played by the polysaccharides of the membrane in the Gram reaction.

So substances other than magnesium ribonucleate influence the appearance of the gram-positive character. Moreover, the actual participation

of magnesium ribonucleate in the Gram reaction is contested. Mitchell and Moyle (1950), confirming the earlier observations of Gutstein (1925) and of Schumacher (1928), showed that certain microorganisms do not loose their gram-positive character even when 90% of their RNA is removed. On the other hand, they think that there is a constant correlation between the Gram character and the presence in the bacteria of an acidic component, which is probably a phosphoric ester. The susceptibility of the gram-positive organisms towards lytic agents and penicillin should also be related to the presence of this phosphoric ester. In reply to Mitchell and Moyle (1950), Jones, Muggleston, and Stacey (1950) insist upon the fact that magnesium ribonucleate combined with a particular protein gives the Gram character.

Bartholomew and Mittwer (1952c) made a general review of the problem and reached the conclusion that there is no evidence for the attribution of the production of the Gram character to one chemical component or to one complex; too many substances of the bacteria can be demonstrated to play a part in this reaction. They think that the mechanism of this staining could be explained by the differences of permeability of the membrane as a whole. All bacteria should stain with gentian violet by a linkage between the acidic groups of the bacteria and the alkaline groups of the dye. Iodine, then, would form a complex with the dye and this complex would be finally dissociated by the alcohol. If the alcohol passes easily through the membrane, the decolorization will be rapid (gram-negative bacteria). It will be very slow or it will not occur if the membrane is hardly or not at all penetrated by the alcohol (gram-positive bacteria).

So the Gram staining cannot be considered at the present time as a specific reaction for some substance, but it could reveal certain physicochemical properties of the bacterial membrane.

III. The Bacterial Nucleus

The controversies upon the existence or the nonexistence of a nucleus in bacteria lasted for a long time because purely morphological studies were not able to allow any conclusion. It was only from chemical and cytochemical data that the concept of a bacterial apparatus was established. Since deoxyribonucleic acid (DNA) is the substance characteristic of the nucleus, the demonstration of the presence of DNA in all the bacteria and, on the other hand, the evidence of a body bearing this DNA in the bacteria, was enough to demonstrate the existence of a nuclear apparatus in bacteria, even if its morphology is not exactly the same as that of the classical cell nucleus.

1. *Chemical Studies on the Nucleic Acids of Bacteria*

Chemical studies (Chargaff, 1947) showed that in bacteria two nucleic acids are constantly present, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA), just as in higher organisms. Ribonucleic acid was known to be an important component of the cytoplasm while deoxyribo-

TABLE I

AMOUNTS OF RIBONUCLEIC AND DEOXYRIBONUCLEIC ACIDS IN VARIOUS BACTERIA[a]

Bacteria studied	DNA per cent dry weight	RNA per cent dry weight	RNA/DNA
Staphylococcus pyogenes (S72)[b]	2.8	8.7	3.1
Anthrax bacillus[b]	1.1	3.2	2.9
Salmonella typhi (0-901 smooth)[b]	3 7	9 1	2 4
Salmonella aerthryck (smooth)[b]	3.0	5.4	1 8
Shigella dysenteriae (smooth)[b]	3.1	6.8	2.2
Shigella dysenteriae (rough)[b]	2 8	5.4	1 9
Escherichia coli (C^1 smooth)[b]	4.4	8.7	1.9
Escherichia coli (C^1 rough)[b]	4.2	10.4	2.5
Vibrio cholerae[c]	2 1	3 7	1.7
Salmonella enteritidis (K64)[c]	2 9	5 8	2 0
Pasteurella pestis (E.V.)[c]	4 1	6.9	1.7
Bacterium pyocianeum[c]	4 9	9 3	1.8
Bacillus subtilis (with spores)[c]	1 5	5.9	3.9
Necrophorus fusiformis[c]	2.3	8.9	3.8
Corynebacterium diphteriae[c]	3 5	13.3	3.8

a 24-hour cultures
b Boivin et al (1947a)
c Leonardi, 1949.

nucleic acid is strictly located in the nucleus. Table I shows that the bacteria contains more RNA than DNA, which is often found to be so in the cells of higher organisms. Moreover, measurements of nucleic acids in developing cultures showed that the RNA increases considerably when the bacteria are in their phase of exponential multiplication; DNA, on the contrary, appears much more stable (Boivin and Vendrely, 1943). This behavior can be compared to that of the nucleic acids of rapidly growing tissues (e.g. embryonic tissues, meristems of plants) where the amount of RNA is much higher than in normal tissues, whereas the DNA content does not seem to be affected. The studies with ultraviolet light of Malmgren and Heden (1947) allow a measurement of the concentration of the nucleotide systems in a bacteria at the various periods of development. The ultraviolet radiations are absorbed by the nucleic acids at the wave-

length of 2600 Å. However, only an approximate estimation of the amount of total nucleic acid is possible. They showed that this amount is considerably increased during the phase of active division of the bacteria, which is in full agreement with the chemical data.

During the same period, the generalization of the method of Piekarski (1937, 1938, 1939) by Robinow (1945) allowed the demonstration in all the microorganisms of a central body very clearly defined and which was interpreted as a nucleus. The explanation of this method in terms of chemistry confirmed this interpretation.

2. *Cytochemical Demonstration of the Bacterial Nucleus*

a. The Technique of Piekarski-Robinow. This technique is universally employed since the beautiful photomicrographs of Robinow were published. It is derived from the Feulgen method. After a hydrolysis in 1 *N* hydrochloric acid at 60°C. for 10 minutes, the bacteria are treated with the Schiff reagent as in the Feulgen technique, but the color developed is then so pale that it cannot be conclusive. Piekarski tried a surcoloration by the Giemsa stain and the results were very satisfactory, and were the same when no Schiff reagent at all was used. So the method developed by Robinow consists of a hydrochloric acid hydrolysis followed by a staining with Giemsa on osmium-fixed bacteria.

From a chemical point of view, Vendrely and Lipardy (1946) showed that hydrochloric acid hydrolysis of bacteria, as practiced by Robinow, removes nearly the whole of the RNA of the bacteria but does not affect the amount of DNA very much. For instance, a *Staphylococcus* during the hydrolysis lost all its RNA and only 7.5% of its DNA; similarly, a *Colibacillus* lost 85 to 90% of its RNA and 37% of its DNA. The DNA which remains after the hydrolysis has lost its purine bases but the molecule has kept its power of intensely binding the basic dyes. These alterations of the nuclear chemical complex might, however, cast some doubt upon the reality of the nuclear picture thus obtained. We shall see further that certain authors have seen in some bacteria a complex nuclear structure with a "cortex" and a "medulla" which may perhaps be a result of these chemical alterations.

The control bacteria (nonhydrolyzed) are colored evenly by the Giemsa stain, a complex basic dye which is fixed by the basophilic elements of the cell and particularly the nucleic acids. In a bacterium the total nucleic acid concentration is very high (up to 15% of the dry weight) and the staining by the Giemsa stain is intense; no structure can be seen in the bacterial body. On the contrary, after the hydrolysis the bacterium, free from its RNA, is no longer stained by the Giemsa as a whole; only one or

several basophilic granules, containing the DNA, can be seen in a clear nonbasophilic cytoplasm. These granules are interpreted as nuclei. This chemical interpretation of the technique of Robinow was confirmed by the enzymatic method of Tulasne and Vendrely (1947) for the demonstration of the bacterial nucleus.

b. The Use of Enzymes for the Demonstration of the Bacterial Nucleus. Ribonuclease, an enzyme which specifically attacks ribonucleic acid, is applied to bacteria fixed in the original method with the fixative of Chabaud (1942) (osmic acid fixatives prevent the action of the enzyme). The diluted ribonuclease is allowed to act on smears from 15 to 60 minutes at 60°C. It destroys the ribonucleic acid, as does HCl, but more slowly and specifically, and a staining with Giemsa afterwards gives good pictures of nuclei. If deoxyribonuclease is used together with the ribonuclease, the nucleus is no longer stained and the bacteria have lost all their basophilia.

The direct action on the nucleus of deoxyribonuclease alone presented some difficulties, for the nucleus is situated in the middle of the bacteria and surrounded everywhere by the cytoplasm, and some authors thought that the deoxyribonuclease was not able to act without a previous action of the ribonuclease. However, a preliminary observation on *Fusiformis fusiformis*, the nucleus of which is visible without the action of ribonuclease (and which still contains ribonucleic acid), showed that under the direct action of deoxyribonuclease the nucleus is no longer stained (Robinow, unpublished data). On *Escherichia coli* also, the deoxyribonuclease destroys the DNA of the nucleus, which appears as a clear area in the middle of a still basophilic cytoplasm after staining with Giemsa.

This enzyme technique was used in a number of bacteria; bacilli and cocci always gave good pictures of nuclei (Boivin *et al.*, 1947b). Since the action of the enzymes is less drastic than that of the HCl, the pictures obtained with them might be considered as more valuable. Indeed, we noted that HCl hydrolysis removes, in certain bacteria, 37% of the DNA, and one could fear that the picture of the nucleus may be modified in its morphology as well as in its staining properties by such a treatment. A comparison between the results of the technique of Robinow and the Tulasne-Vendrely technique shows that the pictures are nearly the same in the two cases and so the hydrochloric acid hydrolysis does not seem to cause marked artifacts. Peters and Wigand (1953), in a detailed study of the use of enzymes in bacterial cytology, found a good agreement between the results of observations of the bacterial nucleus with phase contrast, electron microscopy, and Giemsa or light green staining combined with various enzymatic treatments.

But, in the particular case of the resting spore nucleus, hydrochloric acid hydrolysis causes marked artifacts. The nucleus of the spore appeared outside the cytoplasm. This position was illogical.

Bisset and Hale (1951) postulated that the nuclear material, being in the center of the cytoplasm in early germinating spores, must be at the same place in resting spores. The acidic treatment might cause a rise of pressure in resting spores which forces the nucleus to the surface and finally extrudes the nucleus from the spore. Robinow (1953) succeeded in demonstrating the real morphology and site of the resting spore nucleus in *Bacillus megatherium* and *Bacillus cereus*. He made very thin sections of the spores and thus avoided the use of hydrochloric acid and stained with Giemsa directly or after the preliminary action of ribonuclease. Fitz-James (1953), on the other hand, stained the whole spores cracked by shaking with minute glass beads in acetone or alcohol. The findings obtained by these various ways leads to the following conception: the interior of the spore is almost entirely filled by a core of dense and very basophilic cytoplasm (containing ribonucleic acid, as shown by the ribonuclease treatment); nuclear deoxyribonucleic acid elements are disposed at the periphery of this core forming a ring, a crescent, or a v-shaped figure.

c. The Feulgen Technique in Bacteria. The techniques just described allow for a cytochemical localization of the nucleic acids by indirect means, i.e. the removal of RNA by hydrochloric hydrolysis or with the nuclease makes possible the selective staining of the DNA *in situ*, but the staining by itself has no specificity and its action is not well known. On the contrary, the Feulgen reaction has been well studied and analyzed more particularly by Bauer (1932, 1933) and Stacey *et al.* (1946) and more recently by Di Stephano (1948), and the value of this method seems at the present time to have been so completely admitted that it has been a routine application in quantitative cytochemistry (Pollister, Swift, and Alfert, 1951). The results thus obtained on the DNA content of the cell nuclei are in good agreement with the chemical data. In the Schiff reagent the fuchsin decolorized by sulfurous anhydride is recolorized in the cell by the aldehyde groups of the DNA, which have been liberated by the previous hydrolysis. Other substances containing aldehydes can react with the Schiff reagent, but they are already stained in a control preparation which has not been hydrolyzed and so are easily recognized. Under these conditions the Feulgen reaction is a very specific indicator of the DNA.

However, studies devoted to the Feulgen reaction in bacteria gave most contradictory results. This method cannot easily be applied to bacteria and some authors tried to modify it in order to obtain good and constant results. Stille (1937) obtained a positive Feulgen reaction in a group

of bacilli only after a hydrolysis at 40°C. At 60°C., the temperature at which it is commonly carried out, the reaction was diffuse in the cell. Delaporte (1939) fixed the bacteria in alcohol for 48 hours, and after hydrolysis at 60°C. for 10 minutes she allowed the Schiff reagent to act for 6 hours. She thinks that the long time of fixation and the prolonged contact with the Schiff reagent is very important for obtaining positive results. The age of the culture must also be considered; the lag phase preceding multiplication is the most favorable for the staining of the nucleus, which is then relatively large.

More recently, DeLamater (1948, 1950) published a modified Feulgen technique for yeasts and bacteria. After hydrolysis, the bacteria are treated by 1% formalin before the action of the Schiff reagent. Formalin would act as a mordant and reinforce the staining, but the reaction is less specific for the membrane also stains under these conditions. This technique was applied to bacteria, with some modifications, by Smith (1950).

The three techniques just described, Robinow, Tulasne-Vendrely, and Feulgen, generally give the same pictures, but the Feulgen stains a nucleus more thinly than the other two techniques. Delaporte (1939) described slender Feulgen-positive filaments in certain cases.

The differences in size of the pictures obtained by the Feulgen technique and the other techniques could possibly be attributed to the presence of special proteins fixing the Giemsa stain, or to variations of the degree of degradation of the DNA of the nucleus, or else to the differences in the size of the particles of dye.

Let us quote here the comparative study made by Hoffman (1951) of the three methods described above on a *Lactobacillus* and on *Clostridium septicum*. He thinks that the pictures described as nuclei are composed of a "cortex" made of RNA, and of a central part, the "medulla" containing the DNA. The cortex would represent the small amount of RNA which Vendrely and Lipardy (1946) showed would remain after the acid hydrolysis, or which is not destroyed by the ribonuclease (Loring, Carpenter, and Roll, 1947) in the case of the enzymatic technique. This would explain why the nucleus demonstrated by the Feulgen technique is smaller than that found by the techniques of Robinow and of Tulasne-Vendrely. On the other hand, Clifton and Ehrhard (1952) observed bacteria stained by basic fuchsin after hydrochloric acid hydrolysis and also directly by phase contrast, and they describe spherical nuclei with a dense center and a less dense periphery. But the peripheral part of the nucleus is not necessarily made of RNA resistant to hydrolysis. Knaysi, Hillier, and Fabricant (1950) think that, in the case of *Mycobacterium tuberculosis*, the cortex would be composed of DNA. Clark and Webb (1953) studied the vesicu-

lar nuclei in *Gaffkya tetragena, Micrococcus aureus,* and *Nocardia corallina* and saw no correlation between the appearance of these forms and the stage of development of the culture. They conclude that the vesicular form does not represent a resting stage, as thought by Knaysi and other authors. In *Nocardia corallina,* on the contrary, it is characteristic of the coccoidal stage (active division stage). This form could be obtained by the precipitation of the stain on a nuclear membrane or it could be a mere artifact. It could also be the real morphology of the nucleus which would be stained as a whole in ordinary preparations and needs a thorough differentiation to appear as a vesicle.

d. Other Techniques for the Demonstration of the Bacterial Nucleus. The Feulgen method, the technique of Robinow, and the enzymatic method were fundamental for the demonstration of a nuclear apparatus in bacteria, but this organelle can be seen with other techniques which confirm and complete the data already obtained.

For instance, DeLamater and Mudd (1951), after a fixation with osmic acid vapors and hydrochloric acid hydrolysis, stain the bacteria with a solution of thionine or azure A with a few drops of thionyl chloride (which is a source of SO_2). The stain, thionine-SO_2 (or azure A-SO_2) would combine specifically with the aldehydes (Atwood and Oristein, 1949) just as the Feulgen reagent does, but the color is more intense. They mount the preparation in balsam after a dehydration in absolute alcohol at a very low temperature (about —70°C.). This procedure would avoid the distortion effect due to the ordinary mounting in balsam. But DeLamater (1953) also notes that "unreacted basic thiazin dye in the stain mix stains acid moieties within the cell, thus giving essentially a double stain. This accounts in part for the simultaneous staining of the centrioles, spindle element, cell membrane and mitochondria in addition to the nuclei." This fact, which is an advantage for morphological study, impairs the specificity of this method for DNA, and from a strictly cytochemical point of view the Feulgen technique is more reliable.

With this method DeLamater and Mudd (1951) were able to describe in *Bacillus megatherium* a typical mitotic process similar to the process occurring in the cells of higher organisms with a prophase with the appearance of filamentous chromosomes, a metaphase, an anaphase with the spindle and centrioles, and a telophase. DeLamater and Woodburn (1952) found similar results in *Micrococcus cryophilus.* DeLamater (1953), at the 6th International Congress of Microbiology, presented a great number of pictures interpreted as bacterial mitosis. Hunter (1953), with the same technique, demonstrated phases of meiosis.

Bisset (1953b) does not accept these interpretations. He thinks that

dehydration at a low temperature provokes distortions and artifacts which are interpreted as spindle fibers. On the other hand, the growing points of the septa in formation might be mistaken for centrioles, especially in *Micrococcus cryophilus*. Moreover, Bisset (1953b) noticed that stains which are admitted as specific nuclear stains (methyl green) are able to stain the basophilic septa and growing points of septa. He estimates that a great number of authors have wrongly identified these areas as nuclei. This would be rather an objection against the specificity of these stains. Finally he drew attention to the importance of multicellularity in bacteria. If this multicellularity is not taken into account, portions of bacterial membranes might be taken for nuclei.

These remarks of Bisset must be considered constantly for the interpretation of complex nuclear structures. The simultaneous use of nuclear staining and a staining of the membrane is very important as well as a close criticism of the specificity of the nuclear stains.

However, the technique of DeLamater, compared with the other techniques for the demonstration of the bacterial nucleus, seems able to give valuable results. But at the present time the attractive interpretations of this author should be considered only as a working hypothesis, needing further confirmation; other interpretations are possible.

Other techniques use reagents other than hydrochloric acid for the hydrolysis and the elimination of RNA. Cassel (1950) used diluted perchloric acid, which is known to destroy the RNA at a low temperature (Ogur and Rosen, 1950). The treatment of the bacteria by 10% perchloric acid at 4°C. for 30 to 40 hours removes the RNA of the cells and the nucleus can be stained specifically by basic fuchsin. The DNA is removed by perchloric acid acting for 20 minutes at 70°C.

Cold perchloric acid was also used by Robinow (1953) on sections of spores and by Fitz-James (1953) on cracked spores and gave the same results as ribonuclease, avoiding the artifacts caused by hot hydrochloric acid used in the classical Piekarski-Robinow procedure.

Nermut (1950) used 10% sodium hydroxide at 95°C. for 5 minutes and stained the nuclei afterwards with Giemsa. Welsch and Nihoul (1948) treated the bacteria a few hours at 60°C. with Na_2HPO_4 ($M/30$) or NaOH ($N/10$). The alkali, as Vendrely (1947) noted, dissolves the cytoplasmic RNA more rapidly than the DNA of the nucleus; it also dissolves some protein fraction.

Scaletti, Cleverdon, and Newcomer (1952) put the preparations in a 1% aqueous solution of potassium permanganate for 5 minutes; they washed it with water and oxalic acid and stained with the mixture orcein-carmin of Newcomer (1952). They think that under these conditions the RNA

of the cytoplasm is altered, but this process is not yet clear from the chemical point of view.

The demonstration of bacterial nuclei by detergents, as made by Delmotte (1950) with a solution of 5% sodium lauryl sulfate or lauryl sulfonate, could be explained by a denaturation of the ribonucleoproteins by the detergents, which are known to denature the proteins.

Summarizing, the general principle of these techniques is the removal of the cytoplasmic RNA and subsequent staining of the DNA of the nucleus. Let us point out that these conditions are sometimes spontaneously realized in bacteria by physiological or pathological changes, which result in an important decrease of the RNA; the nuclei then become apparent with any basic dye in a less basophilic cytoplasm. In old cultures, for instance, certain elements still living exhibit their nucleus without any hydrolysis (Boivin, 1948; Peshkov, 1945) and in dead bacteria the autolytic process by the very enzymes of the bacteria affects first the RNA of the cytoplasm, whereas the degradation of the DNA is much slower.

Again, in bacteria cultivated on a nitrogen-free medium or a medium with little nitrogen, the nucleus is easily demonstrated without any hydrolysis (DeLey, 1951; DeLamater and Mudd, 1951). This decrease of the cytoplasmic RNA in bacteria cultivated in nitrogen-free medium has been very useful for the observation of bacterial nuclei with the electron microscope, for in the normal condition no internal structure can be seen in bacteria by this technique. In such bacteria, Knaysi and Baker (1947) demonstrated the existence of dark bodies in a cytoplasm transparent for the electrons. The comparison of such pictures with preparations stained by Robinow's technique allowed the identification of these bodies as nuclei. Mudd and Smith (1950) have also obtained some electron micrographs of bacterial nuclei after hydrolysis. Lembke (1951) studied young forms of *E. coli* submitted to aeration and ultraviolet radiation, so that they become transparent to electrons by the decrease of their RNA, and observation of the evolution of the nucleus is possible. Bartholomew and Mittwer (1952a) also noted this clearing of the cytoplasm under the action of the ultraviolet radiations, which makes the study of internal structure easier. Bringmann (1951) used bacteria cultivated on corn meal agar where the metabolism of the RNA is disturbed and, with the electron microscope, demonstrated in them the bacterial nucleus.

Let us also quote the technique described recently by Bradfield (1954), which is a technique of Feulgen modified for electron microscopy. The aldehydes produced by the hydrolysis reduce ammoniacal silver hydroxide

and the silver metal is precipitated on the nucleus which becomes more opaque to the electrons.

e. Phase Contrast Microscopy as a Control for the Staining Techniques. All the techniques for the histochemical demonstration of the bacterial nucleus described above must fulfil two principal requirements: a certain degree of specificity for the DNA, and a staining of morphological structures existing in the living cell. On the latter point, the use of phase contrast microscopy is very valuable. It makes possible the observation of living bacteria without any treatment. The comparison of the phase contrast pictures and the histochemically stained bacteria is necessary to eliminate the possibility of artifacts of fixation, hydrolysis, etc. (Tulasne, 1949a, b; Stempen, 1950). Tulasne's observations on *Proteus* and *E. coli* by phase contrast showed the bacterial nucleus appearing in clear gray in a dark cytoplasm. He was also able to follow the division of this nucleus or, more exactly, the fissuring of rods resembling chromosomes. Also, he compared living bacteria and bacteria fixed with osmic acid or the fixative of Chabaud, and bacteria hydrolyzed by HCl at 60°C., or treated by enzymes. He noted that all these treatments do not markedly affect the image of the bacterial nucleus. After a Giemsa staining of these bacteria, the pictures are "impasted" and are less precise than in the unstained bacteria. Therefore this dye, fixed on the nuclear structure, makes it thicker and masks some details.

Summarizing, the different techniques (chemistry, histochemistry, phase contrast) have demonstrated in bacteria the existence of one or more organelles which are composed of DNA. Since the DNA is a substance highly characteristic of the nucleus and probably an important component of genes (Vendrely, 1952), we are able to conclude that the bacteria possess a nucleus. Some authors have calculated the amount of DNA contained in one bacterial nucleus. Webb (1953) finds for *Clostridium welchii* that DNA phosphorus is 2.32×10^{-12} mg. (i.e. DNA $= 24.5 \times 10^{-12}$ mg.). Caldwell and Hinshelwood (1950), Morse and Carter (1949) showed that the bacterial cell in the resting phase contains a constant amount of DNA. Now, in higher organisms, the cell nucleus is known (Boivin, Vendrely, and Vendrely, 1948; Vendrely and Vendrely, 1948) to contain a constant amount of DNA in resting tissues of the same animal species and this amount is of the order of magnitude of 10^{-9} mg. (6.0×10^{-9} mg. for humans, for instance), that is to say about two hundred times more than in bacteria. Considering the high complexity of the genetic characters in mammals as compared with bacteria, the ratio of their DNA contents per nucleus seems to be valuable as reflecting the differences in the number of genes. Let us point out here that in bacteria,

as in metazoan cells, the nucleic acid is combined with proteins, but we do not possess any cytochemical data on this point. Belozersky (1947), measuring chemically the basic amino acid content of bacteria, concludes that these organisms do not possess basic proteins (protamines and histones). Is this organelle, bearing the DNA of the bacteria, a typical nucleus? Piekarski (1950) points out that it possesses neither membrane nor nucleolus nor the typical mitosis which are characteristics of the true nucleus, and like a number of other authors (Robinow, Kleinenberger-Nobel, Badian, Bisset) he has preferred to interpret the bacterial "nucleoids" as chromosomes.

IV. The Cytoplasmic Bodies

Bacteria are known to contain granules which in some species are quite characteristic. Their histochemical reactions allow their identification; they are Feulgen negative and do not stain with Robinow's technique, so they cannot be confused with the nuclei. It has been possible to show the presence of granules of *glycogen* in bacteria, staining blue with iodine. The *lipidic inclusions* are stained by Sudan III for instance, and we have seen that they are constant and numerous in some bacteria, less numerous or absent in others. But other granules are more complex and their chemical composition as well as their biological significance are not quite clear.

1. The Metachromatic Granules

The metachromatic granules of *Corynebacterium diphteriae* are considered as identical with the volutin granules of yeasts (Oeding, 1949). They are present in more or less large quantities in bacteria, according to the age of the culture and the chemical composition of the culture medium. They are probably related to the metabolic activity of bacteria.

They exhibit the property of metachromasia which consists of their being stained red or purple by methylene blue or toluidine blue, while the other basophilic components of the bacteria stain blue. The meaning of this metachromasia is still obscure. Studies on volutin granules of yeasts showed the part played by phosphorus in these granules; inorganic phosphates are necessary for their formation (Van Herveden, 1918) and organisms which normally have no metachromatic granules (*B. pseudodiptheriae*) are capable of forming these granules when they are cultivated on a medium rich in phosphorus. Therefore these granules are made of a substance containing phosphorus. Caspersson and Brandt (1940) showed that the material of these granules in yeast absorbs ultraviolet radiations in the wavelength of 2600 Å; since they are also Feulgen-negative, they concluded that ribonucleic acid is concerned. Delaporte and Roukhelman

(1939) confirmed this opinion. They treated bacteria with a dilute solution of sodium bicarbonate (0.02%) and thus made the granules disappear. They thought that this treatment removed the RNA from the metachromatic granules without killing the yeasts. But Vendrely and Sarciron (1945) and Wiame (1946) showed that this procedure only removes some part of the polysaccharidic hapten of the cells and does not extract any ribonucleic acid. On the other hand, Minck and Minck (1949) pointed out that ribonuclease removes the metachromatic granules, but Wachstein and Pisano (1950) noted that ribonuclease has no action on the metachromatic granules. So the only strong argument for the ribonucleic nature of the metachromatic granules is the fact that they absorb the ultraviolet light in the wavelength of 2600 Å. On the other hand, Wiame (1946, 1947a, b) performed chemical analyses on yeasts and showed that the volutin granules contain a polymetaphosphate of a high molecular weight. This substance when isolated gives a strong metachromatic reaction. Ebel (1951) confirmed this result and noted that the granules in *Corynebacterium diptheriae* seem to be constituted with polymetaphosphates. The polyphosphates can be demonstrated *in situ* by a cytochemical reaction. Wachstein and Pisano (1950) and Macary (1951) tried to apply to bacteria the reaction of Gomori for the demonstration of acid phosphatase. This reaction consists of putting a phosphorus substrate (glycerophosphate) in contact with the histological preparation. The enzyme, if present in the cells, releases the phosphoric ions from the substrate. The phosphorus is then demonstrated by the formation of a phosphate by a combination with a metallic salt (lead or calcium). But they noted a positive reaction on the control slides without any glycerophosphate; the metachromatic granules appear stained black. This staining is evidently due to the polyphosphates which reacted with the metallic salt. By this method, granules of polyphosphates have been shown in *Corynebacterium, Escherichia coli, Salmonella typhosa,* and other organisms.

Therefore, the metachromatic granules may contain both polymetaphosphates and RNA. But the presence of RNA is under discussion at the present time. Krieg (1954) recently noted that the metachromatic granules of yeast fixed the respiratory poison "berberin" and then became fluorescent and are readily seen in the fluorescent microscope. We shall see below that the metachromatic granules could represent a particular case of the cytoplasmic bodies interpreted as mitochondria.

2. *The Equivalents of Mitochondria*

Metachromatic granules do not exist in all bacteria and at every stage of their development but, as many authors have pointed out, the bacteria

seem to possess constant cytoplasmic granules distinct from the nuclei, which are very dense by electron microscopy. They persist during phage infection while nuclei are broken down. Mudd (1953) interpreted these granules as mitochondria on the basis of their cytochemical reactions.

The possibility of the presence of mitochondria in microorganisms was first suggested by the works of Ephrussi (1949) and his co-workers. They showed in yeast the existence of cytoplasmic granules which seemed to condition the cytochrome oxidase activity of the cells. Mutants which lacked these granules had no cytochrome oxidase activity. The Nadi reaction indicates the presence of an oxidative enzyme by the formation of blue indophenol; it is an indicator for cytochrome oxidase. Ephrussi (1949) and Slonimski and Ephrussi (1949) assumed that the Nadi-positive granules rich in respiratory enzymes could represent in yeast the equivalent of the mitochondria of animal and plant cells.

Mudd *et al.* (1951a, b) showed that the electron-scattering granules of bacteria are probably analogous to the granules of yeasts studied by Ephrussi; a number of species were found to contain Nadi-positive granules. These granules seem to bear a coordinated system of oxidative and reducing enzymes. Besides the cytochrome oxidase demonstrated by the Nadi reagent, they exhibit a reducing activity shown by the intravital reduction of the tetrazolium salts to their colored formazans. Triphenyltetrazolium, neotetrazolium, and blue tetrazolium chlorides are reduced by granular inclusions of the bacterial cell (Antopol, Glaubach, and Golman, 1948; Bielig, Kausche, and Haardick, 1949; Mudd *et al.*, 1951a; Winterscheid and Mudd, 1953). These granules exhibit in certain cases a degree of heterogeneity. In *Corynebacteria*, Rosenfeld, Davis, and Mudd (1953) noted that the larger polar and central granules reduced triphenyltetrazolium preferentially. Blue tetrazolium and neotetrazolium preferentially stained the peripheral granules disposed along the axis of the cells. Only a few granules stained metachromatically. From a chemical point of view, several enzymes have been identified which are able to reduce the tetrazoles. Brodie and Gots (1952) isolated from *Escherichia coli* a flavoprotein mediating the oxidation of reduced diphosphopyridine nucleotide and capable of reducing the tetrazoles.

Weibull (1953a) studied the staining of *Bacillus megatherium* with triphenyltetrazolium during the development of the culture. The formazan stains first a number of very small granules scattered all over the bacteria. These granules grow larger and fuse and form at last one or two large secondary granules. Under these conditions, these granules would not represent the real sites of reduction of the triphenyltetrazolium. This reagent perhaps is not quite suitable for the observation of intracellular

structures in bacteria. As Weibull points out, the size and appearance of the stained granules are likely to depend largely on the physico-chemical behavior of the insoluble reaction product. The exact morphology of the reducing centers in bacteria could be determined exactly with the tetrazolium salts.

Janus green B (diethylphenosafraninazodimethylaniline) has long been known as a characteristic stain for mitochondria. It was shown to stain granules in bacteria (Mudd *et al.*, 1951a, b; Winterscheid and Mudd, 1953; Davis *et al.*, 1953). From a cytochemical point of view, Lazarow and Cooperstein (1953) showed that this reaction would depend on a nonspecific adsorption of Janus green B on the cell protein and its rapid reduction throughout the cell, except on the mitochondria, where the presence of the cytochrome system prevents reduction and maintains the color of the dye. This interpretation has been criticized by Brenner (1953).

Cytoplasmic granules were also shown to contain alkaline phosphatase in a number of species, especially in *Escherichia coli* K12 by Schaechter, Treece, and DeLamater (1954). These authors used a modification of Gomori's technique. But in some species the enzyme appeared located on the cell wall, or throughout the entire cytoplasm. Bayliss, Glick, and Siem (1948) had already noted that Gomori's method for alkaline phosphatase and lipase stained localized areas within the bacterial cells.

Therefore the electron-scattering granules seem to contain a great number of oxidative and reductive enzymes, just as do mitochondria of animal and plant cells. In addition they are also rich in phospholipids and stain by Baker's method (1946), which gave good results in the study of mitochondria of the cells of higher organisms. In this method, the phospholipids are first mordanted by calcium dichromate. The complex, phospholipid-chrome, thus obtained is then stained by hematein and differentiated by a solution of borate and ferricyanide. The phospholipids as well as certain proteins appear blue-black in color. This technique is not specific, but if the phospholipids are first removed with pyridine in another preparation, a comparison of the two pictures makes possible the precise localization of phospholipids. These substances appear often in the cytoplasm in the form of polar granules.

It is possible also to stain bacterial granules with a citric acid-Sudan black B which reveals masked lipids (Davis *et al.*, 1953).

Mudd (1953) has put together an impressive number of reasons for identifying the cytoplasmic granules of bacteria and the mitochondria. The most serious criticism against this interpretation was formulated by Knaysi (1951, 1952) who studied the granules of *Mycobacterium thamnopheos* by phase contrast and found that they behave like nuclei (divisions

and fusions) and are also slightly Feulgen-positive. But Winterscheid and Mudd (1953) demonstrated in this organism and also in *Mycobacterium tuberculosis* var. *hominis* two distinct types of granule, one staining with the nuclear stains and the other with the mitochondrial stains. Prenner, Von Prittwitz, and Gaffron (1952) demonstrated also, side by side, the Feulgen-positive nuclei and the distinct reductive granules in various Enterobacteria.

Bisset (1953a) thinks that the so-called mitochondria are in fact the site of formation of intercellular septa. From the morphological point of view, the polar granules could indeed represent a zone of separation of two bacteria and a site of intense enzymatic activity. Mudd and Winterscheid (1953) answer this objection by the simultaneous demonstration, with the electron microscope, of the cell wall and of granules in *Mycobacterium thamnopheos* quite distinct from the septum.

Summarizing, the bacteria have been shown to contain granules rich in phospholipid compounds and which exhibit a strong enzymatic activity by their cytochemical reactions. But these cytochemical techniques are still criticized and their results must be interpreted very cautiously. The various arguments put forward by Mudd and his group for the mitochondrial nature of these granules are impressive and deserve further study, especially of the reliability of the cytochemical techniques.

V. The Isolation of Structural Elements of Bacteria

The techniques of isolation of organelles by grinding of tissues, followed by differential centrifugation, led to interesting data on the chemical composition of nuclei, mitochondria, and microsomes of higher organisms. In bacteria, the problem is more difficult. The grinding of bacteria in, for example, ball-mills, is too drastic and merely gives a mixture of fragments which cannot be identified. The use of chemical reagents such as citric acid, which give excellent results in animal tissues, is ineffective with bacteria, probably due to the thickness of the bacterial envelopes. But other methods are efficient for the isolation of bacterial structures: enzymatic lysis, lysis due to infection by bacteriophage, chemical procedures, physical actions (heating, ultrasonic disintegration), and mechanical action (grinding). These various procedures allowed a separation of the cell walls, nuclei, and cytoplasmic granules and, in certain cases, a chemical study of these isolated elements. This modern aspect of the cytochemistry of bacteria is of the greatest importance for obtaining data confirming and completing the indications of the classical cytochemical studies on intact bacteria and for the preparation of material for electron microscopy.

1. *Isolation and Chemical Study of the Cell Wall*

Cell walls partially or completely freed of cytoplasm have been observed by a number of authors in sonically vibrated preparations of bacteria, but the first efficient method of isolation of the cell wall was Dawson's (1949). This author, following the report by King and Alexander (1948) of the lethal effects of shaking various organisms with minute glass beads, showed that this principle could be used to prepare cell wall suspensions of *Staphylococcus aureus*. Hotchin, Dawson, and Elford (1952) gave a detailed protocol for the isolation of cell walls. The shaking is made with minute glass beads in a phosphate-citrate buffer and the bacterial cell walls are separated by differential centrifugation.

The heating of bacteria was shown by Heden and Wyckoff (1949) to be able to disrupt the cell walls. Salton and Horne (1951a) studied this effect with the electron microscope. Suspensions of *E. coli* were subjected to temperatures ranging from 55 to 100°C. Below 70°C. the cytoplasm becomes granulated and shrinkage occurs from 70 to 100°C. These effects are accompanied by a rupture of the cell walls. Similar results were obtained with *Salmonella pullorum* (the rupture occurred from 70 to 75°C.) and with *Pseudomonas fluorescens* and *Pseudomonas pyocyanea*. In *Streptococcus faecalis*, a small proportion of the cell walls were ruptured after heating at 100°C.

Finally Salton and Horne (1951b) proposed to combine their heating method and the shaking with glass beads.

Chemical reagents are not very efficient. Salton and Horne (1951b) treated *E. coli* and *Salmonella pullorum* with 1 *N* NaOH for 48 hours at 37°C., but the residue examined in the electron microscope differed markedly from the pure cell wall preparations obtained by the other methods. Dawson and Stern (1954) incubated the bacteria in 0.01% cetyl trimethyl ammonium bromide at 37°C. for 24 hours. The obvious retraction of the cytoplasm from cell walls and also from the central septa allows the observation of these structures in the electron microscope.

Finally, digestion by trypsin and by lysozyme is able to liberate the cell walls (Weidel, 1950).

Chemical studies of the isolated cell walls were made by Salton (1952, 1953) in some gram-positive and gram-negative bacteria. Cell walls of gram-negative bacteria were found to contain a much greater amount of lipids than gram-positive bacteria. *E. coli* and *Salmonella pullorum* contain 19 to 22.6% of lipids while *Bacillus subtilis*, *Micrococcus lysodeikticus*, and *Sarcina lutea* hold only 1.2 to 2.6% of lipids. The membrane of gram-positive bacteria bears neither aromatic nor sulfur-containing amino

acids; the membrane of gram-negative bacteria, on the other hand, contains all sorts of these acids: proline, arginine, cysteic acid, methionine, tyrosine, phenylalanine, and diaminopimelic acid. In *Streptococcus pyogenes* the type-specific M protein is a part of the membrane. If this protein is removed, the membrane of *Streptococcus* is as poor in amino acids as the membranes of the other gram-positive bacteria. The C substance (specific polyoside of this group of *Streptococcus*) is also present.

On the other hand, Salton and Horne (1951b) studied the ultraviolet absorption spectra of isolated cell walls and found no nucleic acid or purine and pyrimidine compounds.

2. *Isolation of Nuclear Structures*

Marshak (1951) submitted *E. coli* suspended in 5% citric acid to ultrasonic disintegration. After 45 minutes of this treatment, the centrifugation allowed the separation of a heavy fraction composed of particles of 0.96 $\times$ 0.63 μ, which corresponds approximately to the size of the Feulgen-positive bodies of the intact bacteria. Electron micrographs of these large particles showed that they contain helical structures. After treatment with trichloracetic acid (removing the free nucleotides) with lipid solvents, and with perchloric acid (which extract the nucleic acids), Marshak determined the relative amounts of purine and pyrimidine bases and found for 1 mg. of dry matter: 10.6 μg. adenine, 15.3 μg. guanine, 12.1 μg. cytosine, 4.46 μg. uracil, and 2.34 μg. thymine. He concluded that these large particles might represent something comparable to chromonemata of animals and higher plants.

Weibull (1953c) did not succeed in isolating the nuclear apparatus of bacteria by action of lysozyme. In 0.015 *M* sodium chloride, plus 0.01 *M* phosphate he got the nuclei in the form of a gel, and in a sucrose (0.03 *M*)-phosphate medium obtained a viscous solution. The choice of a suitable medium would perhaps allow an isolation of these organelles with their morphology preserved.

3. *Isolation of Cytoplasmic Elements*

Cytoplasmic granules were extracted from yeast by Brachet and Jeener (1943) and by Chantrenne (1943) who, after grinding and differential centrifugation, found in them various enzymes and considerable amounts of ribonucleic acid. More recently, Nyman and Chargaff (1949) isolated from yeasts submicroscopic particles rich in lipoproteins (22 to 26% lipids), the unsaponifiable portion of which proved rich in ergosterol. They also contained 34 to 35% nucleic acid. The chemical composition of

these granules resembles that of cytoplasmic particles of the cells of higher organisms.

In bacteria, the study of the destruction by phage showed that the phage multiplication occurs in the area of nuclear structures of bacteria and finally, cytoplasmic granules are liberated (Militzer *et al.*, 1949, 1950; Georgi *et al.*, 1951; Burns and Militzer, 1953). Hartman *et al.* (1953) showed that the reductase activity toward 2,3,5-triphenyltetrazolium remained at a constant level during the latent period of T2 phage infection of *E. coli*, growing in unaerated medium. The nuclei are disrupted early in the latent period but the cytoplasmic granules remain morphologically intact and functionally active, and after lysis are strewn through the debris of the lysed cells. Mudd *et al.* (1951b) and Militzer *et al.* (1949, 1950) identified the granules liberated after phage lysis as mitochondria by their characteristic cytochemical reactions.

Lysozyme also proved efficient for the separation of the bacterial structures. Kern *et al.* (1951) observed the rupture of the cell membrane and the dispersion of the cell contents of *Staphylococcus aureus* and *Micrococcus lysodeikticus* by action of lysozyme. They demonstrated with the electron microscope the existence of small bodies resistant to the enzyme. Georgi *et al.* (1951) and Burns and Militzer (1953) isolated granules from a thermophilic bacterium after treatment with lysozyme, and found a number of enzymes in them. These granules reduced triphenyltetrazolium chloride. But, on the other hand, Weibull (1953b, c) performed a controlled lysis of *Bacillus megatherium* with lysozyme in a sucrose medium. Under these conditions, he obtained first spherical bodies which are the protoplasts. These protoplasts represent the protoplasm of the bacterial cell freed from its envelope. By a subsequent lysis, which occurs when the solvent is diluted, the protoplasts are destroyed and two fractions can be isolated by differential centrifugation, a fraction made up of lipid-containing granules staining with Sudan black B and a pigmented yellow fraction composed of the "ghosts" of bacteria. Weibull thinks that they represent cytoplasmic membranes and he found that the cytochrome system of *Bacillus megatherium* sediments with the ghost fraction in differential centrifugation.

We must also point out a particular case of simplification of bacteria which realized something resembling an isolation of the minimum structures necessary for life. The L forms of bacteria are produced under the influence of noxious action on the cultures and particularly of antibiotics. The morphological changes of bacteria (Tulasne, 1953) are represented by the formation of giant forms which are spheres of 5 to 25 μ in diameter containing small granules and also by the small granules (250 to 175 mμ)

which are liberated in the medium by the disruption of the giant forms. The small granules (L forms), notwithstanding their small size, are able to become normal bacteria again or to multiply under their simplified form. From a chemical point of view, Vendrely and Tulasne (1953) showed that the L forms contain more DNA than RNA (in normal bacteria, the RNA is more abundant than the DNA) and contain a considerable amount of phospholipids (24%). As for histochemistry, these forms are easily stained by the Feulgen reaction (the absence of a membrane facilitates the staining which is very faint in normal bacteria) and by the method of Piekarski-Robinow, so these forms represent nuclei. They are certainly surrounded by a thin cytoplasmic layer, but their small size makes the observation of this cytoplasm difficult. Under certain conditions these L forms are able to reduce the tetrazolium salts (Somerson and Morton, 1953).

VI. Conclusion

The recent progress of histochemistry of bacteria has allowed the demonstration of a remarkable internal complexity in the microorganisms. The existence of a bacterial nuclear body is now admitted. The constitution of the bacterial envelopes is becoming known. As for the centers of enzymatic activities in bacteria, their study is in full development and the data already obtained make apparent a great similarity between certain bacterial granules and the mitochondria of animals and plants. These first approaches in the field of cytochemistry in bacteria are not, however, definitive; progress is wanted especially concerning the precision and specificity of cytochemical techniques applied to these particular organisms, which at present are situated at the limits of our possibilities of investigation. This brief review of the present state of the question cannot reach any definite conclusion on many points; we can only stress the complexity of the problems and the great interest of their future developments.

VII. References

Antopol, W., Glaubach, S., and Golman, L. (1948) *Publ. Health Repts. (U.S.)* **63**, 1231.

Atwood, L., and Orinstein, L. (1949) A.A.A.S. Exhibit, New York City (abstr. in program).

Baker, J. R. (1946) *Quart. J. Microscop. Sci.* **87**, 441.

Bartholomew, J. W., and Mittwer, T. (1952a) *J. Bacteriol.* **64**, 1.

Bartholomew, J. W., and Mittwer, T. (1952b) *J. Bacteriol.* **63**, 779.

Bartholomew, J. W., and Mittwer, T. (1952c) *Bacteriol. Revs.* **16**, 1.

Bauer, H. (1932) *Z. Zellforsch. u. mikroskop. Anat.* **15**, 225.

Bauer, H. (1933) *Z. mikroskop. anat. Forsch.* **33**, 143.

Bayliss, M., Glick, D., and Siem, R. A. (1948) *J. Bacteriol.* **55**, 307.

Belozersky, A. N. (1947) *Cold Spring Harbor Symposia Quant. Biol.* **12**, 1.

Bielig, H. J., Kausche, G. A., and Haardick, H. (1949) *Z. Naturforsch.* **4b**, 80.
Bisset, K. A. (1950) "The Cytology and Life-History of Bacteria." Livingstone, Edinburgh.
Bisset, K. A. (1953a) *J. Gen. Microbiol.* **8**, 50.
Bisset, K. A. (1953b) Symposia Bacterial Cytology, *6th Intern. Congr. Microbiol.*, Rome, 1953, p. 9.
Bisset, K. A., and Hale, C. M. F. (1951) *J. Hyg.* **49**, 201.
Bisset, K. A., and Hale, C. M. F. (1953) *Exptl. Cell Research* **5**, 449.
Boivin, A. (1948) *Compt. rend soc. biol.* **142**, 1258.
Boivin, A., and Vendrely, R. (1943) *Compt. rend. soc. biol.* **137**, 432.
Boivin, A., Vendrely, R., and Tulasne, R. (1947a) *Bull. Acad. natl. med.* **131**, Nos. **1** and **2**, 39.
Boivin, A., Tulasne, R., Vendrely, R., and Minck, R. (1947b) *Arch. sci. physiol.* **1**, 307.
Boivin, A., Vendrely, R., and Vendrely, C. (1948) *Compt. rend.* **226**, 1061.
Brachet, J., and Jeener, R. (1943) *Enzymologia* **11**, 196.
Bradfield, J. R. G. (1954) *Nature* **173**, 184.
Brenner, S. (1953) *Biochim. et Biophys Acta* **11**, 480.
Bringmann, G. (1951) *Zentr. Bakteriol. Parasitenk Abt. I. Orig.* **156**, 35.
Brodie, A. F., and Gots, J. S. (1952) *Science* **116**, 588.
Burdon, K. L. (1946) *J. Bacteriol.* **52**, 665.
Burns, L., and Militzer, W. (1953) *Proc. Soc Exptl Biol. Med.* **82**, 411.
Caldwell, P. C, and Hinshelwood, Sir Cyril (1950) *J. Chem. Soc.*, p. 1415.
Caspersson, T., and Brandt, K. (1940) *Protoplasma* **35**, 508.
Cassel, W. A. (1950) *J. Bacteriol* **59**, 185
Chabaud, A. (1942) *Ann. Inst. Pasteur,* **68**, 106.
Chance, H. L. (1953) *Stain Technol.* **28**, 205.
Chantrenne, H. (1943) *Enzymologia* **11**, 213.
Chargaff, E. (1947) *Cold Spring Harbor Symposia Quant. Biol.* **12**, 28.
Clark, J. B., and Webb, R. B. (1953) *J. Bacteriol.* **66**, 498.
Clifton, C. E., and Ehrhard, H. B. (1952) *J. Bacteriol.* **63**, 537.
Davis, J. C., Winterscheid, L. C., Hartman, P. E., and Mudd, S. (1953) *J. Histochem. Cytochem.* **1**, 123.
Dawson, I. M. (1949) "The Nature of the Bacterial Surface," p. 119. Oxford Univ. Press, New York.
Dawson, I. M., and Stern, H. (1954) *Biochim. et Biophys. Acta* **13**, 31
DeLamater, E. D. (1948) *Stain Technol* **23**, 161.
DeLamater, E. D. (1950) *J. Bacteriol.* **60**, 321.
DeLamater, E. D. (1953) Symposia Bacterial Cytology, *6th Intern. Congr. Microbiol.* Rome, 1953, p. 108.
DeLamater, E. D., and Mudd, S. (1951) *Exptl. Cell Research* **2**, 499.
DeLamater, E. D., and Woodburn, M. (1952) *J. Bacteriol.* **64**, 793.
Delaporte, B. (1939) Thèse de Doctorat des Sciences naturelles, Paris.
Delaporte, B., and Roukhelman, M. (1939) *Rev. gen. botan.* **51**, 449.
De Ley, J. (1951) *Antonie van Leeuwenhoek J. Microbiol. Serol.* **17**, 15.
Delmotte, N. (1953) *Antonie van Leeuwenhoek J. Microbiol. Serol.* **19**, 117.
Dianzani, M. U., and Maccari, A. (1950) *Nuova Ann. Ig. Microbiol.* **1**, 324.
Di Stefano, H. S. (1948) *Chromosoma* **3**, 282.
Dreyer, G., Scott, S. G., and Walker, E. W. A. (1911) *J. Pathol. Bacteriol.* **16**, 146.

Dufrenoy, J., and Pratt, R. (1947) *J. Bacteriol.* **54**, 283.
Ebel, J. P. (1951) Thèse de Doctorat des Sciences Physiques, Strasbourg.
Eisenberg, P. (1910) *Zentr. Bakteriol. Parasitenk. Abt. I. Orig.* **53**, 481.
Ephrussi, B. (1949) *Colloq. intern. centre natl. recherche sci. (Paris) Abt. I*, p. 165.
Fitz-James, P. C. (1953) *J. Bacteriol.* **66**, 312.
Georgi, C. E., Militzer, W., Burns, L., and Heotis, J. P. (1951) *Proc. Soc. Exptl. Biol. Med.* **76**, 598.
Gutstein, M. (1925) *Zentr. Bakteriol. Abt. I. Orig.* **95**, 1.
Hale, C. M. F. (1953) *Lab. Practice* **2**, 115.
Hartman, P. E., Mudd, S., Hillier, J., and Beutner, E. H. (1953) *J. Bacteriol.* **65**, 706.
Heden, C. G., and Wyckoff, R. W. G. (1949) *J. Bacteriol.* **58**, 153.
Henry, H., and Stacey, M. (1943) *Nature* **151**, 671.
Hoffman, H. (1951) *J. Bacteriol.* **62**, 561.
Hotchin, J. E., Dawson, I. M., and Elford, J. (1952) *Brit. J. Exptl. Pathol.* **33**, 177.
Hotchkiss, R. D. (1948) *Arch. Biochem.* **16**, 131.
Hunter, M. E. (1953) *Abstr. Intern. Congr. Genet.*, Bellagio, Italy.
Hurst, H. (1952) *J. Exptl. Biol.* **29**, 30.
Jarvi, O., and Levanto, A. (1950) *Acta Pathol. Microbiol. Scand.* **27**, 473.
Jones, A. S., Muggleton, P. M., and Stacey, M. (1950) *Nature* **166**, 650.
Kern, R. A., Kingkade, M. J., Kern, S. F., and Behrens, O. K. (1951) *J. Bacteriol.* **61**, 171.
King, H. K., and Alexander, J. H. (1948) *J. Gen. Microbiol.* **2**, 315.
Knaysi, G. (1946) *J. Bacteriol.* **51**, 113.
Knaysi, G. (1951) "Elements of Bacterial Cytology," 2nd ed. Comstock Publishing, Ithaca.
Knaysi, G. (1952) *J. Bacteriol.* **64**, 859.
Knaysi, G., and Baker, R. F. (1947) *J. Bacteriol.* **53**, 539.
Knaysi, G., Hillier, J., and Fabricant, C. (1950) *J. Bacteriol.* **60**, 423.
Krieg, A. (1954) *Experientia* **10**, 172.
Lazarow, A., and Cooperstein, S. J. (1953) *Exptl Cell Research* **5**, 56.
Lembke, A. (1951) *Zentr. Bakteriol. Parasitenk. Abt. I Orig.* **156**, 377.
Leonardi, G. (1949) *Compt. rend.* **229**, 393.
Loring, H. S., Carpenter, F. H., and Roll, P. M. (1947) *J. Biol. Chem.* **169**, 601.
Macary, J. (1951) *Ann. inst. Pasteur* **51**, 652.
Malmgren, B., and Heden, C. G. (1947) *Acta Pathol. Microbiol. Scand.* **24**, fasc. 5-6.
Marshak, A. (1951) *Exptl. Cell Research* **2**, 243.
McKinney, R. E. (1953) *J. Bacteriol.* **66**, 453.
McManus, J. F. A. (1946) *Nature* **158**, 202.
Militzer, W., Sonderegger, T. B., Tuttle, L. C., and Georgi, C. E. (1949) *Arch. Biochem.* **24**, 75.
Militzer, W., Sonderegger, T. B., Tuttle, L. C., and Georgi, C. E. (1950) *Arch Biochem.* **26**, 299.
Minck, R., and Minck, A. (1949) *Compt. rend.* **228**, 1313.
Mitchell, P., and Moyle, J. (1950) *Nature* **166**, 218.
Morse, M. L., and Carter, C. E. (1949) *J. Bacteriol.* **58**, 317.
Mudd, S. (1953) Symposia Bacterial Cytology, *6th Intern. Congr. Microbiol., Rome*, 1953, p. 67.
Mudd, S., Winterscheid, L. C., DeLamater, E. D., and Henderson, H. J. (1951a) *J. Bacteriol.* **62**, 459.

Mudd, S., Brodie, A. F., Winterscheid, L. C., Hartman, P. E., Beutner, E. H., and McLean, R. A. (1951b) *J. Bacteriol.* **62**, 729.
Mudd, S., and Smith, A. G. (1950) *J. Bacteriol.* **59**, 561.
Mudd, S., and Winterscheid, L. C. (1953) *Exptl. Cell Research* **5**, 251.
Nermut, M. (1950) *Scripta medica* **23**, 375.
Newcomer, E. H. (1952) *Stain Technol.* **27**, 205.
Norris, R. F., de Sipin, M., Zilliken, F. W., Harvey, T. S., and György, P. (1954) *J. Bacteriol.* **67**, 159.
Nyman, M. A., and Chargaff, E. (1949) *J. Biol. Chem.* **180**, 741.
Oeding, P. (1949) *Acta Pathol. Microbiol. Scand.* **26**, 886.
Ogur, M., and Rosen, G. (1950) *Arch. Biochem.* **25**, 264.
Pennington, D. (1949) *J. Bacteriol.* **57**, 163.
Peshkov, M. A. (1945) *Am. Rev. Soviet Med.* **2**, 342.
Peters, D., and Wigand, R (1953) *Z. Naturforsch.* **8**, 180.
Piekarski, G. (1937) *Arch. Mikrobiol.* **8**, 428.
Piekarski, G. (1938) *Zentr. Bakteriol. Parasitenk. Abt. I. Orig.* **142**, 69.
Piekarski, G. (1939) *Zentr. Bakteriol. Parasitenk. Abt. I. Orig.* **144**, 140.
Piekarski, G. (1950) *Naturwissenschaften* **37**, 201.
Pollister, A. W., Swift, H., and Alfert, M. (1951) *J. Cellular Comp. Physiol.* **38**, Suppl. 1, 101.
Preuner, R., von Prittwitz und Gaffron, J. (1952) *Naturwissenschaften* **39**, 128.
Robinow, C. F. (1945) Addendum *in* "The Bacteria Cell" (Dubos, ed.). Harvard Univ. Press, Cambridge.
Robinow, C. F. (1953) *J. Bacteriol.* **66**, 300.
Robinow, C. F., and Murray, R. G. E. (1953) *Exptl. Cell Research* **4**, 390.
Rosenfeld, Davis, J. C., and Mudd, S., quoted from Mudd, S (1953) Symposia Bacterial Cytology, *6th Intern. Congr. Microbiol.*, Rome, 1953, p 67.
Salton, M. R. J. (1952) *Biochim et Biophys. Acta* **9**, 334.
Salton, M. R. J. (1953) *Biochim. et Biophys. Acta* **10**, 512
Salton, M. R J., and Horne, R W. (1951a) *Biochim. et Biophys Acta* **7**, 19.
Salton, M. R. J., and Horne, R. W. ((1951b) *Biochim et Biophys. Acta* **7**, 177.
Scaletti, J. V., Cleverdon, R C., and Newcomer, E. H. (1952) *J. Bacteriol.* **64**, 183.
Schaechter, M., Treece, E. L., and DeLamater, E D. (1954) *Exptl. Cell Research* **6**, 361.
Schumacher, J. (1925) *Biochem Z* **166**, 214.
Schumacher, J. (1928) *Zentr Bakteriol. Parasitenk. Abt. I. Orig.* **109**, 181.
Slonimski, P. P., and Ephrussi, B. (1949) *Ann. inst. Pasteur* **77**, 47.
Smith, A. G. (1950) *J. Bacteriol.* **59**, 574.
Somerson, N. L., and Morton, H. E. (1953) *J. Bacteriol* **65**, 245.
Stacey, M., Deriaz, R. E., Teece, E. G., and Wiggins, L. F. (1946) *Nature* **157**, 740.
Stempen, H. (1950) *J. Bacteriol.* **60**, 81.
Stille, B. (1937) *Arch. Mikrobiol.* **8**, 125.
Tulasne, R. (1949a) *Compt. rend. soc. biol.* **143**, 1390.
Tulasne, R. (1949b) *Compt. rend. soc. biol.* **143**, 1392.
Tulasne, R. (1953) Symposia Bacterial Cytology, *6th Intern. Congr. Microbiol.*, Rome, 1953, p. 144.
Tulasne, R., and Vendrely, R. (1947) *Nature* **160**, 225.
Van Herverden, M. A. (1918) *Proc. Acad. Sci. Amsterdam* **20**, 70
Vendrely, C. (1952) *Bull. biol. France et Belg.* **86**, 1.

Vendrely, R. (1947) *Experientia* **3**, 196.
Vendrely, R., and Lipardy, J. (1946) *Compt. rend.* **223**, 342.
Vendrely, R., and Sarciron, R. (1945) *Ann. inst. Pasteur* **71**, 327.
Vendrely, R., and Tulasne, R. (1953) *Nature* **171**, 262.
Vendrely, R., and Vendrely, C. (1948) *Experientia* **4**, 434.
Vendrely, R., Tulasne, R., and Minck, R. (1948) *Compt. rend. soc. biol.* **142**, 239.
Wachstein, M., and Pisano, M. (1950) *J. Bacteriol.* **59**, 357.
Webb, M. (1948) *J. Gen. Microbiol.* **2**, 260.
Webb, M. (1953) *Science* **118**, 607.
Webb, M. (1954) *J. Bacteriol.* **67**, 252.
Weibull, C. (1953a) *J. Bacteriol.* **66**, 137.
Weibull, C. (1953b) *J. Bacteriol.* **66**, 688.
Weibull, C. (1953c) *J. Bacteriol.* **66**, 696.
Weidel, W. (1950) *Z. Naturforsch.* **66**, 251.
Welsch, M., and Nihoul, E. (1948) *Compt. rend. soc. biol.* **142**, 1449
Welshimer, H. J. (1953) *J. Bacteriol.* **66**, 112.
Wiame, J. M. (1946) *Bull. soc. chim. biol.* **28**, 552.
Wiame, J. M. (1947a) *Federation Proc.* **6**, No. 1.
Wiame, J. M. (1947b) *Biochim. et Biophys. Acta* **1**, 234.
Winterscheid, L. C., and Mudd, S. (1953) *Am. Rev. Tuberc.* **67**, 59.

Recent Studies on Plant Mitochondria

DAVID P. HACKETT

*Biological Laboratories, Harvard University, Cambridge, Mass.**

		Page
I.	Introduction	143
II.	Definitions	144
III.	Methods	146
	1. Cytological	146
	2. Isolation	148
IV.	Structure	151
V.	Origin and Distribution	154
VI.	Cytological Observations on Isolated Mitochondria	155
VII.	Chemical Composition	158
VIII.	Respiration	159
	1. Glycolysis	159
	2. Krebs Cycle	162
	3. Electron Transfer	169
IX.	Phosphorylation	174
X.	Biosynthesis	177
	1. Photosynthesis	177
	2. Acetate Activation and Fatty Acid Metabolism	179
	3. Protein Metabolism	180
XI.	Physiology	182
XII.	Genetics, Evolution and Radiation Effects	185
XIII.	Conclusion	188
XIV.	References	188
	Addendum	196

I. Introduction

One of the major recent advances in our understanding of cellular architectonics, the spatial and structural organization of reactions and processes, has been the elucidation of the role of mitochondria in cell metabolism. This discovery has been almost entirely the result of work carried out with mitochondria isolated from animal tissues. Although the similarity, if not identity, of plant and animal mitochondria has long been accepted by cytologists, little was known of the function of plant mitochondria until very recently.[1] In 1951, Newcomer concluded from a review of researches on plant mitochondria that none of the theories as to their functional significance was established with any certainty, in spite of an abundance of descriptive observations. However, in the past three years there has been a burst of research which has revealed the central metabolic role of these

*Present address: Department of Biology, University of Buffalo, Buffalo, New York.

particles in the plant cell. Major advances have been made in our knowledge of the structure and function of plant mitochondria, and the homology with animal particles has been extended to the submicroscopic and molecular levels. Some of this work has been covered in two recent reviews (Millerd and Bonner, 1953; Goddard and Stafford, 1954).

This paper will cover primarily the work published since Newcomer's (1951) general review. The major emphasis is on studies with higher plants, although some references to algae and fungi are included; comparisons are made with results obtained with animal mitochondria. At the outset, definitions and methods of experimental approach, many of which have been recently developed, will be considered. Following a survey of recent cytological studies, the work on isolated mitochondria will be reviewed. The biochemical findings are divided broadly into the analysis of chemical composition, respiration, phosphorylation, and biosynthesis. An attempt has been made to consider the activities of mitochondria in relation to other cellular components and to the cell as a whole. Finally, the implications of this evidence for the physiology of the cell are discussed.

II. Definitions

The nomenclature of the nonplastid cytoplasmic particulates of plant cells has a long and confused history. These particles, visible in the light microscope and characterized by certain sizes, shapes, and staining properties, have been extensively studied in both living and fixed tissues and described by a great variety of terms (see Guilliermond, 1941; Newcomer, 1940, 1951). The confusion stems from the fact that these particles are morphologically heterogeneous (spheres, rods, threads) and very fragile, undergoing great changes in form under altered experimental conditions. Since thread-like forms can fragment into granules and rods change to spheres, a rigid morphological classification of these types is extremely difficult. It may be useful to have loose terms in order to distinguish groups of particles with similar size and shape, but in the present state of knowledge it is probably dangerous to attribute much importance to such differences. The term "mitochondria" has been used both in a general sense and specifically to designate granular forms (Guilliermond, 1941). In the present review, the term is used in a generic sense (cf. "the chondriome" of Guilliermond) and refers to the collection of variously shaped particles, usually 0.5 to 1.0μ in diameter and up to 10μ or more in length, which are composed largely of lipids and proteins (in addition to water) and generally stain with Janus green B.

Recent biochemical studies on isolated animal mitochondria have established these particles as the center of a variety of metabolic reactions.

The localization of similar reactions on plant particles has led to the suggestion that "mitochondria" be defined in biochemical terms, as "cellular particles associated with enzymes of the cytochrome system, the Krebs cycle, fatty acid oxidation, and with oxidative phosphorylation" (Goddard and Stafford, 1954).[1] One is reluctant to define a classical cytological term on a strictly chemical basis, and the evidence to date is not entirely adequate to establish the correspondence between a given type of plant particle, either in intact cells or isolated suspensions, and a specific biochemical activity. Nevertheless, such a definition, by shifting emphasis to the functional role of these particles in the cell, may be of value in encouraging work along these lines. In this connection, it is of interest that recent studies have revealed considerable biochemical heterogeneity within the mitochondrial fraction isolated from animal cells (see Schneider, 1953); it has not always been possible to correlate this with morphological differences. This finding suggests the need for caution in formulating a general biochemical definition such as that of Goddard and Stafford. Various studies have indicated the intimate association of cytochrome oxidase with the mitochondrial structure (Hogeboom and Schneider, 1952; Cleland and Slater, 1953a), and Paigen (1954) has shown that, in several biochemically distinct types of mitochondria, the cytochrome oxidase content is roughly constant. The biochemical definition of mitochondria as "particles associated with cytochrome oxidase" may be more appropriate at this time than the general one quoted above. A less critical operational definition of the mitochondria as particles which can be separated from homogenates by certain centrifugal forces is employed by many workers, and it will be used here in describing their results. In these cases, more work is needed to establish a precise correlation between form and function.

There is at present a real disagreement as to the meaning of the term "microsome". This term is used by the German workers Perner (1953) and Drawert (1952, 1953) and has recently been accepted by Sorokin (1954) to designate spherical cytoplasmic particles with diameters ranging from 0.25 to 1.0μ. On the other hand, the term is used by animal cytologists to indicate the submicroscopic particles which can only be seen in the electron microscope and which are isolated at very high centrifugal forces. Similar particles, 20 to 40 mμ in diameter, have recently been identified in plant cells and in isolated preparations (Robinson and Brown, 1953; Brown and Fitton Jackson, 1953; Sponsler and Bath, 1953), and these will be referred to here as microsomes. The larger particles of Perner and others would be included under the general definition of mitochondria given above. They are always perfect spheres, highly light re-

fractive, and move rapidly in the cytoplasm. Although it is admitted that these "microsomes" are often of a size and shape similar to the mitochondria (Sorokin, 1954), and of similar chemical composition (Perner, 1953), it is claimed that they can be differentiated on the basis of staining reactions. Thus, Janus green B, long used as a mitochondrial stain, does not stain the "microsomes" (Sorokin, 1954), and quantitative differences in staining with fluorescent dyes is reported by Perner (1953). However, Janus green staining, and presumably the reaction with other dyes, is known to be profoundly affected by the morphology of the mitochondria (Lazarow and Cooperstein, 1953) and Drawert (1953) even reports that "microsomes" will fluoresce after staining with Janus green B. On the basis of staining with the Nadi reagent, Perner (1952b) claims that cytochrome oxidase is localized on the "microsomes" rather than the mitochondria. There may well be morphologically distinguishable types of particles, but, in the present state of our knowledge, it seems advisable to include them under the general term, mitochondria.

The origin of the submicroscopic microsomes remains uncertain. Biochemical evidence indicates that they are not disintegration products of mitochondria (Newcomb and Stumpf, 1952; Strittmatter and Ball, 1954). They may be fragments of the endoplasmic reticulum (see Bretschneider, 1952). Animal microsomes are both morphologically (Slautterback, 1953) and biochemically (Hazan *et al.*, 1953) heterogeneous. There is probably not a continuous spectrum of cytoplasmic particles but rather a distinct hiatus between mitochondria and microsomes (see Hogeboom, Schneider, and Striebich, 1953).

III. Methods

1. Cytological

Until recently, investigations on plant mitochondria have relied largely on the classical cytological methods of light microscopy. Although mitochondria have been observed and stained in living tissues, most studies have employed materials which have been fixed and stained by a variety of methods (see Guilliermond, 1941; Newcomer, 1951). Deysson (1952) has recently described a rapid method for staining mitochondria which involves treatment with a solution of 1% orcein in 20% trichloracetic acid, following fixation in 50% neutralized formol or saturated mercuric chloride. Chayen and Miles (1953) have developed an entirely new and simple method which gives excellent preservation of mitochondria. Roots were kept for one day in polyvinyl alcohol, macerated in an aqueous solution of pectinase, stained with very dilute Janus green B, and mounted in a viscous solution of polyvinyl alcohol and glycerine.

Phase contrast, fluorescent, and ultraviolet microscopy have been employed in recent studies. The phase microscope has been used successfully to observe mitochondria in living plant cells (e.g. Perner, 1952b, c) and in fungi (Ritchie, 1953). Sorokin (1954) has used it to extend her earlier study on the use of Janus green B staining to distinguish mitochondria and plastids in living cells. Drawert (1952, 1953) has studied the staining of "mitochondria and microsomes" by Janus green, berberin sulfate, and Nile blue sulfate under the fluorescent microscope.

The major advances in our knowledge of mitochondrial structure have been made with the aid of the electron microscope, which has revealed a complex structure in particles which normally appear homogeneous in the light microscope. Solutions of osmium tetroxide give good cytoplasmic fixation, and the preparation of specimens of extreme thinness by sectioning tissues embedded in medium-hard plastics has greatly increased the possible resolution (see Dalton, 1953; Selby, 1953). Electron microscopic observations have been made on the mitochondria of a variety of plant tissues as follows: egg cytoplasm (Buchholz, 1947), root tips (Rozsa and Wyckoff, 1950; Brown and Fitton Jackson, 1953; Sponsler and Bath, 1953), epidermal cells (Perner, 1952c), and leaf tissue (Palade, 1953). No real improvements on Buchholz's (1947) early pictures were made until the excellent work of Palade (1953) appeared. Isolated plant mitochondria have also been examined with the electron microscope (Stafford, 1951; Anderson, 1952; McClendon, 1952; Perner, 1952c; Farrant, Robertson, and Wilkins, 1953). With suspensions of particles, drying in air may result in the destruction of delicate protoplasmic structures. The method ("critical point") of avoiding this difficulty by removal of water in a series of miscible liquids and final volatilization of liquid CO_2 has been well illustrated by Anderson (1952) with particles isolated from pea seedlings.

Cytochemical methods (see Glick, 1953) have been employed in a few recent studies with intact plant tissues, but their contribution to our knowledge of mitochondrial function has remained very limited (cf. Van Fleet, 1952). The newer techniques of autoradiography and absorption cytospectroscopy have been applied very little to problems of localization within plant cells. Although the exact significance of the observations is as yet uncertain, it is of interest to note the granular distribution of P^{32} (Howard and Pelc, 1951a) and S^{35} (Howard and Pelc, 1951b) in the cytoplasm of root meristem cells. Ball (1953) has studied the cytoplasmic accumulation of C^{14} with callus cultures, and Roche and Yagi (1952) reported a concentration of radioactive iodine in special regions of the cytoplasm. Chayen (1952) found evidence of an absorption of ultraviolet light (265 mμ) by cytoplasmic granules, which could be attributed to both nucleic acids and ascorbic acid.

2. *Isolation*

a. Critique. The new era in mitochondrial research was inaugurated with the discovery and development of a method for the isolation of particles by differential centrifugation from tissue homogenates. During the past 15 years, great advances have been made as the result of the biochemical studies on mitochondria isolated from a variety of animal tissues; these have been extensively reviewed (e.g. Green, 1951; Schneider, 1953). Although cell-free suspensions of chloroplasts and their derivatives have long been used in the study of photosynthesis (see Weier and Stocking, 1952b), little was done before 1950 on the fractionation of plant cell components with a view to the isolation of mitochondria. Hill and Bhagvat (1939), Okunuki (1939b), and Damodaran and Venkatesan (1941) all studied the succinoxidase associated with insoluble elements prepared by centrifugation from homogenates of plant tissues, but no attempt was made to identify the particles with a cytoplasmic component. In 1950, DuBuy, Woods, and Lackey reported their work on the enzymatic activities of mitochondria isolated from leaf homogenates. Since then, a great deal of work, largely along lines developed earlier in the study of animal mitochondria, has been done with similar isolated plant particles.

The fact that results obtained with centrifugally derived intracellular components must be treated with caution is very generally recognized (see Dounce, 1950, 1952; Glick, 1953). Ideally, one should work with unaltered particles in homogeneous preparations, but these conditions are difficult to satisfy completely. The great morphological heterogeneity of the mitochondria in plant cells, their extreme sensitivity to altered environmental conditions, and the possibility of artifacts arising as a result of the disruption of cells, such as the loss of soluble components or the adsorption of extraneous materials, are all factors contributing to the difficulty of obtaining intact and homogeneous preparations. The presence in many cells of a large volume of vacuolar sap, normally separated from the cytoplasm, presents a special problem when plant cells are broken. Localization of an enzyme in a given component should include the determination of activity in all the cell fractions, and this has not always been done. Quantitative estimates of reaction rates in intact cells, based on results with isolated particles, are subject to serious limitations. For example, the demonstration that certain enzymes are "latent" in mitochondria (see Schneider, 1953) makes it difficult to determine the activities of these enzymes *in situ*. Evidence for the correspondence of an isolated component with a similar structure in the intact cell will depend in large part on cytological identifications (see below), and this is made difficult by the small size of the mitochondria.

The whole problem of the localization of enzymes in higher plant cells as revealed by studies on isolated particles has recently been discussed in the critical review of Goddard and Stafford (1954). Perner (1952c, 1953), emphasizing the far-reaching morphological changes and the heterogeneity in isolated preparations from plant tissues, seems to discount almost completely the results obtained by this technique. Other reviewers (Newcomer, 1951; Bogen, 1954; Goddard and Stafford, 1954), while fully aware of the limitations and the as yet partial nature of the findings, nevertheless realize that the analysis of the role of plant mitochondria has received great impetus and has made real advances as the result of the application of these new methods.

b. Technique. Cell-free homogenates of plant tissues have usually been prepared by grinding in a mortar, either with or without sand, or by maceration in a Waring blendor. The milder techniques, such as the use of a glass homogenizer, are difficult to use directly with certain plants due to the toughness of some tissues. This difficulty might be avoided by first separating the tissue into individual cells with the aid of a nontoxic pectinase preparation. The deleterious effects of blending, illustrated by the disorganization of nuclear elements (Weier and Stocking, 1952a) and the solubilization of mitochondrial hexokinase (Saltman, 1953), may be reduced by blending for short periods (e.g. 30 seconds) at low temperatures (Stafford, 1951; McClendon, 1952). All of the preparative procedures should be carried out at temperatures near 0°C. Levitt's (1952) method of freeze-drying and extraction of the resulting powder probably results in more radical changes in the cytoplasmic constituents, and his results are difficult to compare with those of other workers.

Fractionation of cellular components is accomplished by the differential sedimentation of particles of varying size and density in a gravitational field. With low-speed centrifugation the large particles, normally assumed to include tissue fragments, nuclei, plastids, and cell walls, are brought down; at higher speeds, the mitochondria and then the microsomes are precipitated. The supernatant fluid remaining after the final centrifugation is generally assumed to contain the soluble cytoplasmic and vacuolar constituents. Some mitochondria come down at as low as 600 $\times$ g (gravity) (Weier, 1953) and most, if not all, at 8000 $\times$ g (Price, 1952). In general, the first fraction has been brought down by centrifuging at 500 to 1000 $\times$ g for about 10 minutes, and the mitochondrial fraction at 10,000 to 20,000 $\times$ g for 15 to 30 minutes; considerably higher values, 40,000 to 130,000 $\times$ g, and longer times, have been used for the microsome fraction. Some workers have used radically different forces to prepare the "mitochondrial fraction", for example, 6000 to 80,000 $\times$ g (Leopold and

Guernsey, 1953) as compared to 1150 × *g* (Pearson and Robertson, 1954). Such wide variations in method doubtless yield preparations with different characteristics. After centrifugation, it is desirable to wash the particulate fraction by resuspension in the extracting medium and recentrifugation before determining its biochemical characteristics.

The biochemical and morphological properties of isolated cell components depend on the nature of the extraction medium. In the case of mitochondria, the tonicity or osmotic concentration of the medium is of critical importance. It is of interest that a similar requirement for the isolation of the osmometer-like chloroplasts has long been recognized. Although a few workers (e.g. Stafford, 1951) have used water alone, practically all the extractions of plant mitochondria have been made in solutions of sugars or inorganic salts. The pH of the medium is usually regulated near neutrality. Most commonly employed have been phosphate buffers (0.05 to 0.15 *M*) and sucrose solutions (0.2 to 0.5 *M*), used either separately or together; KCl, glucose, and mannitol have also been used.

Several workers have examined in detail the effects of the medium on the activity of mitochondrial reactions (Millerd, 1953; Laties, 1953a; Price and Thimann, 1954a; Mapson, Isherwood, and Chen, 1954). The results obtained depend entirely on the specific reaction under investigation. Thus, Price and Thimann (1954a) found that extraction in water gave optimal activity for some dehydrogenases but only 15% of the maximum succinoxidase activity. Although mitochondria prepared in water show maximal cytochrome oxidase activity, sucrose actually being inhibitory (Stafford, 1951; McClendon, 1953), they show no capacity for oxidative phosphorylation in the presence of succinate (Laties, 1953a). In general, reactions mediated by a multi-enzyme complex, such as complete oxidation from substrate level to oxygen, are impaired by hypotonic solutions. These results suggest that isolated plant mitochondria may behave like osmometers whose physical integrity, maintained by isotonic or hypertonic solutions, is necessary for certain complex reactions but not for the activity of individual enzymes. This has been shown to be the case for animal mitochondria (e.g. Harman and Feigelson, 1952a).

A specific beneficial effect of phosphate in sucrose-containing extraction media has been described by Millerd (1953) and Mapson *et al.* (1954), although it is apparently not a general requirement. These workers found that fluoride, a phosphatase inhibitor, was able to partially replace the phosphate, and Mapson *et al.* obtained maximal activity when arsenate was substituted for phosphate. The phosphate requirement is apparently not directly related to a generation of high energy bonds, and may be connected with the maintenance of mitochondrial integrity. Millerd showed

that one of the effects of phosphate was to eliminate the cytochrome c requirement of organic acid oxidations by mung bean mitochondria. This requirement is also profoundly influenced by the tonicity alone (Laties, 1953a; Price and Thimann, 1954a). The integrated cytochrome system is apparently one of the most sensitive loci in the mitochondrial apparatus; the alterations are probably due to changes in the physical state of the particles rather than to the actual loss of components during preparation.

A number of special substances have been used to activate or stabilize the mitochondria during and after extraction. Price and Thimann (1951, 1954a) reported that protein preparations, such as crystalline bovine serum albumin, 100 mg./l, helped to maintain the mitochondria from *Pisum* stems and greatly promoted dehydrogenase activity. Qualitatively similar effects were obtained with gelatin, asparagine, and the chelating agent "sequestrene," suggesting that these substances may act by binding inorganic ions. McClendon and Blinks (1952) showed that high molecular weight solutes, such as Carbowax and albumins, prevent the swelling of algal plastids even though they burst in sucrose or KCl solutions; they suggested the possible use of these substances to maintain mitochondrial form. Davies (1953) used nicotinamide, a DPNase (diphosphopyridine nucleotidase) inhibitor during grinding. Sharpensteen and Conn (1954) found that the inclusion of cysteine or reduced glutathione in the isolation medium greatly increased the oxidative activity of potato mitochondria. There is an interesting recent report that the enzymatic blackening in a potato extract can be inhibited by the addition of ATP (adenosinetriphosphate) plus isolated mitochondria (Makower and Schwimmer, 1954).

IV. Structure

In 1947, Buchholz published the first electron micrographs of plant mitochondria. Sections of the egg cytoplasm of *Tsuga canadensis* showed rod shaped particles, 1 to 5 μ in length, within which could be seen dark (kernels) and light (vacuoles) areas, suggesting an internal structure. Buchholz also examined the pollen mother cells of maize and found evidence of a thin mitochondrial membrane, as well as an internal structure. With the electron microscope, Rozsa and Wyckoff (1950) were unable to detect an obvious mitochondrial membrane in *Allium* root tips. However, in the mitochondria of *Allium* epidermal cells, Perner (1952c) clearly established the presence of a limiting membrane and a complex inner structure, which could be seen to shrink away from the external layer. Finally, Palade (1953) reported that the characteristic pattern of organization discovered earlier in mammalian mitochondria (Palade, 1952) could be identified in the leaf tissue of two flowering plants, *Lemna viridis* and

Nicotiana tabacum. Electron micrographs of isolated plant mitochondria also show an outer membrane and internal organization (Stafford, 1951; Anderson, 1952; McClendon, 1952; Farrant *et al.*, 1953). Farrant *et al.* estimated the membrane thickness of red beet mitochondria at 27 mμ, which is close to the outer limit (25 mμ) observed by Palade (1953); this value is probably high, due to the angle of sectioning.

The intramitochondrial structural elements which have been described include a limiting membrane, an apparently homogeneous matrix, and a system of internal ridges, cristae mitochondriales (Palade, 1953). The cristae protrude from the inside surface of the membrane towards the interior of the mitochondrion and in many cases are "perpendicular to the long axis of the organelles and occur in series within which they lie parallel to one another at more or less regular intervals." Sjöstrand (1953) has shown in animal mitochondria that both the outer membrane (total thickness, 16 mμ) and the transversally oriented inner membranes are double, probably consisting of two protein layers separated by a lipoid film (Sjöstrand and Rhodin, 1953). It seems likely that the cristae are deep and rigid folds of the second, internal membrane (Palade, 1953).

Examination of mitochondria from a wide variety of vertebrate and invertebrate tissues has led to the discovery of a similar pattern of organization throughout, and it is likely that this extends to lower as well as higher plants. Mitochondria in the chloroplast-containing protozoan *Euglena* show the typical membrane, cristae, and central fluid channel (Wolken and Palade, 1953). Light microscope studies on the fungus *Allomyces* have shown an external membrane and vacuole-like regions within the mitochondria (Ritchie and Hazeltine, 1953). The electron micrographs of Sponsler and Bath (1953) show an apparent outer cortex and inner partitions in the mitochondria of the fungus *Physarum*.

Plant cytologists have long realized that a variety of physical and chemical agents can provoke profound morphological alterations in the mitochondria (see Guilliermond, 1941). In general, the changes produced are of the same kind and are independent of the nature of the agent employed. Three general types of alterations have been described: (1) the fragmentation of filaments into small spheres or granules (granulation), (2) the swelling of particles, with accompanying changes in shape and light refractivity which make them appear as hollow spheres (vesiculation), (3) the coalescence of granules, spheres, or rods to form filaments or threads. Under carefully controlled conditions it has been shown that some of these changes are reversible (Dangeard, 1951b; Geneves, 1951; Buvat, 1953; Dangeard and Parriaud, 1953). In recent cytological studies on the mitochondria of higher plant tissues alterations induced by the following agents

have been observed: acetic acid (Dangeard, 1951b; Puytorac, 1951), chloral hydrate (Dangeard and Parriaud, 1953), low temperature (Geneves, 1951), high temperature (Dangeard, 1951a), Janus green B (Sorokin, 1954), and immersion in water (Puytorac, 1951; Buvat, 1953). Ritchie (1953) and Ritchie and Hazeltine (1953) have observed the mitochondria of the fungus *Allomyces* under the influence of gamma radiation, mechanical pressure, high and low temperature, solutions of NaCl, sucrose, and cyanide.

Although the mitochondria of higher plants and fungi show many similarities with respect to morphology and fragility, certain differences in response to external agents are evident. Ryland (1948) reported that KCN caused fragmentation and coalescence of mitochondria in onion and narcissus root tips, but no evidence of mitochondrial damage was apparent in *Allomyces* treated with 10^{-2} *M* KCN (Ritchie and Hazeltine, 1953). Geneves (1951) found that lowering the temperature to 0°C. caused a reversible granulation and vesiculation in chicory tissue, whereas low temperatures had no effect with *Allomyces* (Ritchie and Hazeltine, 1953). Temperatures between 42 and 50°C. caused the complete destruction of the mitochondria in various higher plant tissues (Dangeard, 1951a); a temperature of 45°C. had no effect on *Achlya* mitochondria (Bhargava, 1950), and destruction of the mitochondria in *Allomyces* did not occur until 75°C. It appears that the mitochondria of these fungi are less sensitive to external agents than are those of higher plants.

Past *in situ* studies on morphological alterations of mitochondria have been largely descriptive, and although little is known of the underlying mechanisms, some recent studies are very suggestive. According to Buvat (1953), the fibrillar mitochondria are built up from elementary granular particles joined together end to end, and fragmentation results when these connections are destroyed. The "elementary particle" might correspond to the mitochondrion whose structure was described above. Ritchie and Hazeltine (1953), on the other hand, consider that fragmentation may be the result of a loss of "toughness" in the external limiting membrane. The swelling or vesiculation of mitochondria is almost certainly the result of an absorption of water (see below). Harman and Feigelson (1952b) have shown that an energy supply (oxidative phosphorylation) is necessary for the maintenance of mitochondrial structure. Phosphorylation in the mitochondrion itself makes possible the "autoperpetuation" of its integrated structure. Therefore, any agent which interferes with the delicate process of oxidative phosphorylation would be expected to provoke a morphological disintegration.

V. Origin and Distribution

The questions of the origin and possible dual nature of the mitochondria have been discussed extensively (see Guilliermond, 1941; Newcomer, 1951). The analysis of these problems, already difficult because of the limited resolving power of the light microscope, has suffered from a lack of clearly defined terms. It is generally accepted by plant cytologists that the mitochondria are self-duplicating elements which do not arise *de novo* but are formed by division of pre-existing particles. In this connection, Eichenberger's (1953) electron microscopic observations on the origin of mitochondria from microsomes in animal cells are of interest. That mitochondria-like particles can differentiate into plastids has been recognized since 1910, and this process is well illustrated in O'Brien's (1951) study on the maturation of starch grains. The controversy concerns whether these proplastids represent a type of mitochondria or constitute an independent organelle system. Strugger (1951, 1953) reports that in young leaves the proplastids can clearly be distinguished from mitochondria; he was unable to find any evidence for the origin of plastids from mitochondria and concludes that this theory must be discarded.

It is to be hoped that new insight into these problems will be provided by electron microscope investigations. Leyon's (1953) study of the differentiation of chloroplast structure in the leaves and root tips of *Vallota* and *Taraxacum* provides support for the theory of duality. He concludes, "If the word mitochondria is used to include all bodies of a certain size in the cytoplasm, the mitochondria of the leaf cells studied here are obviously of at least two morphological types, one of which represents chloroplasts in an early stage of development." Wolken and Palade (1953) found no evidence for the development of chloroplasts from mitochondria, the latter term being used presumably to identify particles with characteristic structural features, such as cristae mitochondriales. The dynamic nature of morphological characteristics, and consequently the hazards inherent in classifications, are well illustrated by the demonstrations that a few hours of light can induce (1) the formation of chloroplasts, with lamellae, in *Euglena* which have been kept in the dark and whose chloroplasts had vanished beyond recognition (Wolken and Palade, 1953) and (2) the conversion, in buds of *Cichorium*, of leucoplasts with a granular internal structure into chloroplasts containing well-defined grana with membranes (De Deken-Grenson, 1954).

Information on the intracellular distribution of mitochondria is of great interest to the physiologist. O'Brien (1951) showed mitochondria-like particles aggregating adjacent to the nucleus in the scutellum of *Triticum*, and Sorokin (1954) refers to the mitochondria associated with the nuclear

surface. Cytological evidence for an intimate relationship, including a possible exchange of substances, between the mitochondria and nucleus has been provided by the microcinematographs of animal tissue cultures (Frédéric and Chèvremont, 1952). Their findings may be compared with those of Sparrow and Hammond (1947) showing an apparent migration of substance from the nuclear membrane into the cytoplasm of plant microsporocytes. Mitochondria may also aggregate next to the outer cell membrane, as shown in *Euglena* (Wolken and Palade, 1953), tulip epidermal cells (Sorokin, 1954), and most dramatically in kidney cells (Sjöstrand and Rhodin, 1953). Light has been shown to affect the differential distribution of mitochondria (Wolken and Palade, 1953; Sorokin, 1954). The localization of *Euglena* mitochondria around the chloroplasts in the light and near the cell surface in the dark has led to the interesting suggestion that local oxygen tension may be a factor in determining the distribution in the cell (Wolken and Palade, 1953). It is generally assumed (e.g. Buvat, 1953) that cytoplasmic streaming plays a major role in this distribution; however, the active, worm-like motions of plant (Ritchie and Hazeltine, 1953; Sorokin, 1954) and animal (Frédéric and Chèvremont, 1952) mitochondria suggest at least a partial independence of movement.

VI. Cytological Observations on Isolated Mitochondria

Isolated cell structures may be identified by either chemical or cytological methods. In those cases where a chemical constituent is restricted essentially completely to a single cellular component, such as the chlorophyll in chloroplasts, analyses for this constituent provide information as to the nature of the isolated cellular fractions (McClendon, 1952). The known chemical composition of the mitochondria does not, as yet, permit any such clear-cut identification. Cytological examination is the most direct means of ascertaining the nature of the components present in the mitochondrial pellet.

The mitochondrial fraction prepared from a variety of plant tissues has been examined in the light, phase, and electron microscopes. In every case, only spherical or granular particles have been reported and no rods or thread-like forms have been detected. Hogeboom, Schneider, and Palade (1947) found that morphologically intact rod-shaped mitochondria could be isolated from rat livers in 0.88 *M* sucrose solution. Sugar solutions of the same concentration did not yield rods from plant tissues (Perner, 1952c; Price, 1952). Two factors must be kept in mind when evaluating this result. First, it cannot be assumed that a single medium is ideal for all types of mitochondria. For example, Recknagel (1950)

has shown that the mitochondria of amphibian eggs are destroyed in 0.88 *M* sucrose, and the morphological integrity of pigeon breast-muscle mitochondria can best be preserved in a complex mixture containing KCl, ATP, $MgSO_4$, and versene (Chappell and Perry, 1954). Consequently, the possibility of isolating rod-shaped mitochondria from plant tissues has not been eliminated; the stabilizing effect of large molecular weight solutes might be investigated in this connection. Second, even *in situ* plant mitochondria are extremely fragile (see above), and it is not surprising that homogenization and centrifugation cause morphological alterations. The fragmentation of thread-like forms into spheres or granules is a common type of transformation, and according to Buvat (1953) may simply represent a dispersion of the elementary units previously joined together. If this is so, fragmentation might not greatly affect the functional capacities of the particles. The hollow, vesiculate particles observed in isolated plant mitochondrial preparations (Perner, 1952c; McClendon, 1952) probably correspond to the "target" and "crescent" animal mitochondria (Harman and Feigelson, 1952a, b; Cleland and Slater, 1953b) which retain the capacity for oxidation and phosphorylation. According to Perner (1952c), the electron micrographs show only coagulated remains within the isolated granules, but this may, in fact, be the internal structure.

Sizes (diameters) of the particles present in the mitochondrial fraction prepared from various tissues have been reported as follows: tobacco leaves, 0.3 to 1 μ (DuBuy *et al.*, 1950) and 1 μ or less (McClendon, 1952); pea seedlings, 0.1 to 7 μ (Stafford, 1951), 1 μ or less (Price, 1952), and 1 μ (Davies, 1953); mung bean seedlings, 0.5 to 2 μ (Millerd *et al.*, 1951); beet root, prepared in sucrose, 0.7 to 1 μ, in KCl, 1 to 2 μ (Farrant *et al.*, 1953). In general, 1 μ is considered a typical value for the diameter of granular plant mitochondria or for the width of rods (Guilliermond, 1941); the dimensions of cylindrical animal mitochondria are given as 1 to 4 $\mu \times$ 0.3 to 0.7 μ (Palade, 1953). It is clear that the isolated plant particles fall within the expected range for mitochondria; lower size limits are not given in several cases, and the values reported by Stafford (1951) are unusually small. Cleland and Slater (1953b) have shown that heart-muscle mitochondria isolated in hypotonic medium may fragment on shaking to give minute spheres which are either single or joined together in clumps or strings. The possibility that the very small spheres seen by Stafford are fragmented mitochondria is made likely by the fact that she used a Waring blendor and water as the medium for preparation. It is possible that the dimensions given for plant mitochondria by Goddard and Stafford (1954), 0.1 to 6 μ, may be misleading with respect to the lower limit. The bead-like structures described as "artifacts"

by Perner (1952c) may correspond to the strings of Cleland and Slater (1953b). The sizes of isolated microsomes have been given for preparations from peanut cotyledons, 20 mμ (Newcomb and Stumpf, 1952), and bean roots, 20 to 40 mμ (Robinson and Brown, 1953) or 40 mμ (Brown and Fitton Jackson, 1953). Slautterback (1953) has shown the presence of three distinctly different elements in mouse liver microsome fractions; the mean diameters were 129 mμ (large), 79 mμ (intermediate), and 22 mμ (small).

The fact that plant mitochondria stain with Janus green B has been known for some time, and Sorokin (1941, 1954) has studied this technique in detail. The mitochondrial fraction isolated by differential centrifugation has been reported by various workers to contain particles staining with Janus green B (Stafford, 1951; Millerd *et al.*, 1951; McClendon, 1952; Newcomb and Stumpf, 1952; Price, 1952; Perner,1952c; Davies, 1953; Brummond and Burris, 1953). It is not at all clear that this sole characteristic can be used for the positive identification of mitochondria, as assumed by some workers (Millerd and Bonner, 1953). Lazarow and Cooperstein (1953) found that a variety of structures, including proteins and ultramicrosomes, adsorb Janus green, and Perner (1952c) found with homogenates of *Allium* epidermis that the dye was accumulated by all protein containing structures. Morphological alterations, such as swelling, which may accompany the isolation of mitochondria, can mask the staining reaction (Lazarow and Cooperstein, 1953).

Relatively little attention has been paid to the cytological homogeneity of mitochondrial fractions isolated from plant tissues. The significance of the presence of a given type of particle is difficult to evaluate in the absence of qualitative and quantitative descriptions of other particles in the fraction. Both Stafford (1951) and McClendon (1952) reported that their mitochondrial fractions contained very heterogeneous mixtures of particles. These workers used a Waring blendor for the initial tissue maceration. Davies (1953) describes his preparation as homogeneous, and Price and Thimann (1954a) have recently reported the isolation of a mitochondrial fraction from pea stems, the homogeneity of which, under the phase microscope, was directly comparable to that of liver mitochondria fractions. It is reported that Jagendorf (1953) has obtained a fraction containing 85% mitochondria and 15% chloroplasts from leaf tissue. Although no completely intact and homogeneous preparation of plant mitochondria has been prepared, the cytological evidence cited above leaves little doubt that the fraction which has been employed in biochemical investigations does contain a large fraction of mitochondria.

VII. Chemical Composition

On the basis of indirect evidence, such as the response to cytological stains and fixatives, it was early realized that mitochondria contain both protein and lipid. Direct chemical analyses of isolated plant mitochondria have now provided more quantitative information as to their composition. Stafford (1951) reported that 30–40% of the mitochondrial dry weight was protein, and the value calculated from Levitt's (1954b) data falls within this range. These values are much lower than the 73% reported for liver mitochondria (Strittmatter and Ball, 1954); that some protein might have been lost during preparation is suggested by the fact that liver mitochondria contain large amounts of soluble protein which are lost following sonic disruption (Hogeboom and Schneider, 1950a). Determinations of the distribution among the various centrifugal fractions has shown 18% (Stafford, 1951) and 10–20% (Price, 1952) of the total homogenate protein was recovered in the mitochondrial fraction; Strittmatter and Ball found a value of 23% for liver cells. Several reports (Robinson and Brown, 1953; Levitt, 1954b; De Deken-Grenson, 1954) indicate that the microsomes of plant cells are richer in protein than are the mitochondria. The lipids in isolated pea and potato mitochondria constitute 25–38% (Stafford, 1951) and 21–22% (Levitt, 1954a) of the dry weight, respectively; a comparable value for liver mitochondria is 27% (Strittmatter and Ball, 1954). On the basis of phosphorus and nitrogen analyses of potato mitochondrial lipids, Levitt (1954b) suggests that they are rich in phospholipids; a similar conclusion has been reached for animal mitochondria (Kretchmer and Barnum, 1951). The values quoted for the protein and lipid content of plant mitochondria account for roughly 70% of the dry weight.

Several reports (Stafford, 1951; McClendon, 1952; Chayen, 1952; Brown and Fitton Jackson, 1953) have established the fact that plant mitochondria contain ribonucleic acids (RNA). The ease with which nucleic acid is washed out of particles (Stafford, 1951; Dianzani, 1953) makes quantitative estimation difficult. Although Stafford (1951) found that RNA accounted for less than 1% of the mitochondrial dry weight, calculations based on the more recent values of 0.064 g. RNA-phosphorus/g. protein nitrogen (McClendon, 1952) and 0.8 mg. RNA/mg. nitrogen (Brown and Fitton Jackson, 1953) suggest that the value is closer to 5–6%. Roughly 60–70% of the total cellular RNA remains in the supernatant after removing the mitochondria (Stafford, 1951; McClendon, 1952). Some of this is associated with the microsomes which are richer in RNA than are the mitochondria (Stafford, 1951; Brown and Fitton Jackson, 1953). While most of the deoxyribonucleic acid (DNA) is in the

nuclei, there is evidence that a small quantity may also be present in plant (Stafford, 1951; McClendon, 1952) as well as animal (Schneider and Ross, 1950) mitochondria. Cytological evidence for the presence of DNA-containing bodies in the cytoplasm of plant cells was reported earlier (Sparrow and Hammond, 1947).

A number of vitamins and cofactors have been shown to be chemical constituents of isolated liver mitochondria (see Schneider, 1953). Seifter (1953) studied the intracellular distribution of coenzyme A in mung bean seedlings and spinach leaves and found it to be most concentrated in the mitochondria. On the basis of silver nitrate reduction in fixed cells, Chayen (1953) has indicated the probable localization of ascorbic acid in the mitochondria of root meristems. Potato mitochondria apparently contain some carbohydrate, measurable as glucose (Levitt, 1954b).

VIII. Respiration

In 1912, Kingsbury first proposed that mitochondria function in cellular respiration. Nearly forty years later, such a function had not been definitely established for the mitochondria of plants (Newcomer, 1951), although there was strong evidence that they were centers of respiratory activity in animal cells. Work during the past few years has shown that plant mitochondria play a similar role. One type of evidence is cytological: for example, elucidation of the mechanism of Janus green B staining (Lazarow and Cooperstein, 1953) establishes the mitochondria as probable centers of oxidation-reduction reactions. A similar conclusion is suggested by the presence of localized areas of tetrazolium reduction in the cytoplasm of plant cells (Dyar, 1953). Perner (1952a) found that berberin sulfate inhibits respiration, and, since it stains the "microsomes," he concluded that these particles are centers of respiratory activity. The vast majority of the evidence is biochemical and comes from work with isolated plant mitochondria. In the following, the function of the mitochondria will be considered in relation to glycolysis, the oxidative reactions of the Krebs cycle, and electron transport mechanisms.

1. Glycolysis

The term "glycolysis" is used here in the most general sense and refers to the disappearance of glucose, without implication as to pathway. We will consider the intracellular localization of starch formation as well as sugar breakdown. Yin and Sun (1949) incubated thin sections of soybean root with glucose-1-phosphate, and, following staining with iodine, found starch exclusively in the leucoplasts. Using a similar technique, Dyar (1950) observed starch formation both in the nucleus and in nonplastid

cytoplasmic granules of pea root tips. Thus, the cytochemical evidence suggested that phosphorylase might be present in any of three different particulate structures. In sharp contrast, Stafford's (1951) analysis of cell fractions isolated by centrifugation indicated that both phosphorylase and amylase were present exclusively in the soluble fraction. Stocking (1952) examined this problem in leaf tissue; cytochemical tests did not reveal polysaccharide formation in the chloroplasts but rather in chains or groups of small granules dispersed throughout the cytoplasm; biochemical tests indicated, in agreement with Stafford, that phosphorylase was present in the soluble fraction rather than in the particles. On the other hand, Paech and Krech (1953) have shown, using sections of wheat leaves, that starch formation takes place in the chloroplasts and this finding has recently been confirmed by Shaw (1954). Isolated chloroplasts were unable to synthesize starch when incubated with glucose-1-phosphate, and Paech and Krech concluded that the phosphorylase normally localized in chloroplasts is solubilized when the cells are disrupted. In maize endosperm, Duvick (1953) has localized starch in both plastids and mitochondria-like structures.

This summary indicates the confusion regarding the localization of phosphorylase in the intact plant cell and emphasizes the critical importance of the experimental method employed. The limitation of starch synthesis in the cell by some factor other than the availability of phosphorylase might explain the observed differences. If, as suggested by the cytochemical evidence, phosphorylase is localized on plastids, this is probably an extremely labile association. The appearance of starch-containing grains around the plastids has been attributed by Paech and Krech (1953) to the movement of the enzyme out of the plastids. In this connection, evidence that phosphorylase (Siegel, 1952) and amylase (Brakke and Nickell, 1951) are excreted by plant cells, and that sugar absorption may be controlled by phosphorolysis at the outer cell membrane (Street and Lowe, 1950; Brown, 1952) is of great interest. It is possible that phosphorylase is associated with the plastid surface. The reported synthesis of starch in the nucleus (Dyar, 1950) might be correlated with an aggregation of plastids around the nucleus. The fact that mitochondria-like cytoplasmic granules can develop into starch grains (O'Brien, 1951) may explain the observed localization in the nonplastid particulates (Dyar, 1950; Stocking, 1952; Duvick, 1953).

The enzymes involved in the breakdown of glucose to pyruvic acid via the classical Embden-Meyerhof pathway have been identified in higher plants (see Stumpf, 1952). Saltman (1953) has studied the intracellular distribution of hexokinase which catalyzes the initial conversion of glucose

to glucose-6-phosphate. Homogenates of various plant tissues were prepared in 0.5 *M* mannitol, and the mitochondria, isolated by centrifugation, were found to contain between 100% (potato tuber) and 19% (*Avena* seed) of the total hexokinase activity, the remainder being present in the soluble fraction. Saltman suggests that there may be both soluble and insoluble forms of the enzyme, and that the major part is associated *in vivo* with the mitochondria. The hexokinase of animal tissues is also localized on particles (Crane and Sols, 1953). Work with centrifuged homogenates of green leaves indicates that aldolase (Tewfik and Stumpf, 1949), phosphoglyceraldehyde dehydrogenase (Arnon, 1952), and lactic dehydrogenase (Nason, Oldewurtel, and Propst, 1952) are all soluble enzymes. By extracting with a nonaqueous medium, Stern and Mirsky (1952) have been able to show that some of these "soluble" enzymes, including aldolase, phosphoglyceraldehyde dehydrogenase, enolase, and pyruvate kinase, are present in the nuclear fraction isolated from wheat germ. Their report (Stern and Mirsky, 1953) that the nuclear membrane is unable to prevent the outward diffusion of protein when a sucrose medium is used indicates the need for caution in interpreting results obtained with aqueous media.

From the above it appears that the enzymes involved in the conversion of glucose to pyruvic acid in plants are present in more than one cellular fraction. Normally, this conversion probably involves the interaction of the mitochondria with other cellular constituents. That a similar situation exists in animal cells is indicated by the work of LePage and Schneider (1948). They found that lactic acid was produced by the supernatant fraction alone, but that this activity was increased when mitochondria, which alone produced no acid, were added; the nuclear and microsomal fractions also promoted glycolysis. Kaplan, Still, and Mahler (1951) subsequently showed that the enzymes implicated in the oxidation-reductions of glycolysis are at least in part on the mitochondria of kidney cells. More recently, Hesselbach and DuBuy (1953) obtained washed brain mitochondria which could convert glucose to lactic acid. Although glycolysis may be largely a function of the soluble cytoplasm, these findings suggest that the mitochondria may play an important role.

Alternate pathways of glucose metabolism have been explored intensively in recent years (see Racker, 1954). The existence in higher plants of the "hexosemonophosphate shunt" enzymes for the metabolism of glucose-6-phosphate has now been established (Axelrod *et al.*, 1953; Gibbs, 1954). With labeled glucose, Beevers and Gibbs (1954) have provided evidence that this pathway functions in intact plant cells; they found an increasing participation of the "shunt" with age. All of the enzymes of

the "shunt" are present in extracts which have been centrifuged at 18,000 × *g* for 20 minutes (Gibbs, 1954), and there is as yet no evidence of their localization on plant mitochondria. The recent report (Wood and Schwerdt, 1954) that all of the glucose-6-phosphate dehydrogenase in *Pseudomonas fluorescens* extracts is found in the particulate fraction, which also contains the cytochrome components, suggests the possible involvement of mitochondria in the "shunt" pathway.

Apart from the direct enzyme studies, there is abundant evidence of an indirect role of mitochondria in regulating glycolysis. The hypothesis that glycolysis is limited by the supply of phosphate acceptors (see Dickens, 1951) has gained support, and it is known that the availability of these acceptors is intimately related to mitochondrial reactions. Thus, the operation of the Pasteur effect depends on phosphorylation in the mitochondria. Newcomb (1950) and Beevers (1953a) have shown that 2,4-dinitrophenol (DNP), which uncouples phosphorylation from oxidation, increases aerobic fermentation in higher plant tissues. Masacré and Paris (1951) have made both biochemical and cytological studies with a view to directly correlating structure and function. They found that the destruction of root mitochondria by acrolein vapors was paralleled by the hydrolytic production of sugars, suggesting a possible relation between the mitochondria and sugar metabolism.

2. *Krebs Cycle*

a. Demonstration. Many studies with intact tissues have favored the view that a Krebs cycle is operative in higher plants for the metabolism of pyruvate. However, final proof of its existence was only obtained as a result of work with isolated mitochondria. After considering this evidence, the individual reactions of the cycle will be discussed. In 1951, Millerd *et al.* isolated particles from mung bean (*Phaseolus aureus*) seedlings which could carry out the oxidation of pyruvate to CO_2 and water. The particles were identified on the basis of Janus green staining and almost certainly included mitochondria. This mitochondrial fraction oxidized citrate, α-ketoglutarate, and succinate rapidly, while fumarate, malate, and pyruvate were more slowly oxidized. The oxidation of pyruvate was greatly increased by adding small "sparker" amounts of any one of the other five acids, thus providing strong evidence that the enzymes of an integrated Krebs cycle were present in the particles. Working independently in England, Davies (1953) isolated, from *Pisum* seedlings, Janus green-staining particles which oxidized all of the acids listed above as well as isocitrate. He showed that small amounts of malate could "spark" the utilization of pyruvate, measured either by oxygen uptake or

pyruvate disappearance. Using chromatographic methods for the identification of individual acids, Davies went on to show that the particulate preparation could carry out all of the single-step reactions of the Krebs cycle. Brummond and Burris (1953) have provided direct evidence for the participation of the cycle in particles prepared from etiolated white lupine (*Lupinus albus*) cotyledons with the demonstration that radiocarbon from pyruvate-2-C^{14} either appears as $C^{14}O_2$ or is transferred into the intermediates of the cycle. Oxidation of pyruvate-2-C^{14} plus unlabeled malate by the particles resulted in the incorporation of C^{14} into all of the cycle acids, including *cis*-aconitic, oxalosuccinic, and oxalacetic. A final criterion for the operation of the Krebs cycle has been fulfilled with Laties' (1953c) demonstration that malonate inhibits the "sparked" pyruvate oxidation by cauliflower (*Brassica oleracea*) bud mitochondria. Davies (1954a) has recently reported that when oxalacetate and pyruvate are oxidized in the presence of malonate, succinate is accumulated.

These findings leave little doubt that the tricarboxylic acid cycle operates in certain plant tissues and that the enzymes necessary for its integrated activity are present in the cytoplasmic particulates or mitochondria. In addition to the cases cited above, malate-sparked pyruvate oxidation and/or the oxidation of the principal cycle acids have been reported for mitochondrial preparations from the following: hypocotyls of etiolated white lupine seedlings (Dow, 1952); stems of etiolated peas (Price, 1952); peanut (*Arachis hypogaea*) cotyledons (Newcomb and Stumpf, 1952); the fruit of avocado, *Persea americana* (Millerd *et al.* 1953); the sterile portion of the spadix of *Arum maculatum* (Hackett and Simon, 1954).

b. Individual Reactions. Since the "sparked" oxidation of pyruvate by mitochondria is a cyclic process, the overall reaction gives little information about the initial step whereby pyruvate enters the cycle. Davies (1953) showed that pea particles could synthesize citrate from pyruvate and oxalacetate. Brummond and Burris (1953), using C^{14}, found that the molar specific activities of citrate and pyruvate were essentially the same, indicating that the citrate arose directly from the pyruvate and malate supplied. The recent study of Seifter (1953) has shown that mitochondrial preparations from mung bean seedlings can carry out the following reaction:

$$\text{pyruvate} + \text{Co A} + \text{DPN}^{+} \xrightarrow{Mg^{++}} \text{acetyl-Co A} + CO_2 + \text{DPNH}$$

Further experiments with purified preparations demonstrated the cocarboxylase requirement of this reaction, and some evidence was supplied for the participation of α-lipoic acid. Seifter's proposed scheme for pyru-

vate oxidation includes an initial decarboxylation and successive formation of acetyl-dihydrococarboxylase, acetyl-α-lipoic acid, and acetyl-Co A; the reduction of DPN (diphosphopyridine nucleotide) is coupled to the oxidation of reduced lipoic acid. Plant particles are thus able to oxidize pyruvate to "active acetate", known to be involved in a great variety of metabolic reactions (see Novelli, 1953; Ochoa, 1954). One of these reactions, the synthesis of citrate, is mediated by the "condensing enzyme", which Seifter has shown to be present in mung bean mitochondria. In their study of acetate activation in plant systems, Millerd and Bonner (1954) found that an acetone powder of spinach leaves contained very little condensing enzyme. Earlier, it had been shown that, in whole homogenates of potato tubers (Barron *et al.*, 1950) and tomato stems (Link, Klein, and Barron, 1952), citrate is synthesized from acetate and oxalacetate, in the presence of ATP and malonate. In summary, it seems likely that pyruvate enters the Krebs cycle associated with plant mitochondria by a reaction in which acetyl-Co A is first formed and then condensed with oxalacetate to give citrate.

Added citrate is rapidly oxidized by mitochondria prepared from all of the tissues described above and also by particles isolated from apple fruits (Pearson and Robertson, 1954). The presence of aconitase, a hydration-dehydration enzyme, in plant mitochondria is indicated by their ability to convert *cis*-aconitate to citrate and isocitrate to citrate (Davies, 1953; Seifter, 1953). By working at pH 9, Davies (1953) was able to show that oxidation of isocitrate is accompanied by an accumulation of α-ketoglutarate. The expected intermediate in this reaction, oxalosuccinate, is labeled by the C^{14} from pyruvate (Brummond and Burris, 1953). Isocitric dehydrogenase occurs widely in plants and is generally assumed to be a TPN (triphosphopyridine nucleotide)-specific enzyme (Vennesland and Conn, 1952). However, Davies observed a large stimulation by DPN and suggested that either the pea mitochondria are able to convert DPN to TPN or that they possess a DPN-specific isocitric dehydrogenase similar to the one present in yeast (Kornberg and Pricer, 1951). By disrupting isolated mitochondria, Davies (1954b) has now shown that both a TPN and a DPN isocitric dehydrogenase are present on the particles. The soluble fraction of centrifuged plant homogenates contains an active TPN-specific dehydrogenase (Anderson *et al.*, 1952; Davies, 1954a), and in liver cells 80% of this enzyme is soluble (Hogeboom and Schneider, 1950b).

Plant mitochondria which have been prepared in a sucrose-phosphate medium readily oxidize α-ketoglutaric acid. Homogenization in water, or the brief exposure of isolated particles to water, can destroy the ability to oxidize α-ketoglutarate (Laties, 1953a). Brief exposure of cauliflower

mitochondria to a dilute KCl solution does not destroy this capacity, indicating that the hypotonic injury is only evident in the absence of salts or in the presence of soluble cytoplasmic factors. The presence of salts might act to maintain some structural integrity necessary for the oxidation. Using the Thunberg method, Price (1953) could not detect any α-ketoglutaric dehydrogenase in pea particles, although a rapid oxygen uptake was easily demonstrated. Direct measurements showed that α-ketoglutarate disappears at a rate corresponding to only one-half the observed oxygen uptake, indicating oxidation to the level of fumarate; a similar conclusion was reached on the basis of R.Q. determinations with *Arum* particles (Hackett and Simon, 1954). In the presence of malonate, succinate accumulates during α-ketoglutarate oxidation (Davies, 1953). However, the use of malonate to block oxidation at the succinate stage is complicated by a malonate inhibition, possibly due to combination with enzyme-bound magnesium, of the α-ketoglutaric oxidase itself (Price, 1953). Brummond (1954) identified an α-ketoglutaric dehydrogenase in the particulate fraction of green leaves, but no cofactors were demonstrable in the solubilized enzyme.

Marked promotions of the oxygen uptake by plant mitochondria in the presence of α-ketoglutarate have been observed with Mg^{++}, ATP, and DPN (Millerd, 1953; Laties, 1953b; Hackett and Simon, 1954; Price and Thimann, 1954a). Both DPN and ATP more than double the oxidation rate, and these effects are not additive; comparable promotions were given by ADP (adenosinediphosphate) and AMP (adenosinemonophosphate), whereas adenosine and adenine produced no stimulation (Laties, 1953b). Laties suggests that the requirement is for a phosphorylated derivative of adenylate, which could be derived from DPN by the pyrophosphatase. That this requirement may be dual in nature is indicated by the following: in the presence of hexokinase and glucose, the α-ketoglutarate oxidation is more than trebled and the adenylate dependence is shifted to a tenfold lower concentration range. Adenylate may thus be required both in the secondary transfer of energy to form ATP (at high concentrations) and as a primary phosphate acceptor or coenzyme for the oxidation itself. Laties' conclusion is similar to that of Lindberg and Ernster (1952), who proposed that AMP may be involved in the generation and ADP in the transfer of energy-rich phosphate bonds. In his examination of the effects of hypotonic media, Laties (1953a) found that the oxidative and phosphorylative activity associated with α-ketoglutaric oxidase were almost equally sensitive, which further suggests that the oxidation depends on concomitant phosphorylation. Similar findings have been reported for heart-muscle sarcosomes (Slater and Cleland, 1953).

Work during the past few years has established the widespread distribution of the succinate oxidizing system in plants (e.g. Bhagvat and Hill, 1951). In 1939, Hill and Bhagvat, and Okunuki, working with particles isolated from *Phaseolus* seeds and *Lilium* pollen respectively, showed that the oxidation of succinate could be promoted by cytochrome c and inhibited by cyanide or malonate. Shortly after, Damodaran and Venkatesen (1941) used methylene blue to detect succinic dehydrogenase activity, and found it localized entirely in the particulate fraction following centrifugation; they also measured the fumaric acid formed during the oxidation. These early experiments established all the major features of this reaction. In recent investigations, succinate oxidation has been measured quantitatively by following the rate of dye reduction, for which 2,6-dichlorophenolindophenol is more suitable than methylene blue (Millerd, 1951; Price and Thimann, 1951), or of oxygen consumption; spectrophotometric determination of cytochrome c reduction has also been used (Stafford, 1951). The optimal extraction medium varies, depending on the method of assay: water, for the dehydrogenase alone, and sucrose-phosphate medium for manometric methods (Millerd, 1953; Price and Thimann, 1954a). Hypotonic conditions destroy the associated phosphorylation without affecting the oxidation of succinate, indicating that these processes are not obligatorily coupled (Laties, 1953a). The washing of cauliflower buds in water at 25°C. decreases the succinoxidase activity of subsequently extracted particles, suggesting an effect of hypotonic conditions on the mitochondria *in situ* (Laties, 1954). Laties also discovered a soluble succinic dehydrogenase inhibitor in the buds. The exclusive localization of the dehydrogenase on particulate components in pea seedlings has been reported (Stafford, 1951; Price and Thimann, 1954a). On the other hand, Brummond (1954) reports the unusual finding of a soluble succinic dehydrogenase in green leaves.

Direct measurements of the disappearance of succinate have indicated that, in the absence of cofactors, it accounts for nearly 90% of the observed oxygen uptake (Price and Thimann, 1954a). The reaction products have been identified as fumarate and malate (Millerd, 1951; Davies, 1953); oxidation to this level is probably considerably more rapid than the subsequent stages of the Krebs cycle. Magnesium ions and ATP stimulate the oxygen uptake (Millerd, 1953; Laties, 1953b), but only the former affects succinate oxidation *per se*, whereas ATP promotes the oxidation of further intermediates (Price and Thimann, 1954b).

The presence of fumarase on plant particles, indicated by the partial conversion of succinate to malate, has been shown directly under anaerobic conditions by Davies (1953). Malate has been used to spark pyruvate

oxidation and is itself oxidized, though rather slowly, by particulate preparations. Malic dehydrogenase may be readily studied by determining the rate of dye (e.g. 2,6-dichlorophenolindophenol) reduction in the presence of cyanide, used to trap the oxalacetate formed. Particles isolated in water show an active dehydrogenase, but hypotonic media greatly reduce both the malic oxidase activity and its associated phosphorylation (Price and Thimann, 1954a; Laties, 1953a). Malic dehydrogenase is present both in particles and in solution; one fourth of the total activity of the homogenate was localized in pea mitochondria (Price and Thimann, 1954a). The dehydrogenase shows an almost absolute requirement for DPN, whereas the oxygen uptake in the presence of malate, though promoted by added DPN, is appreciable in its absence (Davies, 1953; Laties, 1953b; Price and Thimann, 1954a). This suggests that there may be a particle-bound coenzyme which is removed during the preparation of the dehydrogenase. DPN cannot be replaced in this reaction by either TPN or ATP. The oxalacetate formed during the dehydrogenation of malate can be decarboxylated by plant particles to give pyruvate (Davies, 1953; Laties, 1953c). Malate can be converted directly to pyruvate by the malic enzyme which is apparently present in the soluble fraction of many plants (Arnon, 1951; Anderson *et al.*, 1952) and may also be associated with mitochondria (Davies, 1954b).

c. Discussion. The above evidence establishes the presence, in a wide variety of plant tissues, of particles, probably mitochondria, which possess an integrated enzyme complex capable of carrying out the reactions of the Krebs cycle. Two relevant questions remain: (1) What is the precise role of the cycle and its quantitative importance in respiration? and (2) Is this system present in all plant tissues? The answer to the first question depends in large part on studies with intact tissues which are beyond the scope of this review (see Laties, 1952; Burris, 1953). Such studies, as well as the homology between the enzymatic content of plant and animal mitochondria, strongly support the conclusion that at least in certain plant tissues the Krebs cycle is a major respiratory pathway. Quantitative comparisons of the oxygen uptake of a tissue and the maximum activity of its isolated enzymes should throw some light on the ability of the cycle to mediate respiration. Millerd (1953) claimed that the mitochondria can oxidize succinate at a sufficient rate to account for the total respiration of mung bean hypocotyls. Since oxygen uptake in the presence of succinate is predominantly the result of the one-step oxidation to fumarate, this finding establishes only the sufficiency of this single step in the cycle. Price and Thimann (1954b) have recently made a careful comparison of several mitochondrial enzymes and tissue respiration in *Avena* coleoptiles

and pea stem sections. Assuming there are six respiratory dehydrogenations (five in the cycle and one in glycolysis), they argue that each dehydrogenation needs to proceed at one-sixth of the total rate of oxygen consumption. Experiments indicated that roughly 80% of the tissues' dehydrogenases were extracted. More than adequate amounts of succinic and malic dehydrogenase were recovered; in the latter case, the excess was large enough to suggest that the 75% of the enzyme appearing in the soluble fraction may have a separate function. Ketoglutarate oxidation was somewhat slower than the theoretical value, but truly optimal conditions were probably not established. On the other hand, it is pointed out that in extracted systems pyruvate oxidation is quite variable and proceeds only slowly.

The presence of all the component reactions of the Krebs cycle does not of itself establish this as the main route of respiration. In yeast, the required enzymes are present and are associated with particles (Nossal, 1954), nevertheless, Krebs, Gurin, and Eggleston (1952) have concluded that the cycle is not the main mechanism of oxidation; a similar situation has been described in *E. coli* (Roberts *et al.*, 1953). Recent work with various microorganisms, including *Pseudomonas aeruginosa* (Campbell, Smith, and Eagles, 1953), *E. coli* (Wheat and Ajl, 1954), and *Aspergillus niger* (Cleland and Johnson, 1954) indicates the presence of alternate or modified cyclic mechanisms for the oxidative removal of acetate and pyruvate; a common feature of these schemes is a $C_6 \rightleftharpoons C_2 + C_4$ type of reaction. Since malonate inhibition is commonly used to indicate respiration mediated by the Krebs cycle, the discovery (Bentley, 1952) of large amounts of malonate in some plant cells, probably localized within the vacuole, is noteworthy.

With respect to the second question raised concerning the operation of the Krebs cycle, the situation in green leaves is of special interest. Although many of the cycle intermediates and enzymes have been demonstrated in mature leaves, the C^{14} of $C^{14}O_2$ equilibrates only slowly among the various acids, suggesting a sluggish operation of the cycle (Burris, 1953). Brummond (1954) has recently made a direct attempt to obtain particles possessing the Krebs cycle complex from photosynthesizing tissue. Using methods which are successful with etiolated tissues, Brummond found that the cycle enzymes are not all present in the particulate fraction prepared from lupin leaves. Aconitase, α-ketoglutaric dehydrogenase and a trace of malic dehydrogenase are localized on the particles. The soluble fraction contains (1) TPN-specific isocitiric dehydrogenase, which produces a mixture of α-ketoglutarate and oxalosuccinate, (2) malic dehydrogenase, which utilizes either DPN or TPN and gives rise to oxalacetate, and (3)

nonmitochondrial succinic dehydrogenase, an enzyme which has resisted solubilization by drastic experimental methods. A weak condensing enzyme was found in ground whole leaves. Brummond suggests that photosynthetic activity may be correlated with a disintegration of the mitochondrial complex and that the cycle may not be active in green tissues.

3. *Electron Transfer*

a. To Cytochrome c. We will now consider the cellular mechanisms whereby electrons are transferred from the level of the pyridine nucleotides to oxygen. In animal mitochondria, electrons are passed via a flavin enzyme to the cytochrome system and thence to oxygen (Slater, 1950). Although the terminal cytochrome oxidase of plants has been investigated, intermediate steps in the electron transfer from DPNH and TPNH have been little studied. Lockhart (1939) demonstrated the presence of diaphorase in plants, and Smith (1952) has examined its role in corn embryo extracts. Diaphorase is largely present in the soluble fraction following centrifugation (Nason *et al.*, 1952). Davison (1950) partially purified both DPN- and TPN-cytochrome c reductases from extracts of pea seedlings. The intracellular localization of these enzymes in green leaves was investigated by Rosenberg and Ducet (1951), and though they found active reductases in the supernatant fraction, no activity was detected in the particulate fraction sedimented at 20,000 $\times$ *g*. However, recent experiments have established the presence of DPN-cytochrome reductase in plant mitochondria (Davies, 1954b; Evans, 1954; Hackett, 1954). In potato homogenates, a much more active reductase system is present in the soluble fraction than in the particles, but its nature is not yet clear (Hackett, 1954). It is of interest that Strittmatter and Ball (1954) have shown that there is a high concentration of cytochrome reductase in rat liver microsomes, and this may in part account for the activity of the "soluble" potato fraction.

Recent studies have indicated that heart DPN-cytochrome reductase is an iron-containing flavoprotein (Mahler and Elowe, 1953), in which the iron mediates electron transfer between flavin and cytochrome (Weber, Lenhoff, and Kaplan, 1954). The plant reductase is apparently very similar; Davies (1954b) has demonstrated an iron requirement, and Evans (1954) the flavin content of the enzyme. In some animal mitochondria there is also a BAL- and antimycin A-sensitive factor between the flavin and cytochrome c (Slater, 1950; Potter and Reif, 1952). With potato mitochondria, antimycin A partially inhibits the particulate DPN-cytochrome reductase (Hackett, 1954), suggesting that there are two pathways of DPN oxidation, as in some animal tissues (Reif and Potter, 1954).

Demonstration that cytochrome b_4 can act as a bypass of the factor (Pappenheimer and Williams, 1954) raises the possibility that the similar b_3 of plants (Hill and Scarisbrick, 1951) might play such a role. In liver microsomes, Strittmatter and Ball (1954) have found a hemochromogen, cytochrome m, which is responsible for a general absorption throughout the region 550–565 mμ and can be reduced by DPNH. In many plants, the cytochrome b and c bands appear fused in a single broad band at 550-565 mμ (Bhagvat and Hill, 1951), suggesting that a similar component, which may be concerned with DPNH oxidation, is present in plants. Whatever the pathway, it seems clear that in animal and plant mitochondria the oxidation of DPNH is coupled to a reduction of cytochrome c.

The oxidation of succinate by animal mitochondria does not involve a pyridine nucleotide, electrons being transferred via cytochrome b and the factor to cytochrome c (see Bonner, 1954). The cytochrome components on plant particles can be reduced by succinate (Hill and Bhagvat, 1939; Bhagvat and Hill, 1951) and Stafford (1951) has shown that succinic-cytochrome c reductase is confined to the mitochondrial fraction of a pea homogenate. Antimycin A completely blocks the reductase localized on potato particles, indicating that electrons must pass via the factor between succinate and cytochrome c (Hackett, 1954). Spectroscopic studies on living wheat roots also indicated the presence of an intermediate catalyst between cytochrome b and c (Lundegardh, 1953b). A recent report indicates that succinic dehydrogenase is a complex molecule containing both inorganic iron and hemes (Green *et al.* 1954).

b. Cytochrome Oxidase. Many studies have shown that plant particles contain an active cytochrome oxidase. Hill and Bhagvat (1939) reported that the insoluble residue from a *Phaseolus* cotyledon homogenate can rapidly oxidize reduced cytochrome c. That this oxidation can serve in a respiratory sequence from DPNH to oxygen was early shown by Okunuki (1939b). Spectroscopic examination has shown that a complete cytochrome system (a, b, and c bands) is present in isolated plant particles (Bhagvat and Hill, 1951); using a differential spectrophotometer, Chance (1952) has identified cytochromes a and a_3 in pea particles. Chance's simultaneous determinations of oxygen uptake and optical density changes indicated that the plant cytochrome oxidase is as active as that in heart particulates, although cytochrome c is present in relatively small amounts. Various workers have reported cyanide and azide inhibition of plant particle cytochrome oxidase, and the characteristic carbon monoxide (CO) inhibition and light-reversal have been demonstrated (Goddard and Holden, 1950; Webster, 1952). Mapson *et al.* (1954) have identified the terminal enzyme involved in the oxidation of L-galacto-γ-lactone to L-as-

corbic acid by mung bean mitochondria as cytochrome oxidase on the basis of its light-reversible CO inhibition. They have established two important additional points with these particles: (1) the enzyme has the very high affinity for oxygen characterstic of cytochrome oxidase, and (2) azide inhibition could only be demonstrated in the presence of respiration substrates, such as succinate or α-ketoglutarate, indicating that there is a competition among the various substrates for the terminal cytochrome; the residual oxidase in the presence of 1×10^{-3} M azide is sufficient to allow both endogenous respiration and the increase due to the added lactone. It appears that the cytochrome oxidase associated with plant particles is in all respects—absorption spectrum, inhibitor sensitivity, oxygen affinity, and activity—similar to the enzyme of animal mitochondria.

The very general distribution of particle-bound cytochrome oxidase in plants has been shown by Bhagvat and Hill (1951), Ducet and Rosenberg (1951), and Webster (1952); in the last study, more than 90% of the 54 species examined showed activity. The intracellular distribution has been carefully examined in several cases. Goddard and Holden (1950) and Webster (1952) have shown that essentially all of the activity of the homogenate is present in the $16{,}000 \times g$ pellet; Stafford (1951) recovered between 63 and 100% of the activity in the mitochondrial fractions. Working with a homogenate of green leaves, Rosenberg and Ducet (1949) reported cytochrome oxidase in the "chloroplast" fraction sedimented at $25{,}000 \times g$, and DuBuy *et al.* (1950) found activity in both plastids and mitochondria. More recently, McClendon (1953) has shown that there is little activity in the chloroplasts which sediment with a low centrifugal force and can be followed by determining the chlorophyll content of the fractions. He concludes that the cytochrome oxidase of leaves is probably all in the mitochondria. In fungi, as well as in the higher plants, this enzyme is bound to small particles (Darby and Goddard, 1950; Holter and Pollock, 1952; Sussman and Markert, 1953). Other possible intracellular sites of cytochrome oxidase that have been suggested are the protoplasmic surface (Lundegardh, 1952) and the tonoplast (Butler, 1953), although these have not yet received experimental support. With respect to the intramitochondrial localization of the enzyme, there is evidence which suggests that it is situated on the mitochondrial membrane (Cleland and Slater, 1953a).

c. Alternate Pathways. Studies on the respiration of plant tissues have shown that cytochrome oxidase is the general terminal oxidase (see Hill and Hartree, 1953), and the central role of the mitochondria in respiration is thereby established. However, evidence has also been obtained which suggests that an alternate pathway may be operative in certain

tissues (see James, 1953a). Although polyphenol oxidase has long been proposed as a terminal oxidase in respiration, such a role now appears most unlikely. Plant extracts and isolated enzymes can oxidize DPNH by this pathway (Link *et al.*, 1952; Wosilait, Nason, and Terrell, 1954), but this has not been shown in intact tissues. Fractionation of plant homogenates has demonstrated that polyphenol oxidase is very largely in the supernatant following high speed centrifugation (Goddard and Holden, 1950; Webster, 1952; Nason *et al.*, 1952; Todd, 1953; Sussman and Markert, 1953), which suggests that it is soluble in the cell. Nevertheless, a small amount of activity remains in the particulate fractions, and a mitochondrial localization of the enzyme has been claimed in both animal (Hesselbach, 1951) and plant (Jagendorf and Wildman, 1954) tissues. Polyphenol oxidase is present in the particulate fraction of leaf homogenates (Arnon, 1948; Ducet and Rosenberg, 1951), and McClendon (1953) has shown that it is associated with the chloroplasts. His finding that the enzyme can be washed off the particles emphasizes the need for caution in interpreting all of these results.

Model systems have been constructed, with plant extracts, which will oxidize DPNH or TPNH via ascorbic acid oxidase (Mathews, 1951; Mapson, 1953; Beevers, 1953b; Nason, Wosilait, and Terrell, 1954; Kern and Racker, 1954). Although a respiratory role has been proposed for this enzyme (Waygood, 1950; James, 1953b), this has not yet been established conclusively. Stafford (1951) found that 70% of the total homogenate ascorbic acid oxidase was recovered in the final supernatant. The remaining activity associated with particles was ascribed to an atypical ascorbic oxidase, insensitive to azide and diethyldithiocarbamate. Newcomb (1951, 1954) has fractionated tobacco pith homogenates and found that at least 82% of the ascorbic acid oxidase activity is associated with the cell-wall fraction, which sediments at less than $500 \times g$, whereas cytochrome oxidase is concentrated in the mitochondria. In pith sections, both the formation of ascorbic oxidase and cell enlargement can be inhibited by phenylthiourea with little or no effect on respiration. Newcomb concludes that the enzyme does not act as a terminal oxidase for respiration, and suggests that it may play a role in the metabolism of the wall polysaccharides or the intracellular cement. The surface localization of an atypical, cyanide-insensitive ascorbic acid oxidase has been established in fungus spores (Mandels, 1953). The ability to oxidize ascorbic acid has also been reported for a soluble tomato leaf fraction (Nason *et al.*, 1952) and for mung bean mitochondria (Mapson *et al.*, 1954). Since the acid can be oxidized indirectly by several different oxidases, this information alone is not adequate for a specific localization.

Some reduced flavoproteins can react with oxygen to produce H_2O_2, which may then be decomposed or react with various substrates. An example is the flavoprotein glycolic acid oxidase (Zelitch and Ochoa, 1953), and Zelitch (1953) has suggested that this enzyme may serve in a respiratory role between DPNH and oxygen. The enzyme is very largely soluble (Nason *et al.*, 1952; Tolbert and Cohan, 1953a), although some activity is present in the particles (Tolbert and Cohan, 1953b; Zelitch, 1953). The activation of the oxidase by light (Tolbert and Cohan, 1953a) suggests that it may function in photosynthesizing tissue. The soluble enzymes of the oxidative hexosemonophosphate shunt may be flavoproteins.

The nature of the terminal oxidase mediating respiration in a given tissue can only be determined by a study of the intact cells. Some evidence which has been obtained in this way has been interpreted as indicating the nonparticipation of cytochrome oxidase; however, this conclusion needs to be considered very critically. Hill and Hartree (1953) have discussed the limitations of such evidence as the absence of cyanide and/or CO inhibition of respiration. That cytochrome oxidase may be functional in tissues whose oxygen uptake is apparently unaffected by CO has been shown directly by measuring effects on the R.Q. (Okunuki, 1939a; Ducet and Rosenberg, 1953). A number of cases have been reported in which a tissue with a CO-insensitive respiration possesses an extractable cytochrome oxidase (Okunuki, 1939b; Ducet and Rosenberg, 1951; Darby and Goddard, 1950). The inability to extract the enzyme from wheat (Waygood, 1950; Butler, 1953) and barley roots (James, 1953b), although it is present in embryonic tissue, has been cited as evidence for an alternate oxidase. However, both spectroscopic (Lundegardh, 1952) and recent enzymatic studies (Fritz and Beevers, 1953) have indicated the presence of the enzyme at all stages of seedling development. The inability to demonstrate cytochrome oxidase might be related to the presence of an interfering substance, such as the enzyme polyphenol oxidase (Ducet and Rosenberg, 1951), which is known to increase in amount during development (Newcomb, 1951; Ducet and Rosenberg, 1951; Sussman and Markert, 1953). A final type of evidence which has been used is the demonstration that a tissue, such as the *Arum* spadix (James and Beevers, 1950), has a low oxygen affinity; this is more suggestive of a flavin than cytochrome oxidase. However, it has been shown that the *Arum* spadix contains particles which possess both Krebs cycle (Hackett and Simon, 1954) and cytochrome oxidase (Simon, 1954) activity. The spadix of a related plant, *Philodendron grandifolium*, has, like *Arum*, a rapid, cyanide-insensitive respiration; however, the oxygen affinity is similar to that of cytochrome oxidase (Yocum and Hackett, 1954) when measured in small

vessels. When measured in Warburg vessels, under conditions similar to those used by James and Beevers, the oxygen affinity of *Philodendron* slices is very low. It is suggested that the earlier experiments with *Arum* were complicated by diffusion, and that the argument for a terminal flavin enzyme should be reexamined. It may be concluded that in no case do the published results conclusively eliminate cytochrome oxidase as the terminal enzyme for higher plant respiration.

IX. Phosphorylation

It has been known for some time that, in animal tissues, the energy released during the passage of electrons from substrate level to oxygen is normally trapped in the form of energy-rich phosphate compounds (see Hunter, 1951). Since the reactions of aerobic phosphorylation are localized in the mitochondria, these particles serve as "power plants" of the cell. The key compound in the storage and transfer of oxidative energy is adenosinetriphosphate (ATP), and the isolation of ATP from mung beans (Albaum, Ogur, and Hirshfeld, 1950) suggested that similar systems are operative in higher plants. Direct proof that this is the case was supplied in 1951, when Millerd *et al.* reported that the oxidation of α-ketoglutarate by mung bean mitochondria can be coupled to the incorporation of inorganic P^{32} into ATP. That the mitochondria are not the only "power plants" is suggested by the interesting recent report that chloroplasts can carry out photosynthetic phosphorylation (Arnon, Allen, and Whatley, 1954). In other words, the high-energy phosphate bonds of ATP may be generated either at the expense of energy released during oxidation (involving mitochondria) or by the conversion of light energy (involving chloroplasts).

Bonner and Millerd (1953) have made a detailed quantitative study with mung bean hypocotyl mitochondria. Phosphorylation was determined by following the disappearance of inorganic phosphorus from the reaction mixture in the presence of fluoride, used to inhibit ATPase, and an ATP-trapping system. Since the particles possess an active hexokinase (Saltman, 1953), the addition of glucose alone served to trap the ATP as hexosemonophosphate. The aerobic generation of ATP is dependent on a supply of adenylate (AMP, ADP, or ATP). Simultaneous determination of phosphate and oxygen uptake permits the calculation of a P/O ratio (moles of inorganic phosphate converted to organic form/atoms of oxygen consumed), which is a measure of the efficiency of oxidative phosphorylation. In 38 experiments with α-ketoglutarate as substrate, Bonner and Millerd obtained a mean P/O ratio of 0.93. The phosphorylation associated with individual steps of the Krebs cycle was determined and in

each case the P/O ratio was approximately one. A comparison of the activity of particles prepared from different organs of the mung bean seedling showed a tenfold greater phosphate uptake for the hypocotyls than for the roots, leaves, and cotyledons.

Using cauliflower mitochondria, Laties (1953a) has obtained maximum P/O values of 2.36 with α-ketoglutarate as substrate, 1.59 with malate, and 1.42 with succinate. Biale (1953) reported a P/O of 2.4 for avocado mitochondria oxidizing α-ketoglutarate, and Bonner, Bandurski, and Millerd (1953) obtained a value of 1.26 with artichoke tuber particles. The probable value for ketoglutarate in these plant systems is 3.0. With animal mitochondria, values indicating 4.0 have been recorded (Hunter, 1951), although in a recent paper Slater and Holton (1954) conclude that it is 3.0 for the one-step oxidation of ketoglutarate. In both plant and mammalian systems, the P/O ratio for succinate oxidation is apparently two. The general conclusion seems justified that the energy-trapping mechanisms in these plant and animal mitochondria are equally efficient. The low values recorded with mung beans suggest that their phosphorylating machinery may be very labile. Hackett and Simon (1954) and Simon (1954) have been unable to get P/O values greater than 0.34 with *Arum* particles; it remains to be seen whether this is the result of inadequate experimental methods or reflects an inefficient phosphorylation mechanism.

The nature of the preparation medium exerts a marked influence on the phosphorylative capacity of mitochondria. Laties (1953a) has shown that the phosphorylation coupled to succinate oxidation is largely destroyed by exposure of cauliflower mitochondria to a hypotonic environment, whereas the oxygen uptake is essentially unaffected. With malate or α-ketoglutarate, oxidation and phosphorylation are reduced by roughly the same amount; Laties suggests that, with these substrates, phosphorylations occur at loci not shared in common with the succinoxidase system. It is possible that the phosphorylations associated with substrate and DPNH oxidation are less labile than those in the cytochrome region. That the effects of hypotonic media are related to mitochondrial structure is clear from the study of Harman and Feigelson (1952b) on heart muscle. The ability to phosphorylate depends on a certain structural integrity, which is itself dependent on the supply of high-energy phosphates.

Plant mitochondria can only generate ATP when exogenous adenylate, which serves as phosphate acceptor, is added (Bonner and Millerd, 1953; Millerd *et al.*, 1953; Bonner *et al.*, 1953; Biale, 1953). In turn, the phosphorylative capacity limits the rate of oxidation, so that mitochondrial oxidations also show an adenylate requirement. The intimate gearing of the oxidative machinery to phosphorylation is well illustrated by the

following: addition of either hexokinase or ADP to cauliflower mitochondria increases the respiratory rate and simultaneously permits the demonstration of a malonate-inhibition of malate, pyruvate, and citrate oxidation (Laties, 1953c). Thus, by overcoming the limitation imposed by endogenous phosphorylating systems, one-step oxidations are replaced by more rapid, cyclic oxidations of these substrates. A number of agents can disrupt this gearing mechanism by dissociating phosphorylation from oxidation; the effectiveness of one of these, 2,4-dinitrophenol (DNP), has been demonstrated both on intact plant tissues (Newcomb, 1950; Beevers, 1953a; Simon, 1953) and on plant mitochondria (Bonner and Millerd, 1953; Millerd *et al.*, 1953; Bonner *et al.*, 1953; Biale, 1953; Laties, 1953b). DNP can decrease the exogenous adenylate requirement, increase the respiratory rate, and abolish oxidative phosphorylation by mitochondria. These effects may be due to the decomposition of a primary phosphate acceptor (Hunter, 1951) which prevents the generation of ATP and re-establishes acceptor sites. DNP can stimulate oxidation in the absence of exogenous adenylate (Millerd *et al.*, 1953), although with cauliflower mitochondria a small amount of adenylate must be added (Laties, 1953b). Laties suggests that adenylate may act as the primary phosphate acceptor which is subsequently stripped by DNP. The possibility that DNP acts by stimulating ATPase activity was not supported by tests with cauliflower mitochondria.

A number of studies have indicated the presence of ATPase on plant mitochondria. Bonner and Millerd (1953) showed that mung bean particles liberate phosphate from ATP, and this activity can be inhibited by fluoride. In *Phaseolus vulgaris*, the mitochondrial fraction possesses 37% of the total capacity of the homogenate to hydrolyze ATP; the nuclear, microsomal, and soluble fractions have 16, 19, and 28% of the activity, respectively (Webster, 1953a). A fluoride-sensitive ATPase is also associated with cauliflower bud (Laties, 1953b) and *Arum* spadix (Simon, 1954) mitochondria. Courtois and Ai (1953) suggested that some of the phosphatase of leaves is probably associated with particles, either chloroplasts or mitochondria, and may be localized on membranes. When liver mitochondria are disintegrated, the ATPase remains firmly attached to the fragments (Kielley and Kielley, 1953), indicating that the enzyme may be localized in the mitochondrial membrane. It is worth noting that ATP serves as a carrier for phosphate accumulated within the mitochondria (Bartley and Davies, 1954), and this may be the result of a surface ATP hydrolysis.

Brachet (1952a) has supplied evidence for the hypothesis that the nucleus is intimately involved in the coupling of phosphorylation to oxida-

tion. Although enucleation causes little change in the respiratory rate of *Amoeba*, the phosphorylative capacity, indicated by P^{32} uptake, is markedly reduced. Similar effects have been observed with the alga *Acetabularia* (Brachet, 1952b), although in older cells the P^{32} incorporation was not greatly reduced by enucleation. It is apparent that the nucleus affects phosphorylation more directly than oxidation, but the mechanism of this influence is not clear. One possibility is that the nucleus synthesizes a coenzyme necessary for the coupling of oxidation and phosphorylation (Brachet, 1952a). Allfrey, Stern, and Mirsky (1952) have shown that nucleoside phosphorylase is localized in the nuclei, and they suggest a possible role of the nucleus in controlling the synthesis of the coenzymes DPN and TPN. Working with liver cell homogenates, Johnson and Ackermann (1953) have shown that the addition of the nuclear fraction to the mitochondria greatly increases both oxygen and phosphate uptake, thus providing direct evidence of a role of nuclei in oxidative phosphorylation.

X. Biosynthesis

1. *Photosynthesis*

Photosynthesis may be considered in terms of (1) CO_2 fixation mechanisms, (2) the formation of reducing power, (3) the reduction of carboxylated intermediates, and (4) energy relations. Although the mitochondria are centers of CO_2 production, the mechanisms for fixing CO_2 in plants—the synthesis of oxalacetate from pyruvate (Gollub and Vennesland, 1947), oxalacetate from phosphorylenolpyruvate (Bandurski and Greiner, 1953), malate from pyruvate (Arnon, 1951; Anderson *et al.*, 1952)—are apparently largely associated with the soluble fraction. In normal photosynthesis, the carboxyl group of phosphoglyceric acid is the first site of fixation, possibly as a result of ribulosediphosphate carboxylation (Quayle *et al.*, 1954), and Fager (1954) has shown that the carboxylating enzyme is present in the chloroplasts. The whole cycle of carbon compounds connected with photosynthesis may be largely present in the chloroplast and soluble fractions. Clendenning and Gorham (1952) found that, after brief exposure of aquatic plants to $C^{14}O_2$, the C^{14} was mainly recovered in the cell sap from which particles had been removed; they concluded that the intermediates formed during the first few seconds of photosynthesis are already in the free state and become uniformly distributed in the cell by diffusion. Nevertheless, during exposure to light these intermediates do not readily enter the mitochondrial Krebs cycle (Calvin and Massini, 1952; Gibbs, 1953). Calvin and Massini suggest that the light keeps α-lipoic acid reduced, thereby inhibiting the oxidative decarboxylation of pyruvate and its entrance into the cycle.

The photochemical reaction resulting in the reduction of an acceptor molecule and in the liberation of oxygen takes place in the chloroplasts. It has been shown that DPN and TPN can be reduced by illuminated chloroplasts (Vishniac and Ochoa, 1951; Arnon, 1951; Tolmach, 1951), but it is not known whether these substances are the primary acceptors. Chloroplasts contain a cytochrome component, f, resembling c, but with modified properties (Davenport, 1952), and it has been suggested that this may participate in photosynthesis (see Hill and Hartree, 1953). Lundegardh (1954) has reported a light-induced oxidation of cytochrome f, and evidence has been presented that cytochromes may be involved in photochemical hydrogen transport in microorganisms (e.g. Vernon, 1953). These facts suggest that the cytochromes may play similar but reverse roles in mitochondrial oxidations and chloroplast reductions. To extend the parallel, one might expect a flavin enzyme to mediate the reduction of DPN or TPN, and the soluble factor in chloroplast suspensions which catalyzes the reduction of methemoglobin may be such an enzyme (Davenport, Hill, and Whatley, 1952). Photochemically produced DPNH or TPNH can subsequently reduce a variety of substances (Vishniac and Ochoa, 1952a). However, there is good evidence that the reduction of phosphoglyceric acid is the sole reduction step in photosynthesis (Fager, 1954). In green leaves, there are three separate glyceraldehyde phosphate dehydrogenases, two of which require TPN, and any of them might be involved (Arnon, Rosenberg, and Whatley, 1954). According to Arnon (1952), the dehydrogenase is present in the cytoplasmic fluid rather than the particles.

Recent studies indicate a close relationship between photosynthesis and phosphorus metabolism (see Brown and Frenkel, 1953). Illumination causes a rapid drop in cellular orthophosphate and a rise in the ATP level; P^{32} studies indicate that ATP is the first product formed from inorganic phosphate. Using mung bean mitochondria, Vishniac and Ochoa (1952b) demonstrated an oxidative phosphorylation coupled to the photochemical reduction of DPN. It has been suggested that high-energy phosphate compounds may not only be generated, during photosynthesis, but also utilized, e.g. for the conversion of substances from a low to a high reduction potential (Strehler, 1952). The ATP generated in the mitochondria might be used to raise the potential of the primary acceptor, which is reduced in the chloroplast. Such a possible close interplay between different cytoplasmic particles recalls the evidence that, in the light, *Euglena* mitochondria aggregate around the chloroplasts (Wolken and Palade, 1953). On the other hand, ATP may be generated by the chloroplasts themselves (Arnon *et al.*, 1954). Fundamentally different energy-

trapping mechanisms may be involved in the light and dark, and it is relevant that the mitochondrial Krebs cycle apparently disintegrates in the light (Brummond, 1954).

2. *Acetate Activation and Fatty Acid Metabolism*

Acetate is known to be involved in a wide variety of biosynthetic mechanisms, giving rise in large part to the skeletons of many complex substances. Recently, Millerd and Bonner (1954) have studied acetate activation and acetoacetate formation in plant systems. They established the ability of an acetone powder of spinach leaves and other tissues to carry out the following reaction:

$$\text{acetate} + \text{ATP} + \text{Co A}^* \longrightarrow \text{acetyl-Co A} + \text{AMP} + \text{PP}^*$$

The acetyl-Co A thus formed can condense with oxalacetate to form citrate, or react with another molecule of acetyl-Co A to form acetoacetyl-Co A, which gives rise to acetoacetate. The acetate activating enzyme is probably associated with the mitochondria: protein solubilized from oat and avocado mitochondria showed five times greater activity than whole leaf protein, and no activity was detected in the nonmitochondrial fractions. The spinach preparation is capable of activating a variety of higher fatty acids, suggesting that it contains more than one enzyme. Millerd and Bonner point out that acetoacetate may be an intermediate in fatty acid metabolism and in the biosynthesis of such branched-chain compounds as the isoprenoids.

Fatty acid synthesis and oxidation in peanut cotyledons has been the subject of an interesting study by Newcomb and Stumpf (1952). Slices of cotyledons were incubated with a variety of labeled substrates, and acetate was found to be by far the most effective precursor of fatty acids. Mitochondria isolated by centrifugation showed a negligible oxidation of acetate, palmitate, and other lower fatty acids, although they did show Krebs cycle and cytochrome oxidase activity. This is in contrast to animal mitochondria which show high fatty acid oxidase activity (Green, 1951). On the other hand, the peanut microsomes, submicroscopic yellow particles roughly 20 mμ in diameter, readily oxidize palmitate and stearate, without the participation of the Krebs cycle. Similar particles prepared from potato, mung bean, and avocado did not oxidize these acids. The fact that peanut microsomes contain cytochrome oxidase suggests either that mitochondrial fragments are present or that these microsomes are closely related to the mitochondria of other plant and animal cells. The possibility remains that in other plant cells the mitochondria are the centers of fatty acid oxidation.

* Co A = coenzyme A; PP = pyrophosphate.

3. *Protein Metabolism*

Protein metabolism will be considered in terms of (1) the incorporation of nitrogen, (2) the formation of peptides, and (3) the synthesis of proteins. Elliott (1951, 1953) showed that homogenates or extracts of *Lupinus* seedlings and green peas are able to carry out the glutamine synthetase reaction, in which nitrogen is directly incorporated:

$$\text{glutamic acid} + NH_3 + ATP \longrightarrow \text{glutamine} + ADP + P$$

Webster (1953a) found that the enzyme is widely distributed in plants, and that the mitochondrial fraction of bean seedling homogenates possesses 61% of the total activity, the remainder being distributed equally in the microsomal and soluble fractions. When the Waring blendor was used to homogenize the tissue, 95% of the activity appeared in the soluble fraction. It seems likely that the glutamine synthesizing system of the intact cell is largely associated with mitochondria. According to Webster and Varner (1954a), the initial step in the synthesis involves a direct phosphorylation of the enzyme by ATP. Many amino acids are oxidized by animal mitochondria (Green, 1951), and it seems likely that both the oxidation and synthesis of amino acids, from organic acids and ammonia, are localized in plant mitochondria. This conclusion is supported by a recent report that glutamic dehydrogenase is present in pea mitochondria (Davies, 1954b).

Amino acids may also be formed by transamination. Stumpf (1951) reported that aqueous extracts of various plants are able to catalyze the transfer of amino groups to α-ketoglutaric acid, and King, Wilson, and Burris (1953) found that isolated barley particles possess this ability. Both particulate and soluble preparations from lupin and barley can transfer the amino nitrogen of 17 acids to α-ketoglutarate (Wilson, King, and Burris, 1954). With the particulate preparation, aspartic acid and arginine are the most active. Incubation of amino acids with C^{14}-labeled ketoglutarate gives rise to C^{14}-glutamic acid, and experiments with N^{15} showed the reversibility of the reactions. The localization of transaminases on these particles, and the intimate relation between these enzymes and the Krebs cycle intermediates support the view that the mitochondria are important centers of amino acid synthesis.

Little is known of peptide-bond synthesis in higher plants, although the recent work of Webster has made an important beginning in this field. Using labeled glutamic acid, it was shown that dialyzed extracts of acetone powders of *Phaseolus vulgaris* could form γ-glutamyl-cysteine (Webster, 1953b). The reaction may proceed by an initial phosphorylation of the enzyme (Webster and Varner, 1954b). The tripeptide glutathione is known to occur in plants, and Webster (1953c) has investigated its syn-

thesis both by intact bean hypocotyls and isolated cell fractions. The synthesis of glutathione in whole homogenates is promoted by citrate plus cytochrome c, and this promotion can be inhibited by either cyanide or DNP; the stimulation given by ATP is not inhibited by cyanide. Of the total synthetic activity, 51% was recovered in the mitochondrial fraction and the remainder largely in the soluble fraction. These findings indicate that the high-energy phosphate generated during respiration is utilized by the mitochondria for the synthesis of glutathione. The enzymes are apparently easily solubilized; glutathione can be synthesized by soluble enzymes of the rat liver (Holleman, 1954). On the basis of relative rates of synthesis, Webster suggests that the dipeptide, glutamyl-cysteine, reacts with glycine to form glutathione. The reaction requires Mg^{++}, is promoted by K^{+}, and inhibited by sulfhydryl inhibitors. Webster (1953d) has reported that the enzymatic synthesis of other peptides, which do not normally accumulate to a detectable level in plant cells, is associated with mitochondria. Peptide-bond synthesis can be accomplished by liver mitochondria (Sarkar *et al.*, 1952).

It is generally assumed that protein synthesis is intimately associated with nucleic acids, and this has very recently been demonstrated with RNA *in vitro* (Gale and Folkes, 1954). The microsomes are rich in RNA, and evidence has been supplied that indicates they are active centers of protein synthesis (see Brachet, 1952a). Siekevitz (1952) has shown, using a rat liver homogenate, that labeled alanine is most rapidly incorporated into the microsomal protein, but there is also considerable incorporation into the mitochondria. In the presence of mitochondria, which presumably act as an energy source, a soluble factor is formed which enables the microsomes to take up alanine. Plant cells contain RNA-rich microsomes (Brown and Fitton Jackson, 1953), and the fact that their size in bean root sections parallels changes in rate of increase of proteins is suggestive (Robinson and Brown, 1953). Working with fragments of *Acetabularia*, Brachet and Chantrenne (1952) found that CO_2 was incorporated at least two times faster into the proteins of chloroplasts than into other cytoplasmic particles. Their finding that this CO_2 incorporation, and therefore protein synthesis, is largely independent of the nucleus does not agree with the report of Giardina (1954) that enucleation causes a large decrease in the ratio protein N/nonprotein N in *Acetabularia*. De Deken-Grenson (1954) has determined the effect of light on the protein content of chicory leaf fractions; he concludes that in the light the plastids are the first seat of protein synthesis. The fractions sedimenting at $4700 \times g$ (chloroplast fragments and mitochondria) and $105{,}000 \times g$ (microsomes) also showed large increases in protein within

4 hours. In detached tobacco leaves exposed to $N^{15}H_3$, isotope activity was greater in the isolated small particles than in the chloroplasts (Jagendorf, Erickson, and Wildman, 1953). Webster (1954) has recently shown that isolated mitochondria, which contain RNA, are able to incorporate labeled amino acids into the mitochondrial protein. This incorporation is dependent on an energy supply, such as is provided by oxidative phosphorylation in the mitochondria. The radioautographs of Howard and Pelc (1951b) indicate a synthesis of protein in both the nucleus and cytoplasmic granules of bean root cells. In conclusion, it appears that microsomes, plastids, and mitochondria are all able to synthesize protein, and the relative importance of these activities in the economy of the cytoplasm under varied conditions remains to be established.

XI. Physiology

It is clear that any energy-requiring process within the cell will be either directly or indirectly dependent on mitochondrial metabolism. This is illustrated by the wide variety of inhibitory effects resulting from the treatment of intact tissues with DNP, which interferes with phosphorylation in the mitochondria, as follows: inhibition of protoplasmic streaming (Sweeney and Thimann, 1938), growth (Bonner, 1949), glucose absorption (Stenlid, 1949), water uptake (Hackett and Thimann, 1950), salt accumulation (Robertson, Wilkins, and Weeks, 1951), and cell division and tissue differentiation (Torrey, 1953). Further, any physiological activity which is linked to the functioning of the Krebs cycle or the cytochrome system may be said to involve the mitochondria. Apart from these indirect connections, there is growing evidence that the mitochondria themselves are directly involved as the instruments by which certain physiologic functions are exercised. Most of the evidence along this line comes from studies on animal mitochondria, and the subject is considered here with the belief that it represents a fruitful field for future botanical research.

The transport of water in living systems is closely geared to respiratory and energy-yielding reactions, and it has been suggested that nonosmotic or active mechanisms may be involved (see Robinson, 1953). Such a possibility has frequently been considered for plant cells (see Crafts, Currier, and Stocking, 1949), and it has recently been examined with reference to the mechanism of auxin action (see Thimann, 1951). Although proof of active water uptake has been claimed (Bonner et al., 1953), conclusive evidence has not yet been obtained (Burström, 1953). If there is a cytoplasmic water-pumping mechanism, it may well be located in the mitochondria. In earlier discussions, it has been pointed out that mito-

chondria, both within the cell and when isolated, behave much like osmometers, and that a supply of energy is required for their morphological integrity. The water content of isolated mitochondria may be determined directly by weighing, or indirectly by following volume changes spectrophotometrically (Cleland, 1952); for liver mitochondria, the value varies from 80 to 90% (Macfarlane and Spencer, 1953). It has been shown that an energy source, such as ATP, plays an essential role in maintaining the water balance of animal mitochondria (Macfarlane and Spencer, 1953; Raaflaub, 1953; Bartley and Davies, 1954; Chappell and Perry, 1954). The swelling of mitochondria in hypotonic solution can be reversed by ATP, and this reversal is prevented by DNP. In the case of plant cells, a mechanism is needed to secrete water from the outside into the vacuole. Such a process might be related to intracellular gradients of ATP-generation. Phase contrast observations on red beet mitochondria suggested that particles prepared in hypotonic media had taken up water (Farrant *et al.*, 1953); visible effects of ATP on isolated pea mitochondria have been interpreted as a maintenance of water gradients (Price, 1954).

It was long ago proposed by plant cytologists that mitochondria may be involved in secretion phenomena (see Newcomer, 1940). The energy-requiring accumulation of salts against a concentration gradient is well known in plant cells, and both phosphorylation and the activity of the cytochrome system are intimately connected with this process (see Robertson, 1951; Sutcliffe, 1953). Ions are concentrated in the vacuole, and it has been suggested that the mitochondria may act as carriers in transporting ions across the cytoplasm (Arisz, 1945; Robertson, 1951). Direct evidence for such a role was early provided by Mullins' (1940) demonstration that radioactive potassium and phosphate are accumulated in the cytoplasmic granules of *Nitella*. A similar concentration of iodine in the thallus of *Laminaria* is suggested by the radioautographs of Roche and Yagi (1952). Long and Levitt (1952) fractionated potato tuber slices which had been in contact with radioactive calcium or phosphate for 24 hours; they found a marked accumulation of ions in the mitochondria and microsomes. They suggest that ion exchange on the surface of granules may be important for accumulation.

Work with isolated animal mitochondria has supplied considerable support for the view that mitochondria are basic units of secretory and absorptive activities (Macfarlane and Spencer, 1953; Spector, 1953; Stanbury and Mudge, 1953; Bartley and Davies, 1954). It has been shown that the concentration of inorganic ions, such as sodium and potassium, can be greater within the mitochondria than in the surrounding medium, and that the maintenance of this gradient depends on active metabolism.

Likewise, there can be an intramitochondrial accumulation of organic acids, such as α-ketoglutarate, fumarate, pyruvate (Bartley and Davies, 1954), and citrate (Schneider, 1953). Using optical methods, the permeability of mitochondria to both electrolytes and nonelectrolytes has been studied with rat heart (Cleland, 1952) and insect flight muscle sarcosomes (Watanabe and Williams, 1953). The marked effects of ATP on KCl permeability illustrate how an intracellular ATP gradient might result in net movements of ions in one direction.

The actual mechanism of ion accumulation is still uncertain. Lundegardh's (1950, 1952) theory involves the participation of members of the cytochrome system as an electron ladder for transporting ions. His suggestion that this is located in or near the protoplasmic surface may be related to the aggregation of mitochondria at the outer cell membrane. Effects of salt on plant cytochromes were observed early (Keilin, 1925) and Lundegardh (1951) has recorded a shift in the redox balance of the cytochromes when intact roots are shifted from water to a salt solution. In root homogenates, KCl has a stimulatory effect on the cytochrome oxidase (Lundegardh, 1953a). Working with isolated pea particles, Butler (1953) has shown the dependence of cytochrome oxidase activity on the ionic environment, and he concludes that "salt respiration" may arise by direct ionic stimulation of the cytochrome system.

The close correlation between plant growth and both the tissue ATP level (Albaum, 1952) and cytochrome oxidase activity (Hackett and Schneiderman, 1953) indicates the intimate dependence of growth on mitochondrial metabolism. Attempts to demonstrate direct effects of the plant growth hormone on oxidations by isolated plant mitochondria have been unsuccessful (Price, 1952; Dow, 1952). Surprisingly, Brody (1952) found that a synthetic hormone, 2,4-dichlorophenoxyacetic acid, effectively uncouples phosphorylation from respiration in rat liver mitochondria. When tissues which have been grown on auxin are fractionated, the respiratory enzymes of the mitochondria show no significant increase (Price, 1952), although enzymatic activities associated with the cell wall fraction, such as ascorbic acid oxidase (Newcomb, 1951) and pectin methylesterase (Bryan and Newcomb, 1954), are markedly greater than in the controls without auxin. The immediate respiratory response induced by plant growth regulators is probably the result of effects on the phosphorylative machinery and ATP utilization rather than on the synthesis of mitochondrial enzymes (Bonner and Bandurski, 1952; French and Beevers, 1953). Leopold and Guernsey (1953) have proposed a new theory of auxin action which involves its reaction with coenzyme A to produce a thiolester, which controls a variety of metabolic reactions. The reaction

with auxin requires ATP, and the necessary enzyme is apparently localized in the mitochondrial fraction prepared from tomato ovaries. Such a mechanism would place the mitochondria in a central role for growth regulation, and this may be an example of the more general phenomenon of hormonal action at the mitochondrial level (cf. thyroxine action).

In view of the probable control of tissue respiration by phosphorylative mechanisms, the climacteric rise in fruit respiration may be the result of changes in mitochondrial metabolism. Studies on avocado (Millerd *et al.*, 1953) and apple (Pearson and Robertson, 1954) support the view that the preclimacteric respiration is limited by the supply of phosphate acceptors. Avocado mitochondria extracted at various stages of development show no significant differences in activity, whereas apple particles show increasing oxidative activity as the tissue approaches the climax. Millerd *et al.* (1953) attribute the change to the action of native uncoupling agents, which increase the supply of phosphate acceptors by inhibiting ATP synthesis. On the other hand, Pearson and Robertson (1954) suggest that there is a change in the ATP ADP ratio within the particles as a result of an increase in synthetic demands and ATP utilization.

Some miscellaneous observations on mitochondrial activity and distribution have been made. McClendon (1952) found a high concentration of mitochondria in the phloem cells of tobacco leaves, suggesting a possible role in transport. Levitt (1952) has made an attempt to correlate dormancy in potatoes with the amount of isolated mitochondria. Changes in cytochrome oxidase activity, e.g. during development (Stafford, 1952) or vernalization (Oparin, 1953), may reflect alterations in the mitochondria. Future work will undoubtedly establish a characteristic pattern of mitochondrial activity and distribution under various developmental and environmental conditions.

XII. Genetics, Evolution and Radiation Effects

The subject of the possible role of mitochondria in heredity has been thoroughly covered in earlier reviews (Newcomer, 1940, 1951; Weier and Stocking, 1952b). Many examples of cytoplasmic inheritance are known in higher plants (see Caspari, 1948), but recent work has added little to the conclusive definition of the agents responsible. Since plant mitochondria are assumed to be self-duplicating particles, they could act as extranuclear hereditary entities. Plastids may also be endowed with genetic continuity, and it has been claimed that mutant mitochondria give rise to plastid abnormalities (DuBuy *et al.*, 1950; Woods and DuBuy, 1951). The growing knowledge of the biochemical functions of mitochondria may help in determining their role in heredity. For example,

cytoplasmically inherited abnormalities in auxin metabolism, respiration, and cellular osmotic value (see Caspari, 1948) may well be due to changes in the mitochondria. Particularly good examples are provided by some recent studies with fungi. The work of Ephrussi (1953) with yeasts has shown that vegetative "littles" are produced by a cytoplasmic mutation which gives rise to profound changes in the cytochrome system. Mitchell, Mitchell, and Tissières (1953) report that the absence of certain cytochrome components in various strains of *Neurospora* is inherited maternally. In view of the fundamental relation of the cytochrome system to mitochondria, these findings are suggestive (cf. Marquardt, 1952). A similar situation is suggested by the finding that a mutation resulting in the absence of α-ketoglutaric oxidase in *Phycomycetes* (Cantino and Hyatt, 1953) can be attributed to a cytoplasmic factor. Although the cytoplasm may possess a degree of genetic autonomy, there must be a continuous interaction of nucleus and cytoplasm during reproduction and development.

The fact that a major function of the mitochondria is to convert oxidative energy into a utilizable form suggests a relation of these organelles to cellular evolution. If it is assumed that the first organisms were strictly anaerobic (see Oparin, 1938), it seems likely that the earliest mechanisms for phosphorylative energy-trapping were of the type encountered in glycolysis. The interesting work of Stern and Mirsky (1952) has shown that wheat germ nuclei possess the glycolytic enzymes necessary for generating ATP, and they suggest that the chromosomes probably function in an energy-yielding environment. The nucleus thus possesses the ability both to reproduce itself and to supply the required energy. Following the evolution of the pigment chlorophyll, plant cells were able to carry on photosynthesis and thereby to produce oxygen. At this evolutionary stage the large redox potential drop between, say, DPNH and oxygen was not utilized for energy production, and subsequent evolution may have represented a gradual increase in the exploitation of this energy. In other words, evolution may have proceeded along the electron transfer chain. Initially appropriate flavin enzymes could have served to accept hydrogen from DPNH and to transfer it to molecular oxygen. Subsequently, the flavins passed electrons to cytochrome components, relatives of molecules such as cytochrome f, earlier evolved to participate in photosynthesis. Finally, the ability to transfer electrons from cytochrome to oxygen was evolved. The change in redox potential between each of these steps may have been dictated by the requirements of the previously evolved mechanism for generating energy-rich phosphate bonds. An interesting correlation is found in the increasing long-wavelength absorption maxima, going

from DPNH to cytochrome a_3, although it is not clear exactly how this molecular characteristic is related to the redox potential. The cytochrome b maximum does not fall in sequence, and it is of interest that this component stands off the main pathway of electron transfer. The later stages of evolution, during which the entire cytochrome system appeared, probably paralleled the development of the highly organized mitochondrial structure. It may be suggested that the evolution of the most complex organisms depended on the development of these systems, which make possible a more efficient use of oxidation energy.

The mechanisms of radiation damage are not yet entirely understood. There is considerable evidence against the "target theory" of direct action and in favor of the view that radiation effects are secondary, possibly caused by products resulting from the ionization of water. Thus, chromosome breakage could be the end result of complex biochemical changes, some of which may be localized on the mitochondria (see Dounce, 1952). Considering the sensitivity of mitochondria to a variety of external influences, it seems likely that they would be extremely susceptible to irradiation. This possibility is supported by Ritchie's (1953) observation that, in *Allomyces*, the first sign of damage caused by gamma radiation is the disruption of the mitochondria. It was shown much earlier that the mitochondria of onion epidermis are the most X-ray sensitive parts of the cell (Nadson and Rochlin, 1933). There is an interesting report that ultraviolet radiation disrupts yeast mitochondria and that this effect is not observed when cells are irradiated in 0.15 *M* glucose (Sarachek and Townsend, 1953). These cytological observations suggest that the mitochondria may be a primary locus of radiation damage. The effects of carbon monoxide and oxygen on the frequency of X-ray induced chromosome aberrations in *Tradescantia* also suggest a close connection with the site of terminal oxidation (King, Schneiderman, and Sax, 1952).

In earlier sections, we have seen that phosphorylation is one of the most sensitive processes in the mitochondria. That ionizing radiations can act as uncoupling agents is suggested by the large promotions of respiration observed in radiated potato tissue (Sussman, 1953). In addition, a variety of energy-requiring processes in plants, such as growth (Quastler, Schertiger, and Stewart, 1952), germination (Sussman, 1953), and ion uptake (Higinbotham and Mika, 1954), are inhibited by ionizing radiations. Some recent studies with animal tissues indicate marked effects on phosphorus metabolism. The irradiation of rats causes marked changes in the skin ATP level (Gros, Mandel, and Rodesch, 1954), a great increase in mitochondrial ATPase activity, and a greatly decreased capacity for phosphorylation (Maxwell and Ashwell, 1953). When irradiated *in vitro*,

normal rat mitochondria are not affected, suggesting that the effects on phosphorylation may be indirect (Potter and Bethell, 1952). In any case, the interference with energy metabolism in mitochondria may be one of the primary causes of radiation damage.

XIII. Conclusion

The results which have been reviewed above emphasize the fact that the plant cell is not simply a bag of haphazardly arranged enzymes. The cell particulates possess a high degree of structural and functional organization which is, under certain conditions, very labile. The fundamental processes carried out by the mitochondria appear to be (1) the terminal transfer of electrons, (2) the coupling of energy-trapping mechanisms (phosphorylations) to oxidations, and (3) the Krebs cycle reactions and the numerous ancillary processes which eventually funnel through the cycle. In spite of the remarkable autonomy of the mitochondria, the overall activities of the cell are the result of intimate interactions among the various cellular components. Glycolysis involves the plastids, the soluble fraction, and possibly the nucleus as well as the mitochondrial enzymes (e.g. hexokinase). Certain enzymes involved in the Krebs cycle (e.g. malic dehydrogenase) and in hydrogen transfer (e.g. cytochrome reductase) are not confined entirely to the mitochondria. The mitochondria interact with the nucleus in phosphorylation, with the chloroplasts in photosynthesis, and with the microsomes in protein synthesis. In close relation to the outer cell boundary, the mitochondria may participate actively in the movement of substances into the cell or in the growth of the cell wall. The movement of particles from one part of the cell to another may bring about an accumulation of substances within an internal compartment, such as the vacuole. The interdependence of the various activities and regions of the cell points up the fact that the real unit is the whole cell itself, a conclusion supported by the study of nucleo-cytoplasmic relations in heredity. "The cell is an integrated unit which can be broken down only for purposes of analysis" (Ephrussi, 1953), and it is this type of analysis which has revealed the remarkable activities of the mitochondria.

XIV. References

Albaum, H. G. (1952) *in* "Symposium on Phosphorus Metabolism." Michigan State College Press, East Lansing.

Albaum, H. G., Ogur, M., and Hirshfeld, A. (1950) *Arch. Biochem.* **27**, 130.

Allfrey, V., Stern, H., and Mirsky, A. E. (1952) *Nature* **169**, 128.

Anderson, T. F. (1952) *Am. Naturalist* **86**, 91.

Anderson, D. G., Stafford, H. A., Conn, E. E., and Vennesland, B. (1952) *Plant Physiol.* **27**, 675.

Arisz, W. H. (1945) *Koninkl. Ned. Akad. Wetenschap. Proc.* **48**, 420.
Arnon, D. I. (1948) *Nature* **162**, 341.
Arnon, D. I. (1951) *Nature* **167**, 1008.
Arnon, D. I. (1952) *in* "Phosphorus Metabolism" (McElroy and Glass, eds.), Vol. 2, p. 67. Johns Hopkins Press, Baltimore.
Arnon, D. I., Allen, M. B., and Whatley, F. R. (1954) *Program Am. Soc. Plant Physiol., Gainesville* p. 24.
Arnon, D. I., Rosenberg, L. L., and Whatley, F. R. (1954) *Nature* **173**, 1132.
Axelrod, B., Bandurski, R. S., Greiner, C. M., and Jang, R. (1953) *J. Biol. Chem.* **202**, 619.
Ball, E. G. (1953) *Am. J. Botany* **40**, 306.
Bandurski, R. S., and Greiner, C. M. (1953) *J. Biol. Chem.* **204**, 781.
Barron, E. S. G., Link, G. K. K., Klein, R. M., and Michel, B. E. (1950) *Arch. Biochem.* **28**, 377.
Bartley, W., and Davies, R. E. (1954) *Biochem. J.* **57**, 37.
Beevers, H. (1953a) *Am. J. Botany* **40**, 91.
Beevers, H. (1953b) *Program Am. Soc. Plant Physiol., Madison* p. 20.
Beevers, H., and Gibbs, M. (1954) *Program Am. Soc. Plant Physiol., Gainesville* p. 40.
Bentley, L. E. (1952) *Nature* **170**, 847.
Bhagvat, K., and Hill, R. (1951) *New Phytologist* **50**, 112.
Bhargava, K. S. (1950) *Cytologia (Tokyo)* **16**, 72.
Biale, J. B. (1953) *Program Am. Soc Plant Physiol., Madison* p. 21.
Bogen, H. J. (1954) *Fortsch. Bot.* **15**, 212.
Bonner, J. (1949) *Am. J. Botany* **36**, 323.
Bonner, J., and Bandurski, R. S. (1952) *Ann. Rev. Plant Physiol.* **3**, 59.
Bonner, J., Bandurski, R. S., and Millerd, A. (1953) *Physiol. Plantarum* **6**, 511.
Bonner, J., and Millerd, A. (1953) *Arch. Biochem. and Biophys.* **42**, 135.
Bonner, W. D., Jr. (1954) *Biochem. J.* **56**, 274.
Brachet, J. (1952a) *Symposia Soc. Exptl. Biol.* **6**, 173.
Brachet, J. (1952b) *Biochim. et Biophys. Acta* **9**, 221.
Brachet, J., and Chantrenne, H. (1952) *Arch. intern. physiol.* **60**, 547.
Brakke, M. K., and Nickell, L. G. (1951) *Arch. Biochem and Biophys.* **32**, 28.
Bretschneider, L. H. (1952) *Survey Biol. Progr.* **2**, 223
Brody, T. M. (1952) *Proc. Soc. Exptl. Biol. Med* **80**, 533.
Brown, A. H., and Frenkel, A. W. (1953) *Ann. Rev. Plant Physiol* **4**, 23.
Brown, G. L., and Fitton Jackson, S. (1953) *Nature* **171**, 1113.
Brown, R. (1952) *Intern. Rev. Cytol.* **1**, 107.
Brummond, D. O. (1954) Doctor of Philosophy Thesis, University of Wisconsin, Madison.
Brummond, D. O., and Burris, R. H. (1953) *Proc. Natl. Acad. Sci. U.S.* **39**, 754
Bryan, W. H., and Newcomb, E. H. (1954) *Physiol. Plantarum* **7**, 290.
Buchholz, J. T. (1947) *Am. J. Botany* **34**, 445.
Burris, R. H. (1953) *Ann. Rev. Plant Physiol.* **4**, 91.
Burström, H. (1953) *Physiol. Plantarum* **6**, 685.
Butler, G. W. (1953) *Physiol. Plantarum* **6**, 662.
Buvat, R. (1953) *Endeavour* **12**, 33.
Calvin, M., and Massini, P. (1952) *Experientia* **8**, 445.
Campbell, J. J. R., Smith, R. A., and Eagles, B. A. (1953) *Biochim. et Biophys. Acta* **11**, 594.

Cantino, E. C., and Hyatt, M. T. (1953) *Am. J. Botany* **40**, 688.
Caspari, E. (1948) *Advances in Genet.* **2**, 1.
Chance, B. (1952) *Nature* **169**, 215.
Chappell, J. B., and Perry, S. V. (1954) *Nature* **173**, 1094.
Chayen, J. (1952) *Symposia Soc. Exptl. Biol.* **6**, 290.
Chayen, J. (1953) *Intern. Rev. Cytol.* **2**, 77.
Chayen, J., and Miles, U. J. (1953) *Quart. J. Microscop. Sci.* **94**, 29.
Cleland, K. W. (1952) *Nature* **170**, 497.
Cleland, K. W., and Slater, E. C. (1953a) *Biochem. J.* **53**, 547.
Cleland, K. W., and Slater, E. C. (1953b) *Quart. J. Microscop. Sci.* **94**, 329.
Cleland, W. W., and Johnson, M. J. (1954) *J. Biol. Chem.* **208**, 679.
Clendenning, K. A., and Gorham, P. R. (1952) *Arch. Biochem. and Biophys.* **37**, 56.
Courtois, J. E., and Ai, T. V. (1953) *Bull. soc. chim. biol.* **35**, 721.
Crafts, A. S., Currier, H. B., and Stocking, C. R. (1949) "Water in the Physiology of Plants." Chronica Botanica Co., Waltham.
Crane, R. K., and Sols, A. (1953) *J. Biol. Chem.* **203**, 273.
Dalton, A. J. (1953) *Intern. Rev. Cytol.* **2**, 403.
Damodaran, M., and Venkatesan, T. R. (1941) *Proc. Indian Acad. Sci.* **B13**, 345.
Dangeard, P. (1951a) *Compt. rend.* **232**, 1274.
Dangeard, P. (1951b) *Botaniste* **35**, 35.
Dangeard, P., and Parriaud, H. (1953) *Compt. rend.* **236**, 260.
Darby, R. T., and Goddard, D. R. (1950) *Physiol. Plantarum* **3**, 435.
Davenport, H. E. (1952) *Nature* **170**, 1112.
Davenport, H. E., Hill, R., and Whatley, F. R. (1952) *Proc. Roy. Soc.* **B139**, 346.
Davies, D. D. (1953) *J. Exptl. Botany* **4**, 173.
Davies, D. D. (1954a) *Proc. Roy. Soc.* **B142**, 155.
Davies, D. D. (1954b) Personal communication.
Davison, D. C. (1950) *Nature* **166**, 265.
De Deken-Grenson, M. (1954) *Biochim. et Biophys. Acta* **14**, 203.
Deysson, G. (1952) *Compt. rend.* **235**, 1529.
Dianzani, M. U. (1953) *Biochim. et Biophys. Acta* **11**, 353.
Dickens, F. (1951) *in* "The Enzymes" (Sumner and Myrback, eds.), p. 624. Academic Press, New York.
Dounce, A. L. (1950) p. 186. Academic Press, New York.
Dounce, A. L. (1952) *J. Cellular Comp. Physiol.* **39**, 43.
Dow, W. A., Jr. (1952) Cited in Burris, R. H., *Ann. Rev. Plant Physiol.* **4**, 91.
Drawert, H. (1952) *Ber. deut. botan. Ges.* **65**, 263.
Drawert, H. (1953) *Ber. deut. botan. Ges.* **66**, 134.
DuBuy, H. G., Woods, M. W., and Lackey, M. D. (1950) *Science* **111**, 572.
Ducet, G., and Rosenberg, A. J. (1951) *Bull. soc. chim. biol.* **33**, 321.
Ducet, G., and Rosenberg, A. J. (1953) *Bull. soc. chim. biol.* **35**, 467.
Duvick, D. N. (1953) *Botan. Gaz.* **115**, 82.
Dyar, M. T. (1950) *Am. J. Botany* **37**, 786.
Dyar, M. T. (1953) *Am. J. Botany* **40**, 20.
Eichenberger, M. (1953) *Exptl. Cell Research* **4**, 275.
Elliott, W. H. (1951) *Biochem. J.* **49**, 106.
Elliott, W. H. (1953) *J. Biol. Chem.* **201**, 661.
Ephrussi, B. (1953) "Nucleo-cytoplasmic Relations in Micro-organisms." Oxford Univ. Press, New York.

Evans, H. J. (1954) *Program Am. Soc. Plant Physiol., Gainesville* p. 37.
Fager, E. W. (1954) *Biochem. J.* **57**, 264.
Farrant, J. L., Robertson, R. N., and Wilkins, M. J. (1953) *Nature* **171**, 401.
Frederic, J., and Chèvremont, M. (1952) *Arch. Biol. (Liege)* **63**, 109.
French, R. C., and Beevers, H. (1953) *Am. J. Botany* **40**, 660.
Fritz, G. J., and Beevers, H. (1953) *Program Am. Soc. Plant Physiol., Madison* p. 39.
Gale, E. F., and Folkes, J. P. (1954) *Nature* **173**, 1223.
Geneves, L. (1951) *Compt. rend.* **232**, 1132.
Giardina, G. (1954) *Experientia* **10**, 215.
Gibbs, M. (1953) *Arch. Biochem. and Biophys.* **45**, 156.
Gibbs, M. (1954) *Plant Physiol.* **29**, 34.
Glick, D. (1953) *Intern. Rev. Cytol.* **2**, 447.
Goddard, D. R., and Holden, C. (1950) *Arch. Biochem.* **27**, 41.
Goddard, D. R., and Stafford, H. A. (1954) *Ann. Rev. Plant Physiol.* **5**, 115.
Gollub, M. C., and Vennesland, B. (1947) *J. Biol. Chem.* **169**, 233.
Green, D. E. (1951) *in* "Enzymes and Enzyme Systems" (Edsall, ed.), p. 15. Harvard Univ. Press, Cambridge.
Green, D. E., Kohout, P. M., and Mii, S. (1954) *Biochim. et Biophys. Acta* **14**, 295.
Gros, C., Mandel, P., and Rodesch, J. (1954) *Compt. rend.* **238**, 1615.
Guilliermond, A. (1941) "The Cytoplasm of the Plant Cell." Chronica Botanica Co. Waltham.
Hackett, D. P. (1954) In preparation.
Hackett, D. P., and Schneiderman, H. A. (1953) *Arch. Biochem. and Biophys.* **47**, 190.
Hackett, D. P., and Simon, E. W. (1954) *Nature* **173**, 162.
Hackett, D. P., and Thimann, K. V. (1950) *Plant Physiol.* **25**, 648.
Harman, J. W., and Feigelson, M. (1952a) *Exptl. Cell Research* **3**, 47.
Harman, J. W., and Feigelson, M. (1952b) *Exptl. Cell Research* **3**, 509.
Hazan, S. J., Singal, S. A., Sydenstricker, V. P., and Littlejohn, J. M. (1953) *Federation Proc.* **12**, 216.
Hesselbach, M. L. (1951) *J. Natl. Cancer Inst.* **12**, 337.
Hesselbach, M. L., and DuBuy, H. G. (1953) *Proc. Soc. Exptl. Biol. Med.* **83**, 62.
Higinbotham, N., and Mika, E. S. (1954) *Plant Physiol.* **29**, 174.
Hill, R., and Bhagvat, K. (1939) *Nature* **143**, 726.
Hill, R., and Hartree, E. F. (1953) *Ann. Rev. Plant Physiol.* **4**, 115.
Hill, R., and Scarisbrick, R. (1951) *New Phytologist* **50**, 98.
Hogeboom, G. H., and Schneider, W. C. (1950a) *Nature* **166**, 302.
Hogeboom, G. H., and Schneider, W. C. (1950b) *J. Biol. Chem.* **186**, 417.
Hogeboom, G. H., and Schneider, W. C. (1952) *J. Biol. Chem.* **194**, 513.
Hogeboom, G. H., Schneider, W. C., and Palade, G. E. (1947) *Proc. Soc. Exptl. Biol. Med.* **65**, 320.
Hogeboom, G. H., Schneider, W. C., and Striebich, M. J. (1953) *Cancer Research* **13**, 617.
Holleman, J. W. (1954) *Compt. rend.* **238**, 1360.
Holter, H., and Pollock, B. M. (1952) *Compt. rend. trav. lab. Carlsberg Ser. Chim.* **28**, 221.
Howard, A., and Pelc, S. R. (1951a) *Exptl. Cell Research* **2**, 178.
Howard, A., and Pelc, S. R. (1951b) *Nature* **167**, 599.

Hunter, F. E., Jr. (1951) *in* "Phosphorus Metabolism" (McElroy and Glass, eds.), Vol. 1, p. 297. Johns Hopkins Press, Baltimore.
Jagendorf, A. T. (1953) Cited in Millerd, A., and Bonner, J., *J. Histochem. Cytochem.* **1**, 251.
Jagendorf, A. T., and Wildman, S. G. (1954) *Plant Physiol.* **29**, in press.
Jagendorf, A. T., Erickson, J., and Wildman, S. G. (1953) *Program Am. Soc. Plant Physiol. Madison* p. 31.
James, W. O. (1953a) *Biol. Revs.* **28**, 245.
James, W. O. (1953b) *Proc. Roy. Soc.* **B141**, 289.
James, W. O., and Beevers, H. (1950) *New Phytologist* **49**, 103.
Johnson, R. B., and Ackermann, W. W. (1953) *J. Biol. Chem.* **200**, 263.
Kaplan, E. H., Still, J. L., and Mahler, H. R. (1951) *Arch. Biochem. and Biophys.* **34**, 16.
Keilin, D. (1925) *Proc. Roy. Soc.* **B98**, 312.
Kern, M., and Racker, E. (1954) *Arch. Biochem. and Biophys.* **48**, 235.
Kielley, W. W., and Kielley, R. K. (1953) *J. Biol. Chem.* **200**, 213.
King, E. D., Schneiderman, H. A., and Sax, K. (1952) *Proc. Natl. Acad. Sci. (U.S.)* **38**, 34.
King, K. W., Wilson, D. G., and Burris, R. H. (1953) *Federation Proc.* **12**, 230.
Kingsbury B. F, (1912) *Anat. Record* **6**, 39.
Kornberg, A., and Pricer, W. E., Jr. (1951) *J. Biol. Chem.* **189**, 123.
Krebs, H. A., Gurin, S., and Eggleston, L. V. (1952) *Biochem. J.* **51**, 614.
Kretchmer, N., and Barnum, C. P. (1951) *Arch. Biochem. and Biophys.* **31**, 141.
Laties, G. G. (1952) *in* "Organic Acid Metabolism in Higher Plants" (Goddard ed.). Iowa State College Press, Ames.
Laties, G. G. (1953a) *Plant Physiol.* **28**, 557.
Laties, G. G. (1953b) *Physiol. Plantarum* **6**, 199.
Laties, G. G. (1953c) *Physiol. Plantarum* **6**, 215.
Laties, G. G. (1954) *J. Exptl. Botany* **5**, 49.
Lazarow, A., and Cooperstein, S. J. (1953) *J. Histochem. Cytochem.* **1**, 234.
Leopold, A. C., and Guernsey, F. S. (1953) *Proc. Natl. Acad. Sci.* **39**, 1105.
LePage, G. A., and Schneider, W. C. (1948) *J. Biol. Chem.* **176**, 1021.
Levitt, J. (1952) *Physiol. Plantarum* **5**, 470.
Levitt, J. (1954a) *Physiol. Plantarum* **7**, 109.
Levitt, J. (1954b) *Physiol. Plantarum* **7**, 117.
Leyon, H. (1953) *Exptl. Cell Research* **5**, 520.
Lindberg, O., and Ernster, L. (1952) *Exptl. Cell Research* **3**, 209.
Link, G. K. K., Klein, R. M., and Barron, E. S. G. (1952) *J. Exptl Botany* **3**, 216.
Lockhart, E. E. (1939) *Biochem. J.* **33**, 613.
Long, W. G., and Levitt, J. (1952) *Physiol. Plantarum* **5**, 610.
Lundegardh, H. (1950) *Physiol. Plantarum* **3**, 103.
Lundegardh, H. (1951) *Arkiv. Kemi.* **3**, 69.
Lundegardh, H. (1952) *Arkiv. Kemi.* **5**, 97.
Lundegardh, H. (1953a) *Nature* **171**, 477.
Lundegardh, H. (1953b) *Nature* **172**, 303.
Lundegardh, H. (1954) *Physiol. Plantarum* **7**, 375.
McClendon, J. H. (1952) *Am. J. Botany* **39**, 275.
McClendon, J. H. (1953) *Am. J. Botany* **40**, 260.
McClendon, J. H., and Blinks, L. R. (1952) *Nature* **170**, 577.

Macfarlane, M. G., and Spencer, A. G. (1953) *Biochem. J.* **54**, 569.
Mahler, H. R., and Elowe, D. G. (1953) *J. Am. Chem. Soc.* **75**, 5769.
Makower, R. V., and Schwimmer, S. (1954) *Biochim. et Biophys. Acta* **14**, 156.
Mandels, G. R. (1953) *Arch. Biochem. and Biophys.* **42**, 164.
Mapson, L. W. (1953) *Vitamins and Hormones* **11**, 1.
Mapson, L. W., Isherwood, F. A., and Chen, Y. T. (1954) *Biochem. J.* **56**, 21.
Marquardt, H. (1952) *Ber. deut. botan. Ges.* **65**, 198.
Masacré, M., and Paris, R. (1951) *Bull. soc. chim. biol.* **33**, 109.
Mathews, M. B. (1951) *J. Biol. Chem.* **189**, 695.
Maxwell, E., and Ashwell, G. (1953) *Arch. Biochem. and Biophys.* **43**, 389.
Millerd, A. (1951) *Proc. Linnean Soc. N. S. Wales* **76**, 123.
Millerd, A. (1953) *Arch. Biochem. and Biophys.* **42**, 149.
Millerd, A., and Bonner, J. (1953) *J. Histochem. Cytochem.* **1**, 254.
Millerd, A., and Bonner, J. (1954) *Arch. Biochem. and Biophys.* **49**, 343.
Millerd, A., Bonner, J., Axelrod, B., and Bandurski, R. S. (1951) *Proc. Natl. Acad. Sci. U.S.* **37**, 855.
Millerd, A., Bonner, J., and Biale, J. B. (1953) *Plant Physiol.* **28**, 521.
Mitchell, M. B., Mitchell, H. K., and Tissières, A. (1953) *Proc. Natl. Acad. Sci. U.S.* **39**, 606.
Mullins, L. T. (1940) *Proc. Soc. Exptl. Biol. Med.* **45**, 856.
Nadson, G. A., and Rochlin, E. J. (1933) *Protoplasma* **20**, 31.
Nason, A., Oldewurtel, H. A., and Propst, L. M. (1952) *Arch. Biochem. and Biophys.* **38**, 1.
Nason, A., Wosilait, W. D., and Terrell, A. J. (1954) *Arch. Biochem. and Biophys.* **48**, 233.
Newcomb, E. H. (1950) *Am. J. Botany* **37**, 264.
Newcomb, E. H. (1951) *Proc. Soc. Exptl. Biol. Med.* **76**, 504.
Newcomb, E. H. (1954) Personal communication.
Newcomb, E H., and Stumpf, P. K. (1952) in "Phosphorus Metabolism" (McElroy and Glass, eds.), Vol. 2, p. 291. Johns Hopkins Press, Baltimore.
Newcomer, E. H. (1940) *Botan. Rev.* **6**, 85.
Newcomer, E. H. (1951) *Botan. Rev.* **17**, 53.
Nossal, P. M. (1954) *Biochem. J.* **57**, 62.
Novelli, G. D. (1953) *Physiol. Revs.* **33**, 525.
O'Brien, J. A., Jr. (1951) *Am. J. Botany* **38**, 684.
Ochoa, S. (1954) *Advances in Enzymol.* **15**, 183.
Okunuki, K. (1939a) *Acta Phytochim. (Japan)* **11**, 27.
Okunuki, K. (1939b) *Acta Phytochim. (Japan)* **11**, 65.
Oparin, A. I. (1938) "The Origin of Life." Macmillan, New York.
Oparin, A. I. (1953) *Bull. soc. chim. biol.* **35**, 67.
Paech, K., and Krech, E. (1953) *Planta* **41**, 391.
Paigen, K. (1954) *J. Biol. Chem.* **206**, 945.
Palade, G. E. (1952) *Anat. Record* **114**, 427.
Palade, G. E. (1953) *J. Histochem. Cytochem.* **1**, 188.
Pappenheimer, A. M., Jr., and Williams, C. M. (1954) *J Biol. Chem.* in press.
Pearson, J. A., and Robertson, R. N. (1954) *Australian J. Biol. Sci.* **7**, 1.
Perner, E. S. (1952a) *Ber. deut. botan. Ges.* **65**, 52.
Perner, E. S. (1952b) *Biol. Zentr.* **71**, 43.
Perner, E. S. (1952c) *Ber. deut. botan. Ges.* **65**, 235.

Perner, E. S. (1953) *Protoplasma* **42**, 457.
Potter, R. L., and Bethell, F. H. (1952) *Fedcration Proc.* **11**, 270.
Potter, V. R., and Reif, A. E. (1952) *J. Biol. Chem.* **194**, 287.
Price, C. A. (1952) Doctor of Philosophy Thesis, Harvard University, Cambridge, Mass.
Price, C. A. (1953) *Arch. Biochem. and Biophys.* **47**, 314.
Price, C. A. (1954) Personal communication.
Price, C. A., and Thimann, K. V. (1951) *Arch. Biochem. and Biophys.* **33**, 170.
Price, C. A., and Thimann, K. V. (1954a) *Plant Physiol.* **29**, 113.
Price, C. A., and Thimann, K. V. (1954b) *Plant Physiol.* in press.
Puytorac, P. (1951) *Botaniste* **35**, 125.
Quastler, H., Schertiger, A. M., and Stewart, W. N. (1952) *J. Cellular Comp. Physiol.* **39**, 357.
Quayle, J. R., Fuller, R. C., Benson, A. A., and Calvin, M. (1954) *Program Am. Soc. Plant Physiol., Gainesville* p. 25.
Raaflaub, J. (1953) *Helv. Physiol. et Pharmacol. Acta* **11**, 142, 157; from (1953) *Chem. Abstr.* **47**, 11271.
Racker, E. (1954) *Advances in Enzymol.* **15**, 141.
Recknagel, R. O. (1950) *J. Cellular Comp. Physiol.* **35**, 111.
Reif, A. E., and Potter, Van R. (1954) *Arch. Biochem. and Biophys.* **48**, 1.
Ritchie, D. (1953) *Botan. Gaz.* **115**, 138.
Ritchie, D., and Hazeltine, P. (1953) *Exptl. Cell. Research* **5**, 261.
Roberts, R. H., Cowie, D. B., Britten, R., Bolten, E., and Abelson, P. H. (1953) *Proc. Natl. Acad. Sci. U.S.* **39**, 1013.
Robertson, R. N. (1951) *Ann. Rev. Plant Physiol.* **2**, 1.
Robertson, R. N., Wilkins, M. J., and Weeks, D. C. (1951) *Australian J. Sci. Research* **B4**, 248.
Robinson, E., and Brown, R. (1953) *Nature* **171**, 313.
Robinson, J. R. (1953) *Biol. Revs.* **28**, 158.
Roche, J., and Yagi, Y. (1952) *Compt. rend. soc. biol.* **146**, 642.
Rosenberg, A. J., and Ducet, G. (1949) *Compt. rend.* **229**, 391.
Rosenberg, A. J., and Ducet, G. (1951) *Compt. rend.* **233**, 449.
Rozsa, G., and Wyckoff, R. W. G. (1950) *Biochim. et Biophys. Acta* **6**, 334.
Ryland, A. G. (1948) *J. Elisha Mitchell Sci. Soc.* **64**, 117.
Saltman, P. (1953) *J. Biol. Chem.* **200**, 145.
Sarachek, A., and Townsend, G. F. (1953) *Science* **117**, 31.
Sarkar, N. K., Beinert, H., Fuld, M., and Green, D. E. (1952) *Arch. Biochem. and Biophys.* **37**, 140.
Schneider, W. C. (1953) *J. Histochem. Cytochem.* **1**, 212.
Schneider, W. C., and Ross, H. E. (1950) *J. Natl. Cancer Inst.* **10**, 977.
Seifter, E. (1953) Doctor of Philosophy Thesis, University of Pennsylvania, Philadelphia.
Selby, C. C. (1953) *Cancer Research* **13**, 753.
Sharpensteen, H. H., and Conn, E. E. (1954) *Program Am. Soc. Plant Physiol., Gainesville* p. 37.
Shaw, M. (1954) *Can. J. Botany* **32**, 523.
Siegel, S. M. (1952) *Botan. Gaz.* **114**, 139.
Siekevitz, P. (1952) *J. Biol. Chem.* **195**, 549.
Simon, E. W. (1953) *Biol. Revs.* **28**, 453.

Simon, E. W. (1954) Personal communication.
Sjöstrand, F. S. (1953) *Nature* **171**, 30.
Sjöstrand, F. S., and Rhodin, J. (1953) *Exptl. Cell Research* **4**, 426.
Slater, E. C. (1950) *Biochem. J.* **46**, 484.
Slater, E. C., and Cleland, K. W. (1953) *Biochem. J.* **53**, 557.
Slater, E. C., and Holton, F. A. (1954) *Biochem. J.* **56**, 28.
Slautterback, D. B. (1953) *Exptl. Cell. Research* **5**, 173.
Smith, F. G. (1952) *Plant Physiol* **27**, 445.
Sorokin H, (1941) *Am. J. Botany* **28**, 476.
Sorokin, H. (1954) *Am. J. Botany* in press.
Sparrow, A. H., and Hammond, M. R. (1947) *Am. J. Botany* **34**, 439.
Spector, W. G. (1953) *Proc. Roy. Soc.* **B141**, 268.
Sponsler, O. L., and Bath, J. D. (1953) *Protoplasma* **42**, 69.
Stafford, H. A. (1951) *Physiol. Plantarum* **4**, 696.
Stafford, H. A. (1952) *Bull. Torrey Botan. Club* **79**, 351.
Stanbury, S. W., and Mudge, G. H. (1953) *Proc. Soc. Exptl. Biol. Med.* **82**, 675.
Stenlid, G. (1949) *Physiol. Plantarum* **2**, 350.
Stern, H., and Mirsky, A. E. (1952) *J. Gen. Physiol.* **36**, 181.
Stern, H., and Mirsky, A. E. (1953) *J. Gen. Physiol.* **37**, 177.
Stocking, C. R. (1952) *Am. J. Botany* **39**, 283.
Street, H. E., and Lowe, J. S. (1950) *Ann. Botany (London)* [N.S.] **14**, 307.
Strehler, B. L. (1952) in "Phosphorus Metabolism" (McElroy and Glass, eds.), Vol. 2, p. 491. Johns Hopkins Press, Baltimore.
Strittmatter, C. F., and Ball, E. G. (1954) *J. Cellular Comp. Physiol.* **43**, 57.
Strugger, S. (1951) *Ber. deut. botan. Ges.* **64**, 69
Strugger, S. (1953) *Ber. deut. botan. Ges.* **66**, 439.
Stumpf, P. K. (1951) *Federation Proc.* **10**, 256
Stumpf, P. K. (1952) *Ann. Rev. Plant Physiol.* **3**, 17.
Sussman, A. S. (1953) *J. Cellular Comp. Physiol.* **42**, 273.
Sussman, A. S., and Markert, C. L. (1953) *Arch. Biochem. and Biophys.* **45**, 31.
Sutcliffe, J. F. (1953) *Intern. Rev. Cytol.* **2**, 179.
Sweeney, B. M., and Thimann, K. V. (1938) *J. Gen. Physiol.* **21**, 439.
Tewfik, S., and Stumpf, P. K. (1949) *Am. J. Botany* **36**, 567.
Thimann, K. V. (1951) Growth, Symposium, Volume 10, p. 5.
Todd, G. W. (1953) *Physiol. Plantarum* **6**, 169.
Tolbert, N. E., and Cohan, M. S. (1953a) *J. Biol. Chem.* **204**, 649.
Tolbert, N. E., and Cohan, M. S. (1953b) *J. Biol. Chem.* **204**, 639.
Tolmach, L. J. (1951) *Arch. Biochem. and Biophys.* **33**, 120.
Torrey, J. G. (1953) *Am. J. Botany* **40**, 525.
Van Fleet, D. S. (1952) *Botan. Rev.* **18**, 354.
Vennesland, B., and Conn, E. E. (1952) *Ann. Rev. Plant Physiol.* **3**, 307.
Vernon, L. P. (1953) *Arch. Biochem. and Biophys.* **43**, 492.
Vishniac, W., and Ochoa, S. (1951) *Nature* **167**, 768.
Vishniac, W., and Ochoa, S. (1952a) *J. Biol. Chem.* **195**, 75.
Vishniac, W., and Ochoa, S. (1952b) *J. Biol. Chem.* **198**, 501.
Watanabe, M. I., and Williams, C. M. (1953) *J. Gen. Physiol.* **37**, 71.
Waygood, E. R. (1950) *Can. J. Research* **C28**, 7.
Weber, M. W., Lenhoff, H. M., and Kaplan, N. O. (1954) *Biochim. et Biophys. Acta* **14**, 298.

Webster, G. C. (1952) *Am. J. Botany* **39**, 739.
Webster, G. C. (1953a) *Plant Physiol.* **28**, 724.
Webster, G. C. (1953b) *Plant Physiol.* **28**, 728.
Webster, G. C. (1953c) *Arch. Biochem. and Biophys.* **47**, 241.
Webster, G. C. (1953d) *Program Am. Soc. Plant Physiol., Madison,* p. 30.
Webster, G. C. (1954) *Plant Physiol.* **29**, 202.
Webster, G. C., and Varner, J. E. (1954a) *J. Am. Chem. Soc.* **76**, 633.
Webster, G. C., and Varner, J. E. (1954b) *Federation Proc.* **13**, 317.
Weier, T. E. (1953) *Protoplasma* **42**, 260.
Weier, T. E., and Stocking, C. R. (1952a) *Am. J. Botany* **39**, 720.
Weier, T. E., and Stocking, C. R. (1952b) *Botan. Rev.* **18**, 14.
Wheat, R. W., and Ajl, S. J. (1954) *Arch. Biochem. and Biophys.* **49**, 7.
Wilson, D. G., King, K. W., and Burris, R. H. (1954) *J. Biol. Chem.* **208**, 863.
Wolken, J. J., and Palade, G. E. (1953) *Ann. N.Y. Acad. Sci.* **56**, 873.
Wood, W. A., and Schwerdt, R. F. (1954) *J. Biol. Chem.* **206**, 625.
Woods, M. W., and DuBuy, H. G. (1951) *Am. J. Botany* **38**, 419.
Wosilait, W. D., Nason, A., and Terrell, A. J. (1954) *J. Biol. Chem.* **206**, 271.
Yin, H. C., and Sun, C. N. (1949) *Plant Physiol.* **24**, 103.
Yocum, C. S., and Hackett, D. P. (1954) In preparation.
Zelitch, I. (1953) *J. Biol. Chem.* **201**, 719.
Zelitch, I., and Ochoa, S. (1953) *J. Biol. Chem.* **201**, 707.

Addendum

Since the completion of this review, some work on isolated plant mitochondria done in Japan has come to the writer's attention. Mung bean particles, which were shown to play a central respiratory role (Maruo and Matsuhashi, 1952), carried out both a CoA-dependent synthesis of citrate from pyruvate (Funahashi and Akazawa, 1954) and oxidative phosphorylation with a P/O ratio as high as 1.62 (Uritani *et al.*, 1954). Soybean seedling mitochondria possess a Krebs cycle system, including CoA and condensing enzyme, for the oxidation of pyruvate (Funahashi *et al.*, 1953). Akazawa *et al.* (1953) showed that these soybean particles are active centers of transaminations, reductive amination, and glutamic decarboxylation. Sweet potato mitochondria, which contain cytochrome oxidase (Uritani and Akazawa, 1953), can carry out Krebs cycle oxidations and the coupled generation of high-energy phosphate bonds (Akazawa and Uritani, 1954).

References

Akazawa, T., Funahashi, S., and Uritani, I. (1953) *J. Agr. Chem. Soc. (Japan)* **27**, 849.
Akazawa, T., and Uritani, I. (1954) *J. Biochem. (Japan)* **41**, 631.
Funahashi, S., and Akazawa, T. (1954) *J. Agr. Chem. Soc. (Japan)* **28**, 574.
Funahashi, S., Akazawa, T., and Uritani, I. (1953) *J. Agr. Chem. Soc. (Japan)* **27**, 842.
Maruo, B., and Matsuhashi, M. (1952) *J. Agr. Chem. Soc. (Japan)* **26**, 440.
Uritani, I., and Akazawa, T. (1953) *J. Agr. Chem. Soc. (Japan)* **27**, 789.
Uritani, I., Akazawa, T., and Uritani, M. (1954) *Nature* **174**, 1060.

The Structure of Chloroplasts

K. MÜHLETHALER

Institut für Allgemeine Botanik, E. T. H. Laboratorium für Elektronenmikroskopie, Zürich, Switzerland

		Page
I.	Introduction	197
II.	Methods of Investigation	198
III.	Shape and Size of Chloroplasts	199
IV.	Composition of Chloroplasts	200
V.	Microscopic Structure of Chloroplasts	201
	1. Algae	201
	2. The Chloroplasts of Higher Plants	201
VI.	Results of Electron Microscope Investigations	203
	1. Homogeneous Flagellatae Chloroplasts	203
	2. Algal Chloroplasts	205
	3. Granular Chloroplasts	207
	4. Chloroplast Membrane	211
	5. Starch Formation in the Chloroplast	212
VII.	The Molecular Structure of the Grana Lamellae	212
VIII.	The Development of Chloroplasts	215
IX.	Continuity of the Chloroplasts and Heredity	217
X.	References	217

I. Introduction

Views on the fine structure of chloroplasts have changed repeatedly under the influence of changing biological thought in the various periods. The first period of 1855–1907 brought the classic works of Mohl (1855), Schimper (1885), and Meyer (1883) who were the first to describe the granular distribution of chlorophyll. In the chloroplasts these authors found an amorphous basic substance, the *stroma*, and green granules termed *grana*.

With the evolution of colloid chemistry at the turn of the century the idea of structure gradually came into disrepute. The structures in cell organelles were considered to be denaturation products caused by injuries to the cell. A view typical of this second period between 1907 and 1932 was that of Liebaldt (1913). The chloroplasts are said to consist of an aqueous and a lipid phase, and both phases are said to be interspersed homogeneously under the microscope. According to Liebaldt (1913), Schürhoff (1924), Guilliermond, Mangenot, and Plantefol (1933), Sharp (1934), Küster (1935), the chloroplast is thus homogeneous in the natural state while a grana structure was said to be a form of precipitation caused by an injury.

The third period opens with Heitz (1932). In his study on the origin of chromocenters he mentions that the chloroplasts display a granular structure *in vivo*. Doutreligne (1935) also confirms the correctness of Schimper-Meyer's grana theory. In a very comprehensive work Heitz (1936) proves, discussing 180 plants in a variety of stages of phyllogenetic evolution, that the theory of homogeneity of chloroplasts does not correspond to the facts. He was the first to observe that the grana are not spheres but discs. Weier (1936) again confirmed the correctness of these findings on a further 100 species of plants. This proved the correctness of the grana theory. Since then, the disposition and size of these discs has been examined in numerous reports (Hubert, 1935; Wakkie, 1935; Strugger, 1936a; Wieler, 1936; Metzner, 1937; Geitler, 1937; Menke, 1940c).

II. Methods of Investigation

In numerous aquatic plants having transparent leaves, such as *Vallisneria, Cabomba,* and *Myriophyllum,* the chloroplasts can be investigated in the living cell directly. With thicker leaves, recourse must be taken to sections or crushed preparations. According to Heitz (1936) the grana structure of the isolated chloroplasts is well preserved in 7, 10, or 20% cane sugar solutions in the extracted juice of the plant, or in paraffinum liquidum. As early as 1883 Meyer considered the best fixing liquids to be OsO_4 solutions. The compound fixatives otherwise commonly used in botanical microscopy, as a rule, yield unsatisfactory results. OsO_4 has, however, the disadvantage that the fixed chloroplasts can no longer be stained selectively. The chloroplasts either stain uniformly or remain a glassy gray without absorbing any more dye. According to Strugger (1951) staining is successful if the tissue fixed in 2% OsO_4 solution is subsequently allowed to swell somewhat in a 1% solution of KOH. After swelling, the grana stain very satisfactorily with rhodamine B solution, 1:1000, or neutral red pH 7 in a 1:500 solution. Observation by means of a light microscope is then best effected in green light. Further selective grana stains suggested by Strugger (1954a) are acid magenta, Victoria blue, methylene blue, gentian violet, safranine T, and thionine. According to Strugger (1936b) rhodamine B, 1:1000, has proved very suitable for fluorescent microscopy.

The chloroplasts are isolated from the cell for chemical examination. One method of isolating the chloroplasts from the plant juice has been described by Granick (1938).

A special imbibition method has been evolved by Frey-Wyssling and Steinmann (1948) for the examination of algal chloroplasts in the polar-

izing microscope. Some filaments of algae were knotted to a hair and drawn into a capillary tube. This tube was fixed to a capillary rotor on the object table of the polarizing microscope with one end connected to a vacuum pump so that the various liquids could be drawn through the tube to the algal filaments. In this manner one and the same chloroplast could be examined from front and side views.

In electron microscopy the best fixative is the one suggested by Palade (1952), i.e. 1% OsO_4 in a 0.028 *M* sodium veronal-acetate buffer at a pH of 7.0. After dehydration in alcohol the leaves are embedded in a mixture of methyl and butyl methacrylate and sectioned.

Recently the ultraviolet microscope has been employed increasingly for examination in order to gain a better insight into the chemical structure (e.g. the nucleic acid contents) with spectroscopic methods.

III. Shape and Size of Chloroplasts

The chloroplasts are situated in the protoplasm and possess a shape typical of the genus. The chloroplasts of algae are the largest; not infrequently their size reaches 100 μ. Usually plate-type pigment carriers are found which are either lobed like amoebas (*Mougeotia*) or ribbon-shaped (*Spirogyra* groups). But net or lattice type plastids, as in *Oedogonium* and *Peridinium*, are known as well (Küster, 1951). In contradistinction to the variegated shapes of the chloroplasts of primitive organisms, those of the higher plants are quite uniform in shape. They are disc-like or flat ellipsoids. Meyer investigated the axis ratio and found that the principal axis averages 3.9 μ, the median axis 2.9 μ, and the shorter axis 1.6 μ. The volume of such a chloroplast is 9.4 μ^3. Möbius (1920) measured the size of chloroplasts of over 200 species and found that they varied between 3 and 10 μ. 75% of the chloroplasts measured were 4–6 μ and 50% were 5 μ. Now and then larger chloroplasts are found, e.g. Schürhoff (1907) measured a diameter of 24 μ for *Peperonica metallica*.

The number of chloroplasts per cell is subject to substantial variation. In *Thallophyta* there may be just a single one, while up to 100 may be found in a single cell of the higher plants. Haberlandt (1882) gives an average of 36 chloroplasts in every palisade cell and 20 in every spongy parenchyme cell for *Ricinus communis*. The number is, therefore, not constant in one and the same plant.

The various plants differ from one another not only in respect of the size of their chloroplasts but in the grana sizes as well. A photograph from Heitz (1936) is reproduced (Fig. 1) which shows the differences in the grana size for various *Phanerogama* (except Fig. 1b).

Heitz (1936) found a grana diameter of only 0.34 μ in *Plectranthus*,

whereas diameters up to 2 μ can be measured in some *Selaginella* varieties. The average diameter ranges between 0.5 and 0.8 μ. Plants growing in shady places have larger grana than those growing in the sun, and an increase is found towards the interior mesophyll cells. Heitz (1936) also

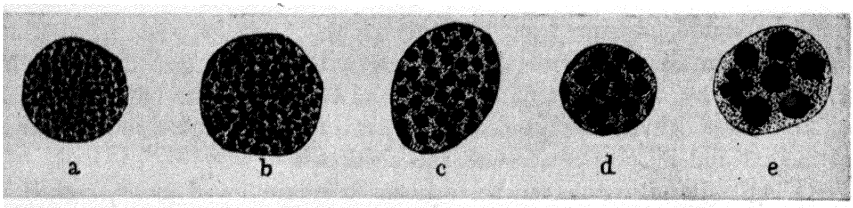


FIG. 1. Size of grana in different phanerogames. (a) *Plectranthus purpuratus*, (b) *Cabomba caroliniana*, (c) *Eucharis grandiflora*, (d) *Eranthemum leuconeurum*, and (e) *Lithops Lesleyi* (from Heitz, 1936)

established variation in accordance with the seasons. In *Lithops*, for instance, grana are of only 2 μ in March. Also the number of grana per chloroplast is subject to substantial variation, e g. between 20 and 100. In *Homidium flaccidum*, Hanson (1939) counted an average of 26 grana per chloroplast.

IV. Composition of Chloroplasts

Chemical analyses have been performed by Chibnall (1924, 1939), Menke (1938, 1940a), Granick (1938), Mommaerts (1938), Neish (1939), Krossing (1940), Bot (1942), Comar (1942), and Galston (1943). A comprehensive survey is found in Rabinowitch (1945).

According to Menke (1938) the chloroplasts of spinach (*Spinacia oleacea*) consist of 31% lipid, 48% protein, and 18% ash. The pigments (chlorophyll a and b, phycobilins, and carotenoids) represent only 5–10%.

The majority of proteins is located in the stroma, and the distribution of the lipids is not uniform. Staining experiments with Sudan (Weier, 1936) and rhodamin B (Strugger, 1936a, b) revealed that the fat-staining dyes leave the stroma unstained. This would indicate that the lipids are located in the grana. Menke (1940b) found that pure fat solvents do not completely remove the grana, and this indicates that they must contain a protein ground mass. Quantitatively, however, the protein is less than the lipid content. This is revealed by the myelin figures which Weber (1933) and Menke (1934a) produced on chloroplasts. The chlorophyll passes over into the myelin tubes thus displaying its lipid solubility.

Today it is also assumed that nucleic acid is localized in the chloroplasts. Metzner (1952b) has attempted to prove the presence of nucleic acid in the chloroplasts by virtue of its stainability and behavior with

respect to proteolytic ferments. His findings are deemed to prove the presence of two nucleic acids in the chloroplasts. The stroma is said to contain only pentose nucleic acid, the grana, pentose and deoxypentose nucleic acids. Rigorous proof would be very desirable, since this is of a certain importance from the point of view of genetics.

V. Microscopic Structure of Chloroplasts

1. Algae

Earlier investigators have reported that algal chloroplasts are homogeneous. But a grana structure has been described for some species of algae (Geitler, 1937; Hygen, 1937). However, these grana are not as clearly visible as in the chloroplasts of higher plants and most of them appear homogeneous under all conditions (Weier, 1938; Beauverie, 1938). Since these chloroplasts display no clear structure, attempts have been made to investigate their structure indirectly by means of the polarizing microscope. The double refraction of chloroplasts was discovered by Scarth in 1924 and generally employed by Küster (1933, 1934), Menke (1934b), Ullrich (1936), Weber (1936, 1937), Frey-Wyssling (1937), Frey-Wyssling and Wuhrmann (1947), and Frey-Wyssling and Steinmann (1948). Kuster (1933) and Menke (1934b) found that the chloroplasts of *Mougeotia* and *Spirogyra* exhibit a negative monoaxial double refraction (the optical basis being normal to the surface of the chloroplast). In *Closterium*, Menke (1934b) found that the double refraction disappears upon immersion in glycerol. This indicates that it is form birefringence, which can be explained by a laminar structure, the planes of the laminae being oriented normally to the short axis of the chloroplasts. Dried chloroplasts show a positive double refraction; extraction of the lipids with ether makes it negative. This implies that the positive double refraction must be an intrinsic property of some regularly arranged anisotropic molecules. Menke (1934b) and Frey-Wyssling (1937) ascribed it to an arrangement of elongated lipid molecules. The oriented lipid layers contain chlorophyll and alternate with protein laminae.

2. The Chloroplasts of Higher Plants

Investigations by Menke and Koydl (1939) on disintegrated microtome slices of *Anthoceros* chloroplasts revealed lamination in the ultraviolet microscope. In chloroplasts which contained accumulated metabolites, the stacks of laminae were pushed apart. They counted 20–40 laminae in one slice. Strugger (1951) investigated isolated chloroplasts in lateral view in distilled H_2O. Shortly after isolation, the chloroplasts swell invariably in a direction normal to the long axis. Desquamation occurs and the grana

become invisible. On the strength of these swelling tests, Strugger (1951) concluded that the grana must be oriented on carrier lamellae parallel to the chloroplast surface, and established the diagram in Fig. 2. Observations with a light microscope have not determined the number or thick-

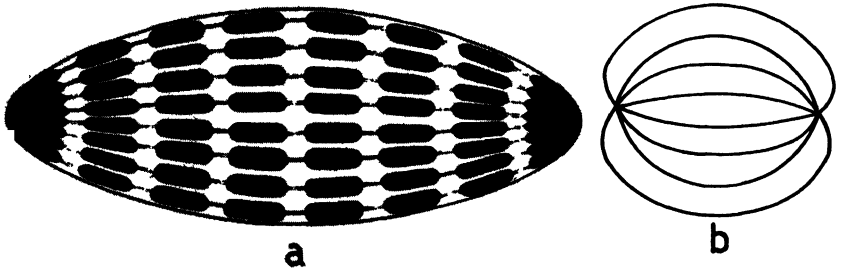


FIG. 2. (a) Scheme of a cross-section of a grana-carrying chloroplast. The grana are connected with a carrier lamella (from Strugger, 1951). (b) Laminated structure after swelling in 0.6 *N* KCNS (from Strugger, 1947). The number of carrier lamellae corresponds to the number of grana in a stack.

ness of the lamellae. Lamination is considered to be typical of both lower and higher plants.

The distribution of the grana in the chloroplasts has been examined by Heitz (1936) and Strugger (1951). Strugger found that the grana discs remain sharply defined when the microscope objective is focused at different levels in a chloroplast. This means that their arrangement is that of a stack of coins. According to Mevius and Düvel (1953), however, the grana lying deeper in the stroma are laterally displaced with respect to the higher ones. Berger (personal communication) thereupon investigated the disposition of the grana in numerous species and found that the coin-stack type of arrangement is found in numerous species of higher plants. Small variations in the degree of swelling can cause considerable displacements. Kaja (1954a, b) observed an exception from the coin-stack type arrangement of the grana in moss chloroplasts. In examinations of a total of 105 *musci* and *hepaticae* varieties, not a single instance of this type of grana arrangement could be found. The number of grana is 60–80 from the front view. Usually they are arranged on three lamellae, infrequently on four. The coin-stack structure is apparently not present in mosses and must be considered to imply higher evolutionary differentiation. In *Mnium hornum* the grana in the protonema are disposed in only two layers arranged parallel to the surface of the plastid.

According to Granick (1949) the spinach chloroplast contains 40–60 grana with a diameter of 0.6 μ and a thickness of 0.08 μ. Thus the volume of the grana is much smaller than the total volume of the chloroplast. According to Frey-Wyssling (1953) it amounts to one-fifteenth of the whole chloroplast. This is obviously not sufficient to hold all the lipids, especially if it is borne in mind that the grana contain protein in addition. The conclusion follows that the stroma is rich in lipids.

The views of Priestley and Irving (1907) and Wieler (1936) that the grana are disposed only on the surface of the chloroplasts is thus disproved. Furthermore, the view of Rezende-Pinto (1952) that the grana are in a helicoidal arrangement and interconnected by a filament must be attributed to the examination of improperly fixed chloroplasts (Strugger, 1951).

VI. Results of Electron Microscope Investigations

1. Homogeneous Flagellatae Chloroplasts

The structure of chloroplasts in algal flagellatae *Euglena gracilis bacillaris* and *Poteriochromonas stipitata* (a phagotrophic chrysomonad) has been investigated in the electron microscope (EM) by Wolken and Palade (1952, 1953). The existence of an orderly, laminated structure below the limit of visibility of the light microscope, which was postulated by investigators using the polarizing microscope, has been confirmed. In *Euglena* (Wolken and Palade, 1952) a stack of lamellae, more or less regularly spaced, is visible (Fig. 3a). There are about 20 bands having a thickness from 180–320 Å. The average thickness is 250 Å. The interlamellar spaces are 300–500 Å and contain homogeneous material of low electron density. Wolken and Palade (1952) noticed that the spacing of the lamellae appears to be affected by the fixative. If the objects are fixed at an acid pH, the lamellae are pulled apart and large free spaces become visible between them. A faint lamination seen in the phase-contrast microscope indicates that a certain variability in the spacing of the lamellae is present *in vivo*. The piling of the lamellae is irregular and occasionally curious patterns can be found in which they appear to converge in a central granule (Wolken and Palade, 1953). The chloroplasts of *Poteriochromonas stipitata* are also arranged in the same pattern. The lamellae are fewer and thicker than in *Euglena* and measure approximately 300 Å. Each lamella appears to have a central layer of lighter material covered by thinner and denser layers on both sides (Wolken and Palade, 1953). These authors also noticed that in flagellates, which have become colorless after being grown in darkness for periods

longer than seven days, the chloroplast changes its structure. The regular lamellation appears to be partly or totally lost. When these dark-adapted colorless *Euglenas* were brought back to light, it was found that, after a 4-hour light exposure, elongated bodies with a characteristic lamination could be seen in the chloroplasts. The first-formed lamellae are thinner and less numerous and less regularly packed than in organisms grown in light. After 72 hours of light exposure the chloroplasts had the same structure as in *Euglena* grown in light for generations.

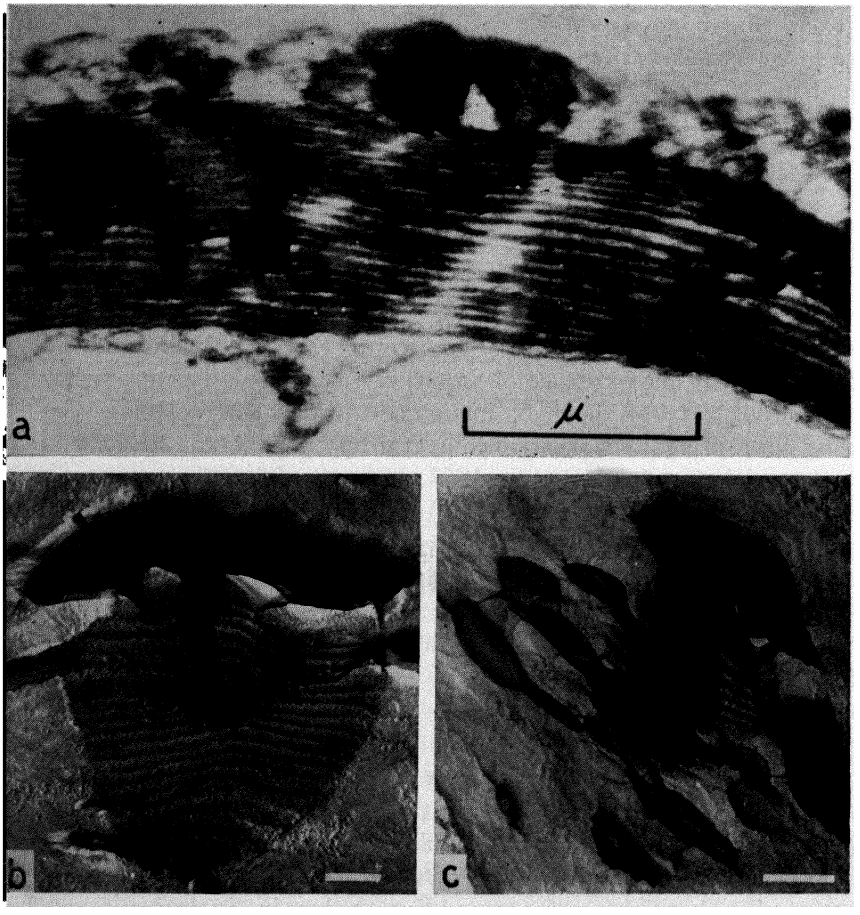


FIG. 3. Electron micrographs of OsO_4-fixed and embedded chloroplasts. (a) *Euglena gracilis* chloroplast consisting of approximately twenty lamellae of an average thickness of 250 Å. Magnification: 30,000 × (from Wolken and Schwertz, 1953). (b) and (c) Pyrenoids of *Mougeotia* with starch grains. Magnifications: 7400 × 9500 ×.

2. *Algal Chloroplasts*

The large algal chloroplasts in *Spirogyra* and *Mougeotia* have been investigated by Steinmann (1952b). Here, too, a pronounced lamination has been found. Measurements of the thickness of the thinnest lamellae in *Spirogyra* have been established as 69 ± 7 Å. Here again the spacing of the lamellae depends upon the method of fixation, similar to the above-mentioned *Flagellatae* chloroplasts. In the planes of the lamellae a fine punctate structure was observed which may be due to a partial separation of the lipid component. In his electron micrographs, Steinmann could not detect any grana structures. The presence of such bodies has been demonstrated in some species of chloroplast-free algae by Geitler (1937). In other species of blue algae, however, no grana could be seen. Thomas (1952) examined several algae (*Chlorella vulgaris, Synechococcus,* and *Porphyrichium cruentum*) and some bacteria (*Chromatium* and *Rhodospirillum rubrum*) and, after grinding and centrifuging, found disc-shaped bodies which he interpreted as grana. However, the question arises whether these bodies are not pyrenoids. Most algae contain pyrenoids, bodies of peculiar shape which are the locus of starch synthesis. The pyrenoids are rich in protein and their size varies within wide limits. The conjugates (up to 18 μ for *Spirogyra*) and peridineae (*Discodinium Pouchetii*) have particularly large pyrenoids (Dangeard 1938). The frequently angular contours have caused the erroneous conclusion that they are crystals (Meyer, 1883; Schimper, 1885). A comprehensive survey of the incidence and the different configuration of the pyrenoids is found in Küster (1951). In the light microscope not much can be found with respect to the structure of the pyrenoids. They appear homogeneous and optically isotropic (Czurda 1929). They often contain small inclusions which Czurda found to be starch granules. Lipids as well as starch may be formed under certain circumstances (Bose 1942–43).

Electron micrographs have been published by Steinmann (1952b), Wolken and Palade (1952), and Leyon (1954). These authors found a marked lamination in the pyrenoids as well, which we have been able to confirm in a number of investigations. A micrograph made in our own examinations of starch formation in *Mougeotia* is shown in Fig. 3b, c. The lamellae of the chloroplast can be followed in some cases through the pyrenoid. Lamination in the pyrenoids are far coarser than in the chloroplasts. Leyon (1954) sets the chloroplasts at 80 ± 20 Å, while the pyrenoid lamella may have a thickness of 300–700 Å. As shown in Fig. 3b, numerous starch granules are usually found around the pyrenoid; some are found even between the lamellae. The formation of these starch granules

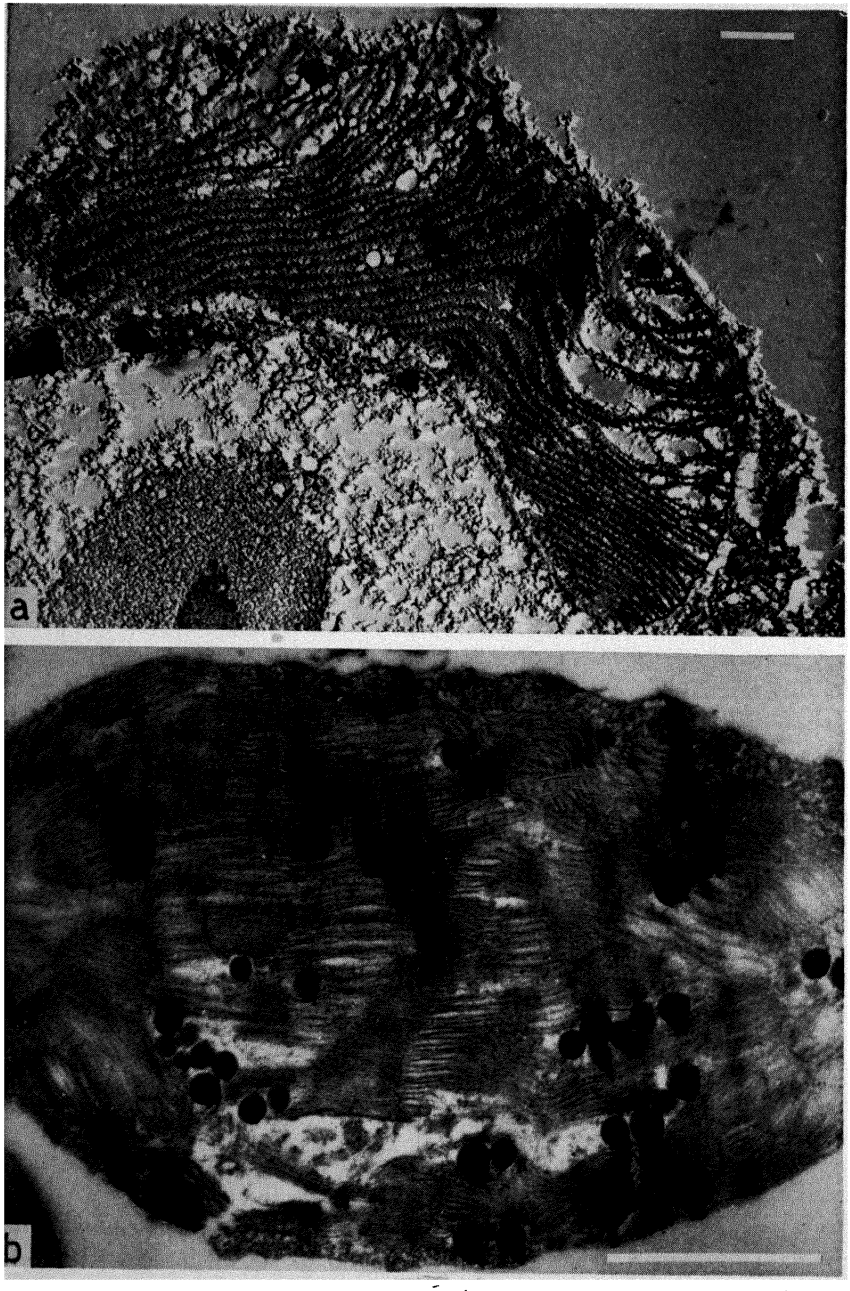


FIG. 4. (a) *Selaginella Watsonii*. Note the fine granulated structure between the dark bands. Magnification: 9500 ×. (b) *Aspidistra*. Structure of a granular-chloroplast. Magnification: 28,500 × (from Finean, Sjöstrand, and Steinmann, 1953).

will be discussed later. The question whether the pyrenoids of the algae are primitive forms of grana of the higher plants is not yet satisfactorily answered.

Some primitive pteridophyta, such as *Selaginella,* still have the same laminated structure as was found in flagellatae, algae, and mosses. Fig. 4a shows a cross-section of a chloroplast of *Selaginella Watsonii.* The grana are not visible in this picture. It is clear that amorphous bands alternate with the less dense lamellae, which are finely granulated. Orientation of the lamellae is not regular, and lamination is often considerably disturbed.

3. Granular Chloroplast

Since the introduction of the EM, numerous reports on the fine structure of the chloroplasts of the higher plants have been published. The first EM investigations were performed by Kausche and Ruska (1940). There followed papers by Algera *et al.* (1947), Granick and Porter (1947), Frey-Wyssling and Mühlethaler (1949), Steinmann (1952a), Thomas, Post, and Vertregt (1954), Cohen and Bowler (1953), and Leyon (1953a, b).

In the preparation for EM investigations numerous artifacts may appear which led to erroneous conclusions, particularly in the first reports. Before ultra-thin sectioning was generally adopted, the method of preparation consisted in isolating the chloroplasts by centrifuging from the press juice. The chloroplasts which had partly burst were subsequently dried directly on the object slide. Apart from well-preserved chloroplasts with numerous grana, they found empty skins, isolated grana, and numerous minute globular macromolecules (Algera *et al.*, 1947; Frey-Wyssling and Mühlethaler, 1949). As reported by Kausche and Ruska (1940), numerous thin lamellae of varying diameters can emerge from the chloroplast (Fig. 5a). These laminae have been regarded as the carrier lamellae postulated by Menke (1940c), and Strugger (1951) and by Kausche and Ruska (1940). Algera *et al.* (1947) first pointed out that they might be dissociation products, and they assume that they are phosphatide bubbles. Menke and Jakob (1940), however, found that the phosphatides are only 2–7% of the total lipid portion of the chloroplasts. These would, therefore, rather appear to be myelin masses growing from the chloroplast, as has already been described by Weber (1933) and Menke (1934a). As shown in Fig. 5b, this tube grows from the stroma. This leads to the conclusion that lipids are present not only in the grana but in the stroma as well. Since lipochromes such as Sudan and rhodamine B are accumulated in the grana only (Wieler, 1936; Weier, 1936; Strugger, 1936a, b), their condition in the two chloroplast portions would have to

be different. Quantitatively, too, it appears impossible that the total lipid content (approximately 31% according to Menke 1938) should be localized in the grana. As regards the grana, there may be free lipids together with lipochromes, while lipoproteins probably appear in the stroma. The

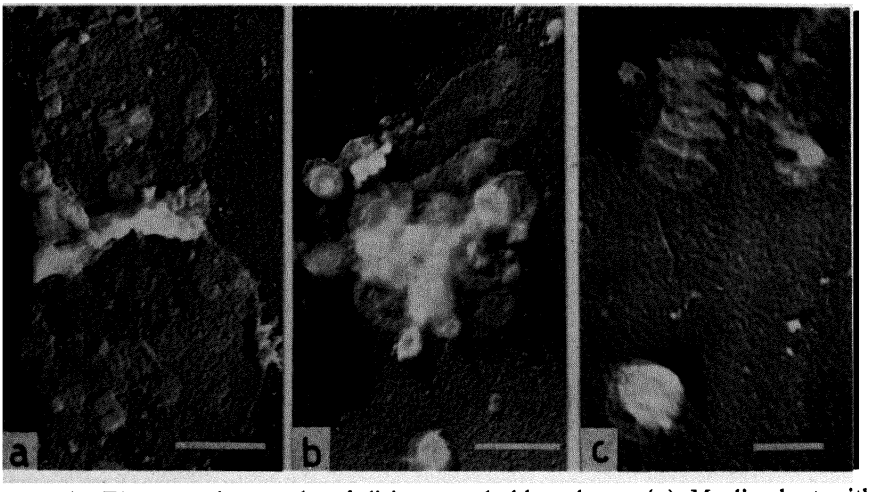


FIG. 5. Electron micrographs of disintegrated chloroplasts. (a) Myelin sheet with numerous grana. Magnification: 12,000 ×. (b) Formation of a myelin sheet in the stroma. Magnification: 11,000 ×. (c) An intact granum and a stack of overturned grana lamellae. Magnification: 20,000 × (from Frey-Wyssling and Muhlethaler, 1949).

binding of stroma lipids with the globular proteins can only be low since such myelin formations occur very frequently.

The grana are formed of disc-like platelets which will easily separate on isolation and disintegrate into the elementary lamellae. Fig. 5c shows an intact granum (below), and a granum disintegrated into lamellae. This disintegration was also found in *Aspidistra* by Frey-Wyssling and Steinmann (1953) and Leyon (1953a). If isolated grana are suspended in 1 *M* cane sugar solution and distilled water is added, the grana will swell. The disintegrated grana lamellae will then blister. Frey-Wyssling and Steinmann (1953) have drawn the conclusion that the elementary lamella in turn consists of two layers. Since the thickness of the elementary lamella is measured as 70 Å, the layer in question would be only 35 Å, which just corresponds to the diameter of the dehydrated protein particles of a Svedberg unit. By means of ultra-thin sections, Cohen and Bowler (1953), Leyon (1953a), and Finean, Sjöstrand, and Steinmann (1953) have found alternating light and dark lamellae in both the stroma and the grana. In the tobacco chloroplasts, they have a thickness of 140–280 Å,

usually about 210 Å for the dark lamellae, and 70–350 Å with an average thickness of about 140 Å for the light lamellae (Cohen and Bowler, 1953). The dark and light lamellae are about 70 Å thick in the grana, and the same value was reported by Steinmann (1952a) for *Aspidistra*. Steinmann has recently published a better picture of an *Aspidistra* chloroplast, which is shown in Fig. 4b (Finean, Sjöstrand, and Steinmann, 1953). Here again the stroma and the grana are laminated throughout. The spacing has been measured directly in the EM and with X-ray diffraction. A clear diffraction band at 250 Å has been obtained from OsO_4-fixed chloroplasts of *Aspidistra* and this spacing is in agreement with the layering in electron micrographs. There are numerous drops stained black by OsO_4 fixation between the lamellae. It is known that apart from starch and similar carbohydrates other visible assimilation products are formed in the chloroplasts, e.g. lipids. Some authors consider the oil drops to be assimilation products while others interpret them as waste products or as symptoms of aging. They are particularly numerous in leaves which are long-lived. Meyer (1917) found that the number of oil drops increases with increasing assimilation activity. These phenomena of fat accumulation described by Meyer (1917) are likely, in part, to be dissociation processes which also occur in degeneration phenomena and are termed 'lipophaneroses.' These findings seem to point to the fact that the droplets visible between the lamellae are such oil drops.

EM examinations revealed that the structural diagram established by Strugger (1951) must be modified with regard to the structure of the carrier lamellae. The numerous submicroscopic lamellae are not, as Strugger presumed, combined into bands in the intergranular areas on which the grana are fixed. As in the primitive flagellatae chloroplasts (Fig. 3a), they are more or less parallel throughout the chloroplast. A diagram based on the EM findings is given in Fig. 6.

In the diagram, the number of the grana and stroma lamellae has been reduced for the sake of clarity. The arrangement of the grana between the carrier lamellae is shown in enlargement in Fig. 6. As has already been mentioned, they have to be interpreted as bubble-type structures on the strength of the swelling tests made by Frey-Wyssling and Steinmann (1953).

The localization of chlorophyll within the chloroplast cannot be studied directly in the electron microscope. Thomas, Post, and Vertregt (1954) used the Molisch reaction (Molisch, 1918) as an indicator for chlorophyll activity. This reaction consists of a blackening of illuminated chloroplasts in the presence of silver nitrate due to the reduction of the salt to metallic silver. The investigations concerning this reduction have been reviewed

by Weber (1938), and Metzner (1952a). The mechanism of the Molisch reaction is not yet fully elucidated. Giroud, LeBlond, and Ratsimamanga (1934) and Dischendorfer (1937) found that ascorbic acid reduces silver nitrate. Metzner (1952a) presumes that chlorophyll displays this effect

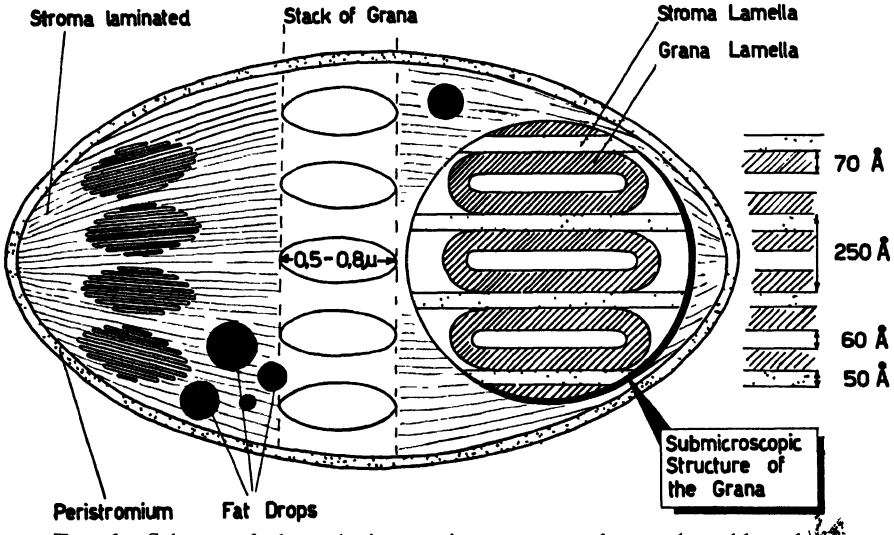


FIG. 6. Scheme of the submicroscopic structure of granular chloroplasts.

as well because the action spectrum established in living cells shows some features in common with the chlorophyll absorption spectrum. The reduced silver deposit could be used as a stain for the electron microscope. Thomas, Post, and Vertregt (1954) were able to show that, under the chosen experimental conditions, silver nitrate reduction is coupled with the presence of excited chlorophyll, the Molisch reaction being used to locate the pigments under the electron microscope. The precipitated metallic silver causes a strong electron scattering, and in this manner it is easily recognized. The silver content was also detected by the electron diffraction diagram. The electron micrographs revealed that this reaction occurs in the grana protein lamellae. From this it was concluded that they are the bearers of the chlorophyll. The stroma of spinach chloroplasts was studied by Thomas, Bustraan, and Paris (1952). They reported on the occurrence of cytoplasmic fibrils in the stroma. As to their shape, they resembled chains of tiny globules linked together by threads. Such structures have been postulated by Monné (1942) (based on his observation of sea urchin eggs) who termed them chromidia and interchromidia. Lehmann and Biss (1949) found the same for *Tubifex* eggs. The section work already discussed does not confirm these findings.

4. *Chloroplast Membrane*

A chloroplast membrane was first mentioned by Nägeli (1846). Meyer (1926), however, rejects the idea of a distinct membrane and speaks of closely adjacent areas of cytoplasm. The existence of a chloroplast membrane, however, is proved by the investigations made by Senn (1908), Lärz (1942), and Schmidt (1951). Senn (1908) observed the formation of pseudopodia on various chloroplasts and assumed that they might be growing from the peripheral layer, the peristromium. This enveloping layer would therefore be the locus of the motility of chloroplasts. Heitz (1936) observed such pseudopodia on *Colchicum Bornmülleri* and reported that such formations occur on other plants as well, irrespective of whether they are examined in water, isotonic cane sugar solution, or in paraffinum liquidum. In *Urginea maritima, Bellevallia romana,* and various *Allium* varieties the motion of these grana-free and colorless pseudopodia may partly be very lively. Lärz (1942) and Schmidt (1951) observed that the chloroplast membrane rises off the stroma like a blister in pathological conditions. This peripheral layer would thus enclose the interior consisting of grana and stroma.

Frey-Wyssling and Mühlethaler (1949) found a membrane using the electron microscope. Later observations, however, seemed to indicate that it was a myelin bubble, in this case, which had been raised from the stroma in preparation. Leyon (1953a) found no membrane around the chloroplast in sections of *Beta saccharifera.* He observed that the stroma continues directly into the cytoplasm outside the plastid and concluded that the boundary is a mere phase boundary. If the phase outside the chloroplast is changed, as may occur during fixation and dehydration, a precipitation of the outer layer of the chloroplast may take place which forms a membrane as an artifact. The more recent section work gives, however, more evidence of a peripheral layer as in the work of Senn (1908). We found that the *Selaginella* chloroplast is separated from the surrounding protoplasm by a pronounced membrane (Fig. 4a). Cohen and Bowler (1953) also mentioned a peripheral chloroplast membrane, but the method of preparation apparently destroyed most of it. Also in the *Aspidistra* chloroplast (Finean, Sjöstrand, and Steinmann, 1953) (Fig. 4b) an unlaminated zone around the plastid is apparent. The thickness of this peristromium zone is subject to considerable variation. The lamella structure such as is revealed by the electron microscope makes it appear unlikely that these inner zones participate in the formation of pseudopodia.

5. *Starch Formation in the Chloroplast*

According to Doutreligne (1953) the bark layer and the interior mass of the plastid must not be considered on a par functionally. On the exterior the early steps of photosynthesis should occur, and in the inner layers starch is being formed. If a starch granule is formed in a certain region of a chloroplast, this does not, therefore, imply that the starch is formed there alone. The entire chloroplast must take part in its formation. As soon as a germ is formed anywhere in the plastid, the total starch substance produced is deposited there. It is possible to study the development of the starch granules in the stroma by means of the electron microscope (Mühlethaler, 1954), but nothing definite can be said on the mechanism of formation of the starch granule. We have no more exact knowledge of whether the starch molecule is formed around the germ, or first formed throughout the plastid, and only subsequently conveyed to the starch granule. It has been shown in connection with the pyrenoids that portions of a plastid are particularly qualified to form starch. It must therefore be determined whether special areas are qualified to form starch in the more highly developed plastid.

Formation of pyrenoid starch has already been studied several times. Klebs (1891) and Czurda (1929) found that pyrenoid starch is the first to appear and the last to disappear in decomposition. Timberlake (1901) and Smith (1916) hold that only the pyrenoids are able to form starch and that a differentiation between stroma and pyrenoid starch is unnecessary. Formation must occur very rapidly because Steinecke and Ziegenspeck (1928) could detect microscopically the first starch granules on *Spirogyra* and *Zygnema* filaments cultivated in the dark after only 30 minutes exposure to light. We have never observed an alteration of the pyrenoid structure during starch formation (Fig. 3a, b). The views of Timberlake (1901) and McAllister (1927) to the effect that the pyrenoid substance converts itself into starch does not tally with the findings.

VII. The Molecular Structure of the Grana Lamellae

Hubert (1935) and Frey-Wyssling (1937) made the first attempt to introduce the molecular elementary units of chloroplasts into the submicroscopic lamellae. Physico-chemical examinations on the behavior of chlorophyll solutions allow certain conclusions to be drawn in respect of the arrangement in the grana. The chlorophyll molecule is like a tadpole in appearance, having a large head and a long tail. Fluorescence measurements of adsorbed chlorophyll monomolecular layers on aluminum hydroxide or globulin led Noack (1927) to the conclusion that it cannot be present in the colloidal state in plastids. This pigment must be present as

a monomolecular film. If Noack interposed lecithin between the chlorophyll layer and the adsorbant, its fluorescence was increased. Based on these results Hubert (1935) and Frey-Wyssling (1937) devised a scheme for the arrangement of the chlorophyll molecules in the grana. These authors assumed that bimolecular films of lecithin and chlorophyll are present in the lipid layers of the grana, the hydrophilic porphyrin "heads" of each chlorophyll molecule extending into the protein complex and the hydrophobic phytol "tail" extending into the lipid layer. Chemical analysis by Bot (1939) and Menke (1940a) proved that the molecular ratio of phosphorus to chlorophyll cannot correspond to that scheme because only a very small portion of the lipids are phosphatides. In a more recent edition of his book, Frey-Wyssling (1953) assumes that the chlorophyll is combined with proteins to form a chromoprotein. Such a complex had previously been postulated by Stoll (1936) who described it as a chloroplastin unit. Viewed in the electron microscope, the grana lamellae display fine granulation (Steinmann, 1952a, Thomas, 1952). If these globules having a diameter of less than 100 Å are considered as macromolecules, they would have to contain fewer than 24 Svedberg units and have a molecular weight below 400,000. Since Stoll (1936) indicates a molecular weight of 5,000,000 for chloroplastin, they cannot correspond to those molecules. According to Frey-Wyssling (1953), the grana contain approximately 9 chlorophyll molecules per Svedberg unit. It is as yet unknown how the chlorophyll is combined with the proteins. Takashima (1952) crystallized a chlorophyll-lipoprotein from an aqueous picoline solution. However, it is still unclear whether they also occur in the chloroplast or whether this is an artificial alliance of a protein with lipide and chlorophyll. In a recent paper Wolken and Schwertz (1953) examined the validity of the monomolecular film hypothesis in plastids of the two algal flagellates *Euglena gracilis* and *Poteriochromonas*. Frey-Wyssling (1937) has already postulated such an arrangement. Whether this pattern is valid for the grana-carrying chloroplasts seems most unlikely, the chlorophyll concentration being too high for such a pattern. It was already mentioned that the chloroplasts of *Euglena gracilis* are built up of approximately 20 lamellae having an average thickness of 250 Å (Fig. 3a). If the assumption that the chlorophyll molecules are arranged in monomolecular layers at the interfaces between the lipide and the aqueous protein complex layers is correct, the average area for a chlorophyll molecule can be calculated if their number is known. First the chloroplast geometry was studied and the width, diameter, number of laminae, etc. accurately measured. In order to investigate the number of chlorophyll molecules in one chloroplast, the number of organisms and chloroplasts per milliliter was counted and

the chlorophyll extracted with 85% acetone, and the concentration determined with a spectrophotometer. Knowing the number of moles of chlorophyll, one can easily calculate the number of chlorophyll molecules in the average cell. Wolken and Schwertz (1953) found an average number of 1.02×10^9 chlorophyll molecules in an *Euglena* chloroplast. According to chloroplast geometry, the available area for one molecule would be 222×10^{-16} cm.^{2} for *Euglena* and 246×10^{-16} cm.^{2} for *Poteriochromonas*, respectively. The cross-sectional area of the hydrophilic "head" of the chlorophyll molecule measures 15×15 Å. This value of 225×10^{-16} cm.^{2} agrees surprisingly well with the available area. By virtue of their investigations Wolken and Schwertz (1953) proposed a model of the molecular arrangement of the chlorophyll molecules in the algal chloro-

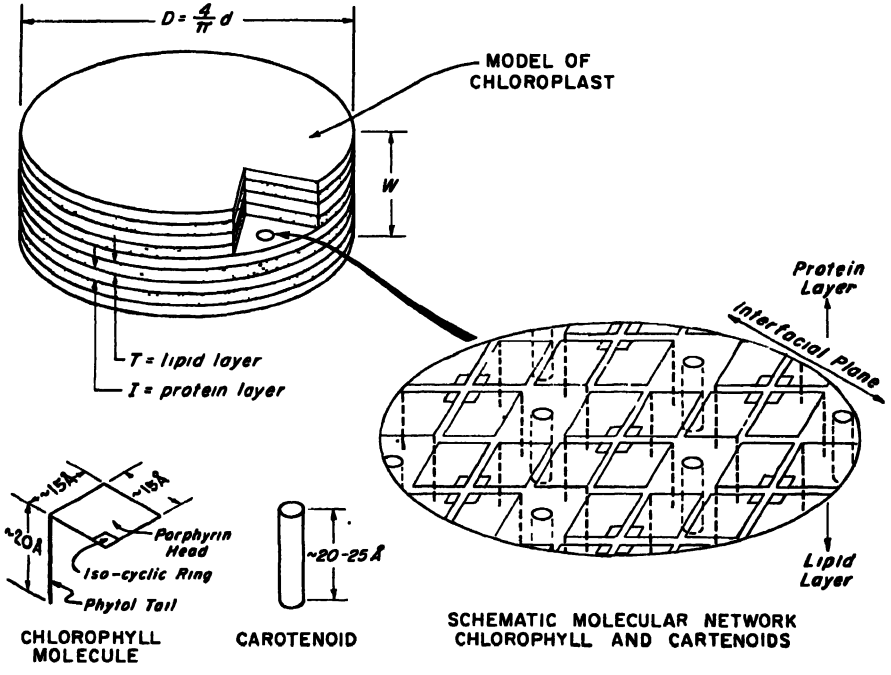


FIG. 7. Schematic molecular lattice for chlorophylls and carotenoids in algal chloroplasts (from Wolken and Schwertz, 1953).

plast. This model is reproduced in Fig. 7. In this figure, the suggestion of Baas-Becking and Hanson (1937) was followed, viz. that four chlorophyll molecules are united to form tetrads in which the reactive isocyclic rings turn toward each other. The carotenoid molecules are arranged between the chlorophyll molecules.

The average number of chlorophyll molecules per chloroplast has also been investigated by von Euler, Bergmann, and Hellström (1934) in a single chloroplast of *Elodea densa.* An average number of 1.7×10^9 or a concentration of 0.1 mole/l. was found. Godnev and Kalishevich (1940) found that the leaf of *Mnium* contained 1.6×10^9 molecules in each chloroplast. Since the volume of an average chloroplast is 4.1×10^{-11} ml., the concentration is 0.065 moles/l. Wolken and Schwertz (1953) found 1.02×10^9 molecules in *Euglena* (chlorophyll concentration 0.025 moles/l.) and 0.11×10^9 for *Poteriochromonas* (concentration 0.016 moles/l.). These values indicate that the number of molecules in different chloroplasts is relatively constant while the concentration increases rapidly with phylogenetic development.

VIII. The Development of Chloroplasts

Apart from evolving the grana theory, Meyer (1883) and Schimper (1885) established the theory of the continuity of the plastids for the phanerogames. They are said never to be formed from the plasma, but are conveyed from cell to cell during cell division. Scherrer (1913) found continuity to apply to bryophytes, and Schmitz (1883) to the algae. This theory of Schimper-Meyer became doubtful when Meves (1904) discovered the chondriosomes in plant cells. Lewitzky (1910), Alvarado (1923), and Guilliermond (1932) considered the chondriosomes to be the precursors of the chloroplasts. Up to very recent times this controversial problem remained unsolved. Lately the genesis of chloroplasts has been thoroughly investigated, in particular by Heitz and Maly (1953), as well as by Strugger (1950, 1954a, b) and Kaja (1954a, b). According to Strugger (1954b) the plastids in the meristems undergo a transformation during partition in respect of their shape, structure, and function. It is only with the lowest algae that no evolutionary metamorphosis is observable. The chloroplasts reproduce by simple division and are thus transferred to the daughter cells. Already with the higher algae species, such as *Fucales, Sphacelariales, Laminariales,* and *Florideae,* transformation can be observed on division (Strugger, 1954b). Owing to the rapid division of the chloroplasts in meristematic tissues the grana number is increasingly reduced. According to Strugger (1950, 1954a, b) it may be reduced to one primary granum in the proplastids of the higher plants. These proplastids can transform like amoeba and it is only the disc-shaped primary granum the shape of which remains constant. In division, it is said to reproduce exactly parallel to the plane. This division could therefore, in principle, be compared to the division of the chromatin filaments in mitosis (Strugger, 1954b). With evolution, division occurs in this man-

ner as well and produces the coin-stack type arrangement already described. Since 40–50 such piles are found in the mature chloroplast, as already mentioned, a lateral distribution must take place as well. Strugger (1951) explains the process as follows: one or more discs slide from the stack laterally and form a new stack by surface division.

These findings were checked by Heitz and Maly (1953) on Strugger's material, *Agapanthus umbellatus, Dracaena draco,* and *D. deremensis.* They found that young chloroplasts fluoresce entirely homogeneously and that a differentiation into stroma and grana occurs only later. From this they conclude that there is no continuous primary granum. They attribute the fluorescence of the proplastids to the chlorophyll content of the stroma. Often Heitz and Maly (1953) found one or two smaller discs beside a large granum in the youngest plastids, which led to the conclusion that they are newly formed in the stroma. Strugger (1954b) attributes these findings, which deviate from his own results, to the method of investigation employed. By means of vital staining with rhodamine B it is said to be impossible for the grana of the proplastids to be observed in the fluorescent microscope, since the dye does not penetrate (Strugger, 1936b). Observation of the primary granum, however, is rendered possible with OsO_4 fixation and with subsequent swelling and staining with rhodamine B. In his comprehensive work Strugger (1954b) found his earlier findings confirmed and considered the above type of reproduction as proved beyond a doubt. Kaja (1954a) has studied the complete development cycle of chloroplasts of *Mnium hornum.* The spores contain plastids of 3–4 μ with 30–40 grana. In places displaying great activity with respect to cell division, the size of chloroplasts can be reduced to a mere 0.8 μ, the grana number being only 4 in the extreme case. The process of inserting a particularly reduced embryonic phase of the plastids becomes the ruling principle here. But reduction of the grana number is not as great in higher plants where only one granum is usually present in the protoplastid (Strugger, 1954a, b).

These investigations further reveal that the thesis established by Guilliermond (1932) regarding the descent of the plastids from the chondriosomes is incorrect. Owing to the minuteness of the proplastids, confusion with the chondriosomes is easily possible.

Investigations by means of electron microscope of the first differentiation of chloroplasts have been reported by Leyon (1953b). Like Heitz and Maly (1953), he reaches the conclusion that the grana are formed at a later stage of plastid development. The first ultrastructure to be seen within the proplastid are a few extended laminae. It is assumed that these laminae become interrupted and the small rounded parts are trans-

formed into grana. These conclusions would appear to be quite uncertain because the electron micrographs indicate that the fixation of the objects is not good, and the details have been lost.

IX. Continuity of the Chloroplasts and Heredity

Independently of morphological investigations, genetics has revealed that non-Mendelian control centers, outside the nucleus in the cytoplasm and in the cell organelles, must be assumed for the performance of the metabolism in the cell (Marquardt 1952). The genes localized in the nucleus would, therefore, have to be interpreted as karyogenomes and distinguished from the genes localized in the plasma for which Wettstein (1928) coined the term plasmon. But if we assume that, above all, the centers important in metabolic activity may be localized in the cell organelles as well, a further division can be effected. It is known that e.g. the mitochondria control cell respiration. Photosynthesis and condensation of sugar into starch takes place in the chloroplasts and Strugger (1954b) considers these results to be a further confirmation of his findings with respect to the continuity of the chloroplasts. He proposes that the term plasmon, coined by Wettstein (1928), should be used only for the totality of all plasmagenes and that the individual gene groups should be subdivided. All controlling elementary constituents in the cytoplasm constitute the cytoplasmon. Strugger (1954b) terms plastidoms the units transferred by the chloroplasts, those of the chondriosomes, chondriomes etc. According to their functions, the elementary constituents for the formation of starch from sugar would have to be localized in the stroma, those causing pigment formation, in the grana, etc.

These views are most interesting as working hypotheses, and it will be the task of future research to investigate them.

X. References

Algera, L., Beijer, J. J., van Iterson, W., Karstens, W. K., and Thung, T. H. (1947) *Biochim. et Biophys. Acta* **1**, 517.

Alvarado, S. (1923) *Ber. deut. botan. Ges.* **41**, 85.

Baas-Becking, L. G. M., and Hanson, E. A. (1937) *Proc. Acad. Sci. Amsterdam* **40**, 752.

Beauverie, J. (1938) *Rev. cytol. et cytophys. végétales* **3**, 80.

Bose, S. R. (1942–43) *Botan. Gaz.* **104**, 633.

Bot, G. M. (1939) "Chloroplast, Proportion of Phosphor." Dissertation, Leiden.

Bot, G. M. (1942) *Chronica Botanica* **7**, 66.

Chibnall, A. C. (1924) *Biol. Chem.* **61**, 303.

Chibnall, A. C. (1939) "Protein Metabolism in the Plant." Yale Univ. Press, New Haven.

Claude, A. (1949) *Advances in Protein Chem.* **55**, 423.

Cohen, M., and Bowler, E. (1953) *Protoplasma* **42**, 414.
Comar, C. L. (1942) *Botan. Gaz.* **104**, 122.
Czurda, V. (1929) *Beih. Botan.-Zentr.* **45**, Abt. 1, 97.
Dangeard, P. A. (1938) *Botaniste* **29**, 153.
Dischendorfer, O. (1937) *Protoplasma* **28**, 516.
Doutreligne, S. J. (1935) *Proc. Koninkl. Akad. Wetenschap. Amsterdam* **38**, 886.
Euler, H. von, Bergman, B., and Hellström, H. (1934) *Ber. deut. botan. Ges.* **12**, 458.
Finean, J. B., Sjöstrand, F. S., and Steinmann, E. (1953) *Exptl. Cell Research* **5**, 557.
Frey-Wyssling, A. (1937) *Protoplasma* **29**, 279.
Frey-Wyssling, A. (1953) "Submicroscopic Morphology of Protoplasm." Elsevier, New York.
Frey-Wyssling, A., and Muhlethaler, K. (1949) *Vierteljahresschr. naturforsch. Ges. Zürich* **94**, 179.
Frey-Wyssling, A., and Steinmann, E. (1948) *Biochim. et Biophys. Acta* **2**, 254.
Frey-Wyssling, A., and Steinmann, E. (1953) *Vierteljahresschr. naturforsch. Ges. Zürich* **98**, 20.
Frey-Wyssling, A., and Wuhrmann, K. (1947) *Helv Chim. Acta* **30**, 20.
Galston, A. W. (1943) *Am. J. Botany* **30**, 331
Geitler, L. (1937) *Planta* **26**, 463.
Giroud, A., Leblond, C. P., and Ratsimamanga, A. R. (1934) *Compt. rend. soc. biol.* **117**, 614.
Godnev, T. N., and Kalishevich, S V. (1940) *Compt. rend. Acad. Sci. U.R.S.S.* **27**, 832.
Granick, S. (1938) *Am. J. Botany* **25**, 558.
Granick, S. (1949) "The Chloroplasts: Their Structure, Composition, and Development, in Photosynthesis in Plants." Iowa State College Press, Ames.
Granick, S., and Porter, K. P. (1947) *Am J. Botany* **34**, 545.
Guilliermond, A. (1932) *Protoplasma* **16**, 291.
Guilliermond, A., Mangenot, G., and Plantefol, L. (1933) "Traité de Cytologie végétale." E. Le François, Paris.
Haberlandt, G. (1882) *Jahrb. wiss. Botan.* **13**, 84.
Hanson, E. A. (1939) *Rec. trav. botan. néerl.* **36**, 180.
Heitz, E. (1932) *Planta* **18**, 571.
Heitz, E. (1936) *Ber. deut. botan. Ges.* **54**, 362.
Heitz, E., and Maly, R. (1953) *Z. Naturforsch.* **8b**, 243
Hubert, B. (1935) *Rec. trav. botan. néerl.* **32**, 323.
Hygen, G. (1937) *Planta* **27**, 379.
Kaja, H. (1954a) "Untersuchungen über die Entwicklung und Struktur der Moosplastiden." Dissertation, Münster.
Kaja, H. (1954b) *Ber. deut. botan. Ges.* **67**, 93.
Kausche, G. A., and Ruska, H. (1940) *Naturwissenschaften* **28**, 303.
Klebs, G. (1891) *Botan. Ztg.* **49**, 789.
Krossing, G. (1940) *Biochem. Z.* **305**, 359.
Küster, E. (1933) *Ber. deut. botan. Ges.* **51**, 523.
Küster, E. (1934) *Ber. deut. botan. Ges.* **52**, 626.
Küster, E. (1935) "Die Pflanzenzelle." Gustav Fischer, Jena.
Küster, E. (1951) "Die Pflanzenzelle," p. 296. Gustav Fischer, Jena.
Lärz, H. (1942) *Flora (Ger.)* **135**, 319.
Lehmann, F. E., and Biss, R. (1949) *Rev. Suisse Zool.* **56**, 264.

Lewitzky, G. (1910) *Ber. deut. botan. Ges.* **28**, 538.
Leyon, H. (1953a) *Exptl. Cell Research* **4**, 371.
Leyon, H. (1953b) *Exptl. Cell Research* **5**, 520.
Leyon, H. (1954) *Exptl. Cell Research* **6**, 497.
Liebaldt, E. (1913) *Z. Botan.* **5**, 65.
Marquardt, H. (1952) *Ber. deut. botan. Ges.* **65**, 198.
McAllister, F. (1927) *Am. J. Botany* **14**, 246.
Menke, W. (1934a) *Protoplasma* **21**, 279.
Menke, W. (1934b) *Protoplasma* **22**, 56.
Menke, W. (1938) *Z. Botan.* **52**, 273.
Menke, W. (1940a) *Z. physiol. Chem.* **263**, 100, 104.
Menke, W. (1940b) *Naturwissenschaften* **28**, 31.
Menke, W. (1940c) *Naturwissenschaften* **28**, 158.
Menke, W., and Jakob, E. (1940) *Z. physiol. Chem.* **272**, 227.
Menke, W., and Koydl, F. (1939) *Naturwissenschaften* **27**, 29.
Metzner, H. (1952a) *Nachr. Akad. Wiss. Göttingen, Math.-physik Kl. IIb. Biol.-phys.-chem. Abt.* No 1, p. 1-5.
Metzner, H. (1952b) *Biol. Zentr.* **71**, 257.
Metzner, P. (1937) *Ber. deut. botan. Ges.* **55**, 16.
Meves, F. (1904) *Ber. deut. botan. Ges.* **22**, 284.
Mevius, W., and Duvel, D. (1953) *Protoplasma* **42**, 287.
Meyer, A. (1883) "Das Chlorophyllkorn in chemischer, morphologischer und biologischer Beziehung." Felix, Leipzig.
Meyer, A. (1917) *Ber. deut. botan. Ges.* **35**, 658.
Meyer, A. (1926) "Morphologische und physiologische Analyse der Zelle der Pflanzen und der Tiere." Gustav Fischer, Jena.
Möbius, M. (1920) *Ber. deut. botan. Ges.* **38**, 224.
Mohl, H. von (1855) *Botan. Ztg.* **13**, 89, 105.
Molisch, H. (1918) *Sitzber. Akad. Wiss. Wien. Math. naturwiss. Kl. Abt. I.* **127**, 449.
Mommaerts, W. F. H. (1938) *Proc. Acad. Sci. Amsterdam* **41**, 896.
Monné, L. (1942) *Arkiv Zool* **B34**, No. 5, 1.
Muhlethaler, K. (1954) *Z. wiss. Mikroskop.* in press.
Nageli, C. von (1846) *Z. wiss. Botan.* **3**, 119.
Neish, A. C. (1939) *Biochem. J.* **33**, 293, 300.
Noack, K. (1927) *Biochem. Z.* **183**, 135.
Palade, G. E. (1952) *J. Exptl. Med.* **95**, 285.
Priestley, J. H., and Irving, A. A. (1907) *Ann. Botany (London)* **21**, 407.
Rabinowitch, E. I. (1945) "Photosynthesis." Interscience, New York.
Rezende-Pinto, M. C. de (1952) *Protoplasma* **41**, 336.
Scarth, G. W. (1924) *Quart. J. Exptl. Physiol.* **14**, 99.
Scherrer, A. (1913) *Ber. deut. botan. Ges.* **31**, 493.
Schimper, A. (1885) *Jahrb. wiss. Botan.* **16**, 11.
Schmidt, H. H. (1951) *Protoplasma* **40**, 209.
Schmitz, F. (1883) *Verhandl. Naturwiss. Verein Rheinland u. Westf.* **40**, 1.
Schurhoff, P. N. (1907) *Beih. Botan. Zentr.* **23**, Abt. 14.
Schurhoff, P. N. (1924) *in* "Handbuch der Pflanzenanatomie" (Linsbauer, ed.), Vol. 1. Abt. I. Borntraeger, Berlin.
Senn, G. (1908) "Die Gestalts- und Lageveranderung der Chromatophoren." Engelmann, Leipzig.

Sharp, L. W. (1934) "An Introduction to Cytology." 3rd ed. McGraw-Hill, New York.
Smith, G. M. (1916) *Ann. Botany (London)* **30**, 467.
Steinecke, F., and Ziegenspeck, H. (1928) *Ber. deut. botan. Ges.* **46**, 678.
Steinmann, E. (1952a) *Experientia* **8**, 300.
Steinmann, E. (1952b) *Exptl. Cell Research* **3**, 367.
Stoll, A. (1936) *Naturwissenschaften* **24**, 53.
Strugger, S. (1936a) *Flora (Ger.)* **131**, 113.
Strugger, S. (1936b) *Flora (Ger.)* **131**, 324.
Strugger, S. (1947) *Z. Naturforsch.* **2b**, 146.
Strugger, S. (1950) *Naturwissenschaften* **37**, 166.
Strugger, S. (1951) *Ber. deut. botan. Ges.* **64**, 69.
Strugger, S. (1954a) *Ber. deut. botan. Ges.* **66**, 439.
Strugger, S. (1954b) *Protoplasma* **43**, 120.
Takashima, S. (1952) *Nature* **169**, 182.
Thomas, J. B. (1952) *Proc. Koninkl. Akad. Wetenschap. Amsterdam* **C55**, 207.
Thomas, J. B., Bustraan, M., and Paris, C. H. (1952) *Biochim. et Biophys. Acta* **8**, 90.
Thomas, J. B., Post, L. C., and Vertregt, N. (1954) *Biochim. et Biophys. Acta* **13**, 20.
Timberlake, H. G. (1901) *Ann. Botany (London)* **15**, 619.
Ullrich, H. (1936) *Planta* **26**, 11.
Wakkie, J. G. (1935) *Proc. Koninkl. Akad. Wetenschap. Amsterdam* **38**, 1082.
Weber, F. (1933) *Protoplasma* **19**, 455.
Weber, F. (1936) *Protoplasma* **26**, 312.
Weber, F. (1937) *Protoplasma* **27**, 280.
Weber, F. (1938) *Protoplasma* **29**, 427.
Weier, E. (1936) *Am. J. Botany* **23**, 645.
Weier, E. (1938) *Botan. Rev.* **4**, 497.
Wettstein, F. von (1928) *Bibl. Genet.* **10**, 1.
Wieler, A. (1936) *Protoplasma* **26**, 295.
Wolken, J. J., and Palade, G. E. (1952) *Nature* **170**, 114.
Wolken, J. J., and Palade, G. E. (1953) *Ann. N.Y. Acad. Sci.* **56**, 873.
Wolken, J. J., and Schwertz, F. A. (1953) *J. Gen. Physiol.* **37**, 111.

Histochemistry of Nucleic Acids

N. B. KURNICK

Department of Medicine, University of California, Los Angeles, and the Research Laboratory of the Paraplegia Service, Veterans Administration Hospital, Long Beach, California

		Page
I.	Introduction	221
II.	Requirements for Quantitative Histochemical Analysis and Sources of Error	222
III.	Fixation of Tissues	229
IV.	Staining Methods	231
	1. General Discussion of Use of Basic Stains and Extraction Procedures	231
	2. Feulgen Nucleal Reaction and Its Variants	235
	3. Methyl Green	241
	4. Pyronin	245
	5. Other Stains "Specific" for Nucleic Acids	248
V.	Physical Methods	252
	1. General Introduction	252
	2. Ultraviolet Spectroscopy	253
	3. Historadiography	256
	4. Infrared Histospectroscopy	257
	5. Electron Microscopy	258
	6. Interferometry	258
	7. Photofluorometry	259
	8. Autoradiography	260
VI.	Summary	260
VII.	References	262

I. Introduction

In recent years, much evidence pointing to the central position of nucleic acids in cellular metabolism has accumulated. The schools of Caspersson and Brachet have emphasized the role of ribonucleic acid (RNA) in protein synthesis and in embryogenesis. The mechanism of the nucleic acid control of these processes remains unclear, but a relationship between RNA content and rate of protein synthesis appears to be a regular finding (Caspersson, 1950; Brachet, 1950; Kurnick, 1955a).

There is good evidence that deoxyribonucleic acid (DNA) is a major part, if not the sole constituent, of the gene. In support of this are (1) the work of Avery, MacLeod, and McCarty (1944) on the chemical nature of the pneumococcus transforming agent, (2) observations on the role of DNA in other bacterial mutations (Boivin *et al.*, 1945), (3) the finding of constant DNA content in nuclei of several organs of a single

species (Boivin, Vendrely, and Vendrely, 1948; Kurnick and Mirsky, 1947; Mirsky and Ris, 1949, 1951), a constancy which was unaffected by nutritional changes which greatly altered RNA and protein content (Campbell and Kosterlitz, 1952; Davidson and McIndoe, 1949; Mandel, Jacob, and Mandel, 1949; Kurnick and Mirsky, 1947), and (4) the discovery that haploid sperm cells contain approximately one-half as much DNA as the diploid somatic nuclei (Boivin *et al.*, 1948; Mirsky and Ris, 1949).

With such important biological roles, the identification, localization, and quantitation of nucleic acids within organelles of cells is of obvious significance. Several excellent reviews on this subject have appeared (Danielli, 1947; Lumb, 1950; Gomori, 1952; Sharma, 1952; Seshachar, 1952; Marinone, 1953; Pearse, 1953; Swift, 1953; Vialli, 1953).

The methods of nucleic acid localization and quantitation may be considered under the headings of macrochemical cellular fractionation and analysis, staining methods, natural absorption, other physical methods and combination of the histochemical methods with extraction procedures. Our discussion will be limited to those methods which may be applied for accurate localization in microscopic tissue sections or smears. We shall not review here the methods of tissue disruption, differential centrifugation, and chemical analysis (see Hogeboom, 1951 for review of these methods).

II. Requirements for Quantitative Histochemical Analysis and Sources of Error

Whereas all the procedures to be considered may be applied for qualitative analysis, only some are suitable for quantitative application. For quantitative analysis of nucleic acids *in situ*, photometric procedures must be used. The requirements for quantitation are that the absorbing material be specific for the nucleic acid and localize it without diffusion, that the reaction (if intrinsic absorption is not utilized) be stoichiometric, and that the material be suitable for photometry. The aspects of specificity and stoichiometry will be considered in turn for each technique as it is discussed. The requirements of photometry, other than instrumental (see Davies and Walker, 1953, for review of instrumentation) are that Beer's and Lambert's laws apply, and that the object photometrized will not give spurius absorption data (or that the data be susceptible to correction for such nonspecific effects on light transmission). Caspersson (1950) has reviewed the effect of the dimensions of the object and errors due to light diffraction and dispersion (see also Wilkins, 1950, who has included discussion of effect of focus, and Davies and Walker, 1953). He has also

emphasized the importance of optical homogeneity (Caspersson, 1939; see also Glick, Engstrom, and Malmstrom, 1951; Davies and Walker, 1953; Danielli, 1947, 1953; Glick, 1953). This may be secured by appropriate treatment of the material in some cases or by the successive analysis of very small areas, thereby reducing the degree of heterogeneity in any single determination. To facilitate the latter method of analysis, automatic scanning devices have been designed (Caspersson, Jacobsson, and Lomakka, 1951; Deeley *et al.*, 1954).

In nuclei, homogeneous dispersion of material is readily obtained by appropriate preliminary treatment (Ris and Mirsky, 1949b; Kurnick, 1949, 1950b; Kurnick and Herskowitz, 1952). However, many studies have been performed on heterogeneous structures. The authors who have photometrized heterogeneous objects have, in general, been aware of the fact that spuriously low absorptions are obtained. However, they have considered that comparative (relative) data could be obtained, or at least that the intensity of two absorbing materials with identical heterogeneous distribution could be compared with validity. The error which may occur from comparing absorptions obtained on heterogeneous structures with different distributions has been discussed repeatedly, as noted above. However, it has not been generally recognized that valid comparisons may not be made, even if the distributions are identical. It may be concluded that "relative" contents (usually expressed as the measured optical density multiplied by the measured area) are comparable only if both the optical densities and distributions are very similar (Ris and Mirsky, 1949b). A simple example will illustrate this fact.

In Fig. 1, the absorbing material in A occupies 75% of the field; in B it is uniformly distributed throughout it. The total areas photometrized

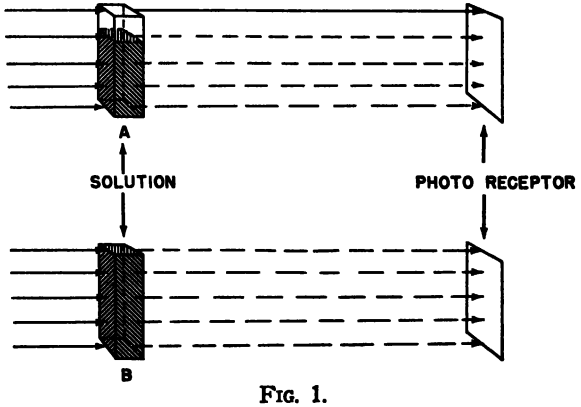


FIG. 1.

are equal and the measured optical density is 0.602 in each case. The conclusion that both contain the same amount of absorbing material would be in error. The optical density (E) of 0.602 corresponds to 25% transmission (T) since $E = -\log T$. In example A, since the empty 25% of the field absorbs no light, the remainder of the field must absorb 100% of the incident light to yield a mean of 25% transmission; i.e., the concentration of absorbing material is infinite. In B, the concentration of absorbing material will be E/ϵ where ϵ is the molar absorption coefficient. If, in our example ϵ is 1, the concentration is 0.6 M. The discrepancy between infinite concentration and 0.6 M is obvious, although the "relative units" are equal. Lesser errors will occur as A approaches B in homogeneity, and at lower optical densities. Thus, if the optical density were 0.301 and the other conditions remained as described, the concentration in B would be 0.3 M and in A, the concentration in the absorbing 75% of the area would be 0.477 M (i.e., E for the absorbing portion is $0.477 \equiv T = 33.3\%$; T for whole field, as measured by the phototube, is one-fourth $\times$ 100% + three-fourths $\times$ 33.3% = 50% $\equiv E = 0.301$). The mean concentration for the entire photometrized area is, then, 0.357 M (an error of 20% as compared with the infinitely large error in the preceding example). An indication of the magnitude of the possible error in the practice of photometrizing heterogeneous objects is found in Pollister and Ris' (1947) analysis of nucleic acid content of thymus nuclei by ultraviolet absorption; they found a content of 1.1×10^{-9} mg. DNA, which was thought to agree with the value obtained by chemical analysis. In fact, however, the result of the chemical analysis (in which we participated), to which the DNA content calculated from photometry was compared, was expressed in milligrams of phosphorus, not of DNA. Thus, the histochemical data was approximately 9 times too low!

The preceding source of error, as we have mentioned, has been recognized (if minimized in importance) by investigators photometrizing inhomogeneous structures. However, they appear to have neglected the fact that errors may occur in the interpretation of data even when the identical heterogeneous distribution is photometrized with two different stains (or the same stain at two different wavelengths at which the molar extinction coefficients differ considerably). Thus, the comparison of Feulgen stain density and of Millon reaction density (Pollister and Leuchtenberger, 1949a; Leuchtenberger, 1950; see Pollister, 1952, for other references) and of Feulgen and methyl green intensity (Korson, 1951a) in the same nucleus has been performed, and the conclusion that the ratio has experimental validity, even though the absolute values do not, has been inferred. The error in this reasoning may be illustrated as follows.

Let us consider the absorbing area in A (Fig. 1) to contain a 10 *M* solution of each of two solutes (e.g., protein and nucleic acid). We now stain with a dye specific for one of these, with a stoichiometry of 1:1 and a molar extinction coefficient of 1.0. The photometer will read 25% transmission (one-fourth × 100% + three-fourths × 10^{-8}%), which corresponds to an optical density of 0.602. We calculate, then, a content of 0.602 *A* "arbitrary" units where *A* is the total area photometrized. We next stain an identical solution with a second dye, specific for the second solute. The stoichiometry is again 1:1, but the molar extinction coefficient (ϵ) is 0.2. We now read 25.75% transmission (one-fourth × 100% + three-fourths × 1%), corresponding to *E* of 0.589. We calculate, then, a content of 0.589 *A* "arbitrary units". Assuming that stoichiometry and molar extinction coefficients are known, the relative contents of solutes 1 and 2 are calculated from the formula

$$\frac{S_1}{S_2} = \frac{E_1 \times A_1 \times k_2 \times \epsilon_2}{E_2 \times A_2 \times k_1 \times \epsilon_1}$$

where S_1 and S_2 are the contents of the solutes 1 and 2, respectively, and k_1 and k_2 are, respectively, the moles of dye 1 bound per mole of solute 1 and of dye 2 bound to solute 2. The calculation results in the conclusion that solutes 1 and 2 are present in a ratio of 0.205. The correct ratio is 1.0.

Usually k_1 and k_2 are not known for the histochemical situation. Since the ratio $(k_2 \times \epsilon_2)/(k_1 \times \epsilon_1)$ is a constant, the ratio S_1/S_2 is, therefore, more commonly expressed in "arbitrary relative values", i.e. $(E_1 \times A_1)/(E_2 \times A_2) = 1.02$. This is then compared to another cell. Let us consider the second cell to be identical in distribution of chromophore to cell A (though this precaution is generally not taken, which greatly compounds the error), but with solute 1 present in 1 *M* concentration in the absorbing 75% of the photometrized area and solute 2 present in 10 *M* concentration as before (i.e., $S_1/S_2 = 0.1$ instead of 1.0 as in the preceding example). $E_1 \times A_1$ now equals 0.488 *A*, and $E_2 \times A_2$ equals 0.589*A*. S_1/S_2 is now calculated to be 0.166 (the correct ratio is 0.1) and $(E_1 \times A_1)/(E_2 \times A_2)$ equals 0.83. Thus, it is concluded that the ratio of S_1/S_2 is 1.23 times as high in the first cell as it is in the second; in fact the ratio has changed by a factor of 10.0. Thus even the comparison of identical distributions in heterogeneous structures by such "arbitrary relative units" is not valid. However reasonable they may appear, the statements that heterogeneity "has little effect upon the ratio between the amounts of two substances with identical distributions" (Pollister and Leuchtenberger, 1949a), and despite inhomogeneity, "one is entirely justified in computing ratios of con-

centrations of different substances within one structure" (Moses, 1950), are erroneous.

Since the values derived from the photometry of heterogeneous material by the one wavelength method just considered have neither absolute nor relative validity, they can hardly be considered to be expressed in meaningful units at all.

Whereas most histochemical photometry has been done on heterogeneous structures at a single wavelength for any given component, and has therefore yielded data which must be examined with appropriate skepticism, the problem is not insurmountable. Nuclei may be rendered homogeneous by appropriate treatment, such as immediate fixation of one-cell thick tissues in carefully neutralized formalin (Kurnick and Herskowitz, 1952) or by immersion in cold 30% sucrose, followed by fixation in neutral formalin (Ris and Mirsky, 1949b; Kurnick, 1949, 1950b). Ornstein (1952) and Patau (1952) have described a technique of two-wavelength photometry which promises to be applicable to many tissues. Their technique requires that the ratios of the molar extinctions of the stained material at two wavelengths be known (preferably the ratio should be 2:1) and that no local areas of very high optical density be present. Very little data has been reported with this technique (Patau, 1952; Setterfield, Schreiber, and Woodard, 1954); but on theoretical grounds, valid data should be obtainable within the limitations mentioned.

The two-wavelength method of Orenstein and Patau permits calculation of content without reference to the geometry of the structure analyzed, since only the area of photocell aperture is required (in this discussion, errors due to erroneous light transmission measurements due to dispersion, refraction, and reflection are considered noncontributory, although these are often significant, particularly at short wavelengths, as discussed by Swift, 1953, and Naora and Sibatani, 1952). Other procedures require the photometry of central cores [this is preferred even with homogeneous structures, since it permits reduction of errors from other sources (Swift, 1953; Kurnick and Herskowitz, 1952)]. The calculation of content from such data requires some information about geometry. Cross sectional parameters may be accurately measured in the microscopic image, but thickness can be determined only with considerable uncertainty. Fortunately, if the geometry is regular, thickness cancels out in the calculation of content. Thus, $C = E/\epsilon t$ where C is concentration, E is optical density, ϵ is molar extinction coefficient, and t is thickness; content is equal to concentration $\times$ volume. For any regular figure, volume is $K \times$ cross-sectional area $\times\ t$, where K is a constant appropriate to the geometry of the figure. Therefore, content $= E/\epsilon \times K \times$ cross-sectional area (for the

entire structure; if a section of a nucleus is examined, of course no information is provided about the entire nucleus unless the volumes of the measured portion and the whole nucleus are known, in which case thickness must be known for both cases). Some estimate of the geometry is necessary for the selection of the appropriate K. For a sphere, or ellipsoid, K is two-thirds, for a cylinder it is 1. Since an ellipsoid represents a reasonably good approximation of most uncompressed nuclei, nucleoli, and cells (Mickle-wright, Kurnick, and Hodes, 1953), K may be used as two-thirds for such preparations. If there has been considerable flattening of the structure, it will approach a cylinder, and, K will approximate 1 more closely (Kurnick and Herskowitz, 1952).

Davies, Wilkins, and Boddy (1954) have proposed a method of cell flattening (of whole cells or nuclei). This converts the photometrized object to a cylinder, excluding thickness from the calculation as above, and making K equal to 1 for all objects. Their principal objective, however, was to reduce the absorbing path length so that no local densities in a heterogeneous object exceed 0.1 in optical density. Reference to our example above (see also Glick, *et al.*, 1951) will demonstrate that as the highest optical density within the inhomogeneous image approaches zero, the error due to averaging the transmissions (as performed by the phototube) rather than averaging the optical densities, approaches zero. Unfortunately, at optical densities outside the range 0.2 to 0.7, photometry becomes inaccurate, and is extremely unreliable at very high transmissions.

In some situations, such as when sections are analyzed, the measurement of thickness becomes necessary for quantitative interpretation of the measured optical densities. The various methods available have been reviewed by Lange and Engstrom (1954). They conclude that the probable error of approximately 10% for the mean of several measurements obtained by focusing on the upper and lower surfaces of microscopic images, is not significantly reduced by other available methods. Certain methods, such as interferometry have theoretical advantages, but are not applicable to the irregular surfaces of microscopic tissue structures (see also Meyer-Arendt and Meyer-Arendt, 1953). Other methods such as shadow casting and stereoscopic techniques were found to be accurate only for thicknesses outside the usual range of histological sections. Instead of using the micrometer screw scale to measure the displacement in focal plane between the top and bottom surfaces, Caspersson (1950) has measured the displacement required in the negative lens of a pancratic system interposed between ocular and objective to change the focal plane. In this manner, the required displacement is multiplied several-fold. In our experience with this apparatus, the advantage of wider range is offset by loss of stability

in the mechanical system. Glimstedt and Hakansson (1951) have devised a "microcator," which mechanically measures thickness without compression. If their observation that a 5 μ paraffin section regularly shows variations in thickness from 2.5 to 6.5 μ is correct for most material, quantitative analysis on sections is practically impossible by any technique for all but very small areas where the thickness can be measured at the point photometrized. It is obvious that with such irregular thicknesses, the fraction of the volume of the total structure represented in the section cannot be calculated, and results cannot be translated from content per unit area to concentration per unit volume or content in the whole structure. The photometry of the whole, unsectioned structure either in smears or squashes, however, appears feasible.

DeSmul and Lecomte (1953) report that after formol-acetic acid-alcohol fixation, the chromatin of nuclei is distributed in a cortical layer. If this is indeed the case, the photometry of the whole area of sectioned nuclei (which would appear as rings) as a basis for the calculation of the content of the whole nucleus from measurements of section thickness becomes invalid. These authors demonstrate, however, that for the whole nucleus, variations in shape from a sphere to a cylinder for such a cortical distribution of absorbing materials, do not alter K (see above) so that the calculation of content in relative units from the formula $E \times A$ introduces very minor errors. DeSmul and Lecomte believe that this may account for the uniformity of results of photometry of nuclei in a smear. However, their interpretation of the distribution of chromatin in a cortical layer is not consistent with the observation that nuclei fixed in similar fluids (such as Carnoy's) reveal greater central than peripheral absorption (Korson, 1951a).

To the sources of error already discussed, further contributions result from dispersion, refraction, and reflection of light (Swift, 1953; Naora and Sibatani, 1952; and others). In the ultraviolet these effects are generally much more significant than in the visible part of the spectrum. Dispersion, in particular, increases in magnitude at shorter wavelengths, probably as the fourth power (Rayleigh, 1871). Svensson (1954) has designed an apparatus to measure dispersion of light about a microscopic object. This apparatus is useful in calculating corrections (which may exceed the specific absorption) and in the selection of the appropriate apertures for condenser and objective. Commoner (1949) has discussed the error introduced into light absorption measurement by molecular orientation in the object. This error may, theoretically, be very considerable if orientation is extreme. However, in practice, it appears to be of very little significance (Caspersson, 1940; Thorell and Ruch, 1951; Pollister and Swift, 1950).

It would appear that much photometric "quantitative" data in the literature is to be regarded as at best qualitative, particularly where sources of error have not been meticulously excluded. This probably accounts for discrepant results by authors working on the same tissues, by similar techniques (cf. Swift, 1950b; Bryan, 1951; cf. Naora, 1951; Swift, 1950a; Thomson and Frazer, 1954).

Visual observations have often been interpreted semi-quantitatively. Even where gross differences appear to exist, inferences must be drawn with the greatest caution. Visual impressions are influenced most strongly by concentration (or density of stain, which is a function of concentration). As a consequence, a large structure with a very low concentration is often interpreted as having less nucleic acid than a small densely stained (or absorbing) structure. This confusion between content and concentration, which occurs repeatedly in the biochemical and histochemical literature must be rigidly guarded against in order to avoid fallacious conclusions. Thus, the observation that DNA concentration is reduced approximately threefold in the livers of cortisone-treated rabbits, and nuclei appear sparser in sections, has been interpreted as evidence that a large amount of DNA has been lost (Lowe, Williams, and Thomas, 1951). It appears probable from the data that content has not been altered, but that dilution by other material (glycogen, fat) has occurred, since the livers of treated rabbits were approximately three times as large as in the controls. Similarly, the increase in concentration in DNA and RNA of the livers of rats during prolonged protein depletion is *not* due to an increase in content, as has been thought (Muntwyler, Seifter, and Harkins, 1950); the former remains unchanged in content and the latter diminishes. The increase in concentration is here due to the reduction of other tissue constituents (primarily protein).

III. Fixation of Tissues

In general, information on localization and quantitation is desired for the living structure, unaltered by the techniques of preparation and examination. That this ideal is unattainable in the physical world is generally accepted (Heisenberg, 1930); it is much less successfully approached in the biological. Very few of the procedures to be considered can be performed on living cells in a physiological environment. Even then, the measuring procedure may not be innocuous (as in the ultraviolet examination of protozoa). For most procedures, fixation will, therefore, be necessary. Freeze-drying has been recommended as the fixative of choice (Caspersson, 1950). In most tissues, deformation is least marked by this procedure (see review by Bell, 1952). Mammalian kidney, however,

undergoes marked changes, characterized by shrinking of the lumina of the tubules with the formation of large spaces between tubules. It is also possible that the formation of ice crystals may cause molecular orientation, which might affect photometric results (Commoner, 1949). Indeed, Pease and Baker (1949) noted that frozen muscle showed an increase in birefringence. However, frozen-dried tissue is not suitable for many procedures without additional chemical fixation. Without such denaturation of tissue proteins, many tissue components would be dissolved during further procedures. Unless the method of drying after sectioning designed by Linderstrøm-Lang and Mogensen (1938) is applied, embedding in paraffin or Carbowax for sectioning is required. The removal of the embedding medium in the latter case, and staining procedures in either case, require contact of the tissue with a solvent which may remove cellular constituents or cause redistribution. Almost without exception, fixation (indeed, even cell death) involves some redistribution and loss of cellular components. In the case of nucleic acids, organic solvents, formalin, and most acid fixatives do not cause significant losses from the cell. Redistribution, is, however, a significant problem. Most fixatives produce some shrinkage. However, where analysis of nucleic acid content in a whole organelle is desired, this has no significance. In the nucleus, chromatin threads and bands are most often artifacts, since they result from post-mortem shrinkage of the chromosomes (Lewis, 1951; Doyle and Metz, 1935; Buck, 1939; Ris and Mirsky, 1949a; Kurnick and Herskowitz, 1952; and others). Again, if total nuclear content is investigated, this is without significance except for the complication which heterogeneity imposes upon the analysis. In some materials, this can be prevented (Kurnick and Herskowitz, 1952) or corrected (Ris and Mirsky, 1949b; Kurnick, 1950b). In the cytoplasm, more serious displacement may result. Chayen and Norris (1953) have reported transfer of nucleic acid from cytoplasm to nucleus (see discussion below under "Feulgen nucleal stain") and Mellors (1950) and Mellors, Berger, and Streim (1950) have reported transfer of nucleic acids from mitochondria to cytoplasmic "ground substance" in acetic acid-alcohol.

Some fixatives, such as Rossman's (Kurnick, 1952a) depolymerize DNA. As noted below, this is important in the methyl green stain and in ultraviolet spectroscopy.

There would appear to be no perfect fixative. However, if our interest is limited to analysis of nucleic acids, neutral (pH 7.0) 10% formalin, dehydration through graded alcohols and xylol, paraffin embedding at low temperature (52–54°C.) and deparaffinizing in xylol and alcohol appears to be satisfactory for most purposes.

IV. Staining Methods

1. *General Discussion of Use of Basic Stains and Extraction Procedures*

Since structures containing nucleic acids are basophilic because of the presence of free phosphate groups (originally free or liberated by the displacement of proteins bound to the nucleic acids), any basic dye in aqueous solution may be applied for the staining of such structures. It is thought that this reaction depends upon the formation of a salt linkage between the acid tissue component and the basic stain (Stearn and Stearn, 1929; Michaelis, 1947). Except for certain stains, such staining will not distinguish between the classes of nucleic acids nor between nucleic acids and other acid substrates. Nevertheless, a rough screening test which will distinguish structures poor in nucleic acids from others which may have a high concentration may be advantageously applied. In our experience, Toluidine blue 0 (1% in 95% ethyl alcohol) is particularly useful for this purpose (Kurnick, 1952a).

Such nonspecific stains may be rendered useful for specific localization by the addition of extraction procedures. Comparison of the original preparation with one stained after extraction by specific procedures will permit the differentiation of basophilia due to nucleic acids from that due to other materials. For the extraction of all nucleic acids, the method of Pollister and Ris (1947) of extraction with hot trichloracetic acid (after Schneider, 1945) is useful. Kaufmann, Gay, and McDonald (1951) have reported that this procedure is not applicable to tissues fixed in Flemming's solution and that the duration of extraction varies with the fixative. Hot perchloric acid (Ogur and Rosen, 1949) is also of value for the extraction of both nucleic acids simultaneously. Differences in solubility in electrolyte solutions, in case of hydrolysis, and in reaction with enzymes, permit selective extraction of the two types of nucleic acids. Differences in solubility of the two types of nucleoproteins (the protein moiety to which the nucleic acids are bound in tissues are undoubtedly important in this connection) in electrolyte solutions were observed by Levene (1901) and extended by Mirsky and Pollister (1942), Panijel (1948), Opie and Lavin (1946), and White (1950b). After certain fixatives, such as Carnoy's solution, ribonucleoproteins (RNP) are extracted by distilled water (Brachet, 1940) and more effectively by approximately 1% NaCl; deoxyribonucleoprotein is extracted by approximately 6% NaCl. The extraction of RNP is accelerated at 40–56°C. We have found (Kurnick, 1952a) that dilute trimethylolaminomethane buffer at pH 7.6 is very effective in removing cytoplasmic basophilia (RNP) without affecting DNA observably. Others have used other alkaline buffers (White, 1947; Stowell and Zorzoli, 1947) and even strong alkali (Sulkin, 1951).

The greater sensitivity of RNA to acid hydrolysis is also useful in its differential extraction. Hydrolysis with 1 *N* HCl as in the Feulgen nucleal reaction removes RNA (Boivin *et al.*, 1947; Vendrely-Randavel, 1949). Its possible effect on DNA will be discussed later; it may be stated that under suitable conditions, in most materials, however, DNA is depolymerized, but removal from its localization in the section is only slight. However, Dempsey, Singer, and Wislocki (1950) have reported complete extraction of both nucleic acids with 1 *N* HCl at 37°C. for 3 hours. Atkinson (1952) has used trichloracetic acid at 60°C. for the extraction of RNA without DNA. Erickson, Sax, and Ogur (1949) and Sulkin and Kuntz (1950) used cold perchloric acid to remove RNA without DNA, then hot perchloric acid to remove both. Koenig and Stahlecker (1952) have confirmed the validity of this procedure. However, Seshachar and Flick (1949), Pearse (1953), and Franz, Weiden, and Meyer-Arendt (1954) have indicated that RNA extraction with cold perchloric acid may be incomplete after certain fixatives, and DNA extraction may occur simultaneously. Wenderoth (1953) found cold perchloric acid unsatisfactory for the extraction of RNA from blood smears, but used 40°C. for 3 minutes successfully. He pointed out that unlike ribonuclease, which may be "poisoned" by stains, perchloric acid may be applied successfully to stained material. Our experience *in vitro* on unfixed and formalin-fixed tissue homogenates have indicated that 10% perchloric acid does not yield quantitative separation of the two nucleic acids, although RNA is predominantly extracted at 0–4°C. (incompletely, and with some DNA). Atkinson (1952) has pointed out that the extractability of RNA by $HClO_4$ varies in different tissues and with different fixatives. In addition, acid mucopolysaccharides, which are basophilic, are also extracted so that the localization of RNA by loss of basophilia is unreliable. Davies (unpublished data, referred to in Davies and Walker, 1953) has observed increase in ultraviolet absorption in fixed chick fibroblasts after cold perchloric acid treatment. The basis for this change was not determined, but it was suggested that change in nonspecific light losses may have been responsible. Bile salts were applied by Henry and Stacey (1943) for the removal of RNA from gram-positive bacteria, and Foster and Wilson (1952) recommended their use for the differential extraction of RNA without DNA from tissues. The mechanism of this reaction is unknown.

The specific enzymes, ribonuclease and deoxyribonuclease, have been extensively used to extract their respective substrates from tissues to permit their localization by the change induced in staining reactions. Danielli (1947) has dismissed their use as too uncertain because of possible lack of specificity, contamination with other enzymes, and interference by

proteins in the tissue with access to the nucleic acid substrate. To these may be added the fact that even in pure systems, RNA (Loring, Carpenter, and Roll, 1947) and DNA (Zamenhof and Chargaff, 1949) contain "cores" which are not attacked by the enzymes. Furthermore, since the nucleases are proteins, if used in sufficient concentration they could, theoretically, bind acid groups nonspecifically, and thus prevent basic staining without, in fact, removing any substrate. Such a reaction is suggested by Loring's observation (1942) that ribonuclease does not attack the nucleic acid of native tobacco mosaic virus, but combines with the virus to form an insoluble and inactive compound. However, much informative data has been obtained with these enzymes (Brachet, 1940; Kaufmann, McDonald, and Gay, 1951). The preparation of crystalline enzymes (Kunitz, 1940, 1950) has obviated the objection of nonspecificity. McDonald (1948) has, in the case of ribonuclease, removed the effect of contaminating enzymes by heating the enzyme solution. The problem of access to the substrate is undoubtedly real in unfixed material (Davidson, Leslie, and White, 1948), as it is with native tobacco mosaic virus. However, in fixed material, the denaturation of protein appears to "uncover" the substrate to the nucleolytic enzymes. Presumably the indigestible cores are sufficiently soluble so as not to interfere with subsequent staining in practice, although they are precipitable by basic stains (Vercauteren, 1950a). Ribonuclease has little, if any, advantage over the use of warm buffer solutions (Kurnick, 1952a). Indeed, in order to demonstrate its specificity, it is necessary to use glass distilled water (Stowell and Zorzoli, 1947; see this reference also for discussion of optimal conditions) or a slightly acid solution (Davidson, Leslie, and White, 1948). If this is not done, the control (solvent alone) removes the RNA. In blood smears ribonuclease is often unsatisfactory (Davidson *et al.*, 1948; Wenderoth, 1953). As Wenderoth has indicated, nonenzymatic extraction (such as with perchloric acid) has the advantage that it may be applied to stained material, whereas ribonuclease may be "poisoned" by stains. Kaufmann, Gay, and McDonald (1951) reported that tissues fixed in Flemming's solution are resistant to ribonuclease. They are also resistant to hot trichloracetic acid, but the effect of alkali was not determined. Crystalline deoxyribonuclease (DNase), in fixed material and in adequate concentration, appears to be very useful in establishing the location of DNA without removing other basophilic compounds not removed by the solvent alone (such as RNA, which may be removed by the buffer; a solvent control is, therefore, essential). Brachet and Shaver (1948), using noncrystalline DNase, found that the addition of magnesium was required to remove DNA from tissues. Although DNase is activated by magnesium (Kunitz, 1950), in our ex-

perience its addition has not been necessary (although advisable) for the extraction of tissue sections, presumably because enough magnesium is present in the tissue. Brachet (1947) detected no action by DNase on sperm cells and some erythrocyte nuclei. However, studies carried out with preparations before the crystalline enzyme became available (Kunitz, 1950) require re-evaluation (see Kaufmann, McDonald, and Gay, 1951 and discussion, and Kaufmann, Gay, and McDonald, 1951). Unlike the inconsistent and contradictory results obtained with the crude preparations (see also Laves, Thoma, and Oberdorfer, 1952), we have had excellent results with crystalline deoxyribonuclease.

In summary, under suitable conditions and with proper controls, the nucleolytic enzymes are useful in the identification of basophilic cellular elements. Substitutes for ribonuclease, which are as effective and much more readily obtained, are available.

Quantitative analysis of nucleic acids by the use of nonspecific basic dyes combined with specific extraction has been attempted. This depends upon microphotometric measurements before and after extraction (Pollister and Ris, 1947) so that the quantity of the specific nucleic acid removed may be determined. However, it is probable that extraction will alter nonspecific light losses both by altering the characteristics of the unextracted components and by the reduction in nonspecific light losses attributable to the extracted components. Furthermore, since the stoichiometry of these stains is, with few exceptions, undetermined, and it is not certain that there is a constant stoichiometry which would permit calculation of the quantity of nucleic acid extracted, such procedure is subject to grave doubt. Herrman, Nicholas, and Boricious (1950) have investigated the stoichiometry of the RNA-toluidine blue reaction *in vitro* and in sections of muscle. They found that at a controlled pH, in the vicinity of pH 4 to pH 6, the intensity of cytoplasmic staining was a function of RNA content. Gallocyanin-chromalum has been more extensively investigated in this regard, and will be considered in a separate section. Since many of the acid groups of nucleic acids are bound to tissue proteins, available groups for dye combination may vary in number, thus complicating quantitative analysis. The competition of proteins with basic dyes has been observed by a number of authors (Hammarsten, Hammarsten, and Teorell, 1928). The magnitude of this problem may be reduced by extraction of proteins preliminary to staining. Histone may be removed from combination with DNA by dilute HCl. Proteolytic enzymes may also be applied, but with the risk of distortion of tissue structures and loss and diffusion of nucleic acids. The completeness of the liberation of reacting groups on the nucleic acid always remains uncertain, and therefore imposes a hazard

upon quantitative analysis with basic stains (see discussion of methyl green below).

Fortunately, there are available specific staining techniques for the nucleic acids. Those which are useful for DNA have also permitted quantitative analysis. The specific stains for RNA have been less satisfactory for quantitative analysis without extraction techniques.

2. *Feulgen Nucleal Reaction and Its Variants*

Probably the specific staining method for DNA which has enjoyed the greatest popularity is the "nucleal reaction" of Feulgen and Rossenbeck (1924). This reaction is thought to depend upon the uncovering of the potential aldehyde group of deoxyribose by hydrolysis of purine-deoxyribose bonds by the HCl (Feulgen and Rossenbeck, 1924; Li and Stacey, 1949; Overend, 1950). Overend and Stacey (1949) have pointed out that removal of purines is not essential for the production of the stain, and suggest that depolymerization may "uncover" deoxyribose involved in polymeric bonds. The uncovered aldehyde of deoxyribose gives the Schiff reaction when treated with leuco-basic fuchsin. The chemistry and stoichiometry of this reaction remain in doubt, and it appears that several complexes are formed, some water soluble, others not (Wieland and Scheuing, 1921; Overend and Stacey, 1949; Di Stefano, 1948a, b; see also Sharma, 1952; Lessler, 1953; Pearse, 1953 for reviews). Lhotka and Davenport (1951) have questioned that the reactive group is an aldehyde; they failed to observe reactions with oxidizing agents (Fehling's solution) or hydrazines, but were able to block the Feulgen reaction by the Cannizaro aldehyde reaction. They considered, therefore, that "steric configurations which fit Schiff's reagent, but not other reagents for aldehydes, may be involved." However, such an interpretation does not appear necessary, since the hydrazine reactions attempted may have been unobserved because the products were uncolored (see the experiments of Danielli and Pearse, below, in a similar test in which coupling was demonstrated). Semmens (1940) found a positive reaction with some purines and considered that the Feulgen reaction depended upon the content of these bases in the DNA and not on the aldehydic sugar. However, Barber and Price (1940) showed that this was an effect of the alkaline pH of the purine solutions and was not relevant to the test. Hillary (1940) found that a positive Feulgen reaction was obtained with a variety of aldehydes or potential aldehydes in tissues, including lignin, suberin, cutin, starch, and glycogen. As Stowell (1945) has pointed out, some of these may be detected without acid hydrolysis, and can therefore be recognized by performing a control test in which this step is omitted. Others are removed

from the section by the acid hydrolysis. However, it appears that a substance in bacterial cell membranes, which is apparently not DNA, gives a positive Feulgen reaction and is not distinguished by the preceding procedures (Knaysi, 1946; DeLamater, 1953). Pearse (1953) mentions interference by plasmals in frozen sections, and recommends their removal by organic solvents.

The Feulgen reaction is sensitive and apparently specific. It is a very useful qualitative test. Many efforts to apply it as a quantitative photometric procedure have been made. All of these are subject to doubt for several reasons in addition to those discussed in Section II above. The stoichiometry and molar extinction coefficients are not known. Indeed, it is doubtful that they are constant since a succession of compounds are probably formed with different stoichiometries and extinction coefficients. Moses (1951) has described two distinct absorption maxima for the Feulgen stain (both with DNA in solution and in nuclei). These maxima varied independently of each other with hydrolysis time, pH, and the presence of protein. It has been claimed that different methods of preparing the leuco-basic fuchsin yield different intensities of stain (Rafalko, 1946; Longley and Lillie, 1951). Even with a given method of preparing the solutions, the intensity of the stain varies with the duration of hydrolysis, increasing to a maximum and then decreasing as hydrolysis proceeds (Di Stefano, 1948b). The time at which this peak is reached is a function of the fixation used (Bauer, 1932; Hillary, 1939). The height of the peak (i.e. color intensity) also varies with the fixative (Sibatani and Fukuda, 1953). Swift (1950a) has noted that, under optimal conditions, the intensity of the reaction in formalin-fixed tissue is double that in Carnoy-fixed material and Naora, Matsuda, and Sibatani (in Sibatani and Fukuda, 1953) have reported much more intense reaction after 20% formalin fixation than after 50% formalin. Lhotka and Davenport (1951) state that rapid fixation from the living state fails to give as intense Feulgen staining as if fixation is delayed after death. All of these factors tend to make the results variable, particularly when tissues are compared which are not prepared simultaneously.

Ely and Ross (1949) have shown that the phenomenon of increasing stain intensity followed by diminution as hydrolysis proceeds (without a distinct plateau) is due to the fact that hydrolysis not only removes purines, but also splits off the deoxyribose which passes into solution. If the purine hydrolysis were complete before that of the sugar occurred at all, an end point might be considered to exist. Di Stefano (1948b) considered this to be the case, but his conclusion is based on an error in his analysis of methyl green staining (Kurnick, 1950a). Thomas (1950, 1952)

however, came to the same conclusion by analyzing for purines, pyrimidines, and aldehyde in the supernatant HCl during hydrolysis. He found no pyrimidines or aldehydes until the hydrolysis of purines was complete. These experiments have been criticized by Sibatani and Fukuda (1953) on the grounds that the high concentrations of DNA used caused occlusion by the formation of a heavy precipitate in acid, so that the experiment is not comparable to the Feulgen reaction in tissues. Ely and Ross' (1949) data indicate that the solution of purines and sugar occur simultaneously, although at different rates. Stedman and Stedman (1950) and Sibatani and Fukuda (1953) have also noted solubilization of the sugar during hydrolysis as in Feulgen staining. The latter authors observed a 10–20% loss of DNA from formalin-fixed tissues hydrolyzed for 13–15 minutes, and the former noted even greater loss after Carnoy fixation. Sibatani and Fukuda (1953) failed to obtain any reaction with the Schiff reagent following the hydrolysis of sections with dilute HCl at 37°C. for 26 hours, a procedure which removes all the purines from DNA *in vitro* (Tamm, Hodges, and Chargaff, 1952). This unexpected observation is not explained, nor is evidence provided that purines are, in fact, removed from tissue sections by this procedure. Di Stefano (1952) has substituted perchloric acid for hydrochloric acid in the hydrolysis. A more quantitative removal of purines without loss of the sugar is claimed. However, since $HClO_4$ removes some DNA from many tissues in the cold, and all the DNA at 90°C., the chance of losing some stainable material at 25°C. seems great. Nevertheless, Di Stefano found that after hydrolysis of Carnoy-fixed rat pancreas nuclei in 10% perchloric acid, the intensity of the stain with the Schiff reagent increased for approximately twelve hours, and then remained constant for at least 12 hours. Sharma (1951) has observed that trichloracetic acid (0.25 *M*) at 65°C. for 50 minutes also yields satisfactory hydrolysis for the Feulgen stain. Hashim (1953) has recommended phosphoric acid for the hydrolysis at room temperature. It is probable that the uniformity of staining required for quantitation is not significantly improved by these modifications of the hydrolysis procedure. Another factor in rendering quantitative analysis uncertain is the effect of proteins on the intensity and color of staining observed by Widstrom (1928), Caspersson (1932), and Sibatani (1950, 1953). The reason for this is unknown. Sibatani and Naora (1952) have observed that the pH of the sulfite rinse after immersion in the leuco-dye influences the intensity of the color development. Fading of the stain upon exposure of the slide to intense illumination as used in microphotometry has been observed to be quite rapid by DeSmul and Lecomte (1953).

However, these authors report that if illumination is reduced and filtered during searching and focusing, the effect can be minimized.

Some questions have been raised about the specificity of localization by the Feulgen stain. Stedman and Stedman (1943, 1947) have suggested that localization is an artifact of diffusion and absorption. Choudhuri (1943) and Carr (1945) have supported this view. They have demonstrated that the "developed" Feulgen stain, obtained by treating hydrolyzed (HCl solubilized) DNA *in vitro* with Schiff's reagent, stains chromosomes. Their conclusion that DNA may not, therefore, be an original component of chromosomes has been refuted by independent evidence (as by ultraviolet absorption) by a number of observers (Callan, 1943; Barber and Callan, 1944; Caspersson, 1944; Stowell, 1946; Dobson, 1946; Brachet, 1946; and others). In the light of these studies, the Feulgen stain has become generally accepted as a reliable indicator of the location of DNA. Chayen and Norris (1953) have, however, recently brought forth well-documented evidence that hydrolysis with HCl may cause the diffusion and faulty localization of DNA in some material. In the root meristem cells of *Vicia faba,* they have found cytoplasmic particles which stain with methyl green and absorb ultraviolet strongly at 2650 Å. Both the stainability and ultraviolet absorption are removed by deoxyribonuclease but not ribonuclease. Even after fixation, these granules are very sensitive to acids; and the authors have demonstrated the transfer of their DNA to the nucleus by such treatment. They are therefore not stained by the Feulgen stain since this requires the use of acid; and the intensity of nuclear staining with this procedure is presumably (as it is after cold dilute acid and methyl green staining) increased by the transfer of their DNA to the nucleus. Thus, in this case, the Feulgen reaction fails to detect DNA in its correct site. A somewhat similar situation has been described by Brachet (1947) in some bird erythrocytes, the nuclei of which readily loose all their DNA on acid hydrolysis so that the Feulgen reaction is negative. Danielli (1947) has attempted to study the problem of localization by the Feulgen reaction by substituting 2,4-dinitrophenylhydrazine for leuco-basic fuchsin as the reagent to detect the uncovered sugar after HCl hydrolysis. Since this complex is less soluble in water than the fuchsin complex, it was anticipated that the colored reagent formed with hydrolyzed DNA in solution might have a different distribution when used as a stain. The same distribution as with the Feulgen "developed" stain, namely primarily chromosomal staining, but also pale cytoplasmic staining, resulted. No conclusions were possible, therefore, as to the specificity of the Feulgen stain. Since the color with dinitrophenylhydrazine is less intense and staining less discrete, the stain has no advantage over the Feulgen stain. Pearse

(1951) has used 2-hydroxy-3-naphthoic acid hydrazide followed by coupling with diazotized *o*-dianisidine after HCl hydrolysis. This gives distribution similar to the Feulgen (but purplish blue); there is also pale pink staining of protein.

The phenylhydrazine compound used by Danielli and the naphthoic acid hydrazide introduced by Pearse, like the Schiff reagent, are known to react with aldehydes. DeLamater (1953) has used two reagents prepared by the addition of sulfurous acid to (1) azure A and to (2) thionine for the detection of DNA in bacteria after HCl hydrolysis. He claims equal specificity for DNA as the Feulgen reaction. DeLamater's reagents are not decolorized, although the color of the basic stain is altered by the SO_2. This may be an effect of the acid pH. Carr (1945) has observed that basic fuchsin changes from red to mauve, as in the "developed" Feulgen stain, in acid. As with basic fuchsin, neither stain is specific for DNA without SO_2 according to DeLamater. The mechanism of the reaction in the presence of SO_2 is unknown. The stains have not gained popularity for animal tissues, nor has their specificity been fully investigated. It appears possible that the specific staining with these basic dyes, unlike the Feulgen, is dependent upon the selective extraction by the HCl hydrolysis of acid cell components (such as RNA), leaving the DNA *in situ* to accept the basic dye. It should be noted that Carr (1945) suggested that this was the mechanism of the Feulgen stain, too, and recommended the use of undecolorized basic fuchsin.

Since only purine-bound deoxyribose is uncovered, variations in the ratio of purines to pyrimidines in different nucleic acids would cause differences in the amount of deoxyribose uncovered for reaction with the leuco-basic fuchsin. Whereas evidence has already been obtained that deoxyribonucleic acids differ in their composition, Chargaff (1951) and co-workers have reported that the ratio of purines to pyrimidines appears to be very close to 1:1 for a number of different DNA's. If this is universally true, this source of potential error in quantitative analysis with the Feulgen reaction may be neglected.

Ris and Mirsky (1949b) have obtained quantitative data with Feulgen staining by including in the slide, reference "standard" nuclei. The latter are nuclei with known DNA contents from biochemical analysis. It may be mentioned that the "standard" nuclei must be of a single, uniform class. In adult mouse liver, for example, there appear to be several classes of nuclei with different DNA contents. Here, the chemical data are not applicable, since they give only a mean content. It was further found necessary that the reference nuclei be similar in size, chromatin distribu-

tion (if not rendered homogeneous), and optical density to the test nuclei for reliable data to be obtained. Although many authors apply the Feulgen reaction and obtain reproducible data which appear to provide a valid basis for comparison, very few regard their data sufficiently secure quantitatively to present the results in absolute terms (by obtaining the factor from analysis of nuclei with known DNA content).

Many variations on the technique of performing the Feulgen nucleal reaction have been described. All the common fixatives, including formalin, alcohol, Carnoy's, and Zenker's, are suitable if adequately rinsed. Hydrolysis is usually performed in 1 *M* HCl at 60°C., as originally described by Feulgen and Rossenbeck (1924). The period of hydrolysis is arrived at empirically to yield the deepest stain, and depends upon the tissue and fixation. The usual range is 8–12 minutes. DeSmul and Lecomte (1953) find 30 minutes optimal after formol-acetic acid-alcohol. Itikawa and Ogura (1954a) have used 5 *N* HCl at room temperature for 15 minutes. In our hands, this gives satisfactory qualitative results, slightly less intense than we obtain with the usual treatment. It is possible that varying the concentration of HCl and the time of hydrolysis might succeed in providing identical results to the 60°C. hydrolysis, subject to the effects of variation in room temperature. Hydrolysis at room temperature has the advantage, as pointed out by the authors, that tissue slices and smears are less frequently washed off the slide than at the higher temperature. After rinsing, the hydrolyzed tissue is transferred to the leuco-basic fuchsin. A number of modifications of the procedures for the preparation of the leuco-dye have been used. Stowell (1945) used bisulfite. Rafalko's method (1946) (also suggested by Feulgen and Rossenbeck, 1924) for preparing the leuco-dye by bubbling SO_2 through the solution is simple and popular. We have found Itikawa and Ogura's procedure (1954b) most convenient. This consists of adding sulfurous acid to the basic fuchsin solution to a pH of 3.0 (we require 3.0 ml. of 6% sulfurous acid/100 ml. 0.5% basic fuchsin solution rather than the 1.5 ml. of 5.6% acid recommended by Itikawa and Ogura), and completing decolorization with activated charcoal after 24 hours. The unbound "stain" is removed from the slide after immersion for 45 minutes by rinsing the slide in two changes of 1.5 ml. 6% sulfurous acid in 100 ml. water, then in water.

For the qualitative localization of DNA, irrespective of its state of polymerization (provided it still meets the criterion of acid insolubility which is characteristic of nucleic acid as opposed to smaller nucleotide complexes), the Feulgen nucleal reaction is probably the stain of choice. Of course, in very low concentrations, as in some oöcyte nuclei, DNA stain-

ing may be so faint as to be indiscernible. Localization is now generally considered to be accurate, although the recent data of Chayen and Norris (1953) on root meristem cells may demand reevaluation of this conclusion.

3. Methyl Green

Methyl green appears to have been introduced into cytology by Balbiani (1881), who applied it in combination with carmine in his classical study of *Chironomus* salivary gland chromosomes. He obtained green stained chromosome bands with red nucleoli. The stain has become more popular in the mixture with pyronin introduced by Pappenheim (1899) as a differential histological stain. Pappenheim's mixture was modified by Unna (1902) by the addition of glycerin and phenol. For qualitative application, phenol serves to adjust the pH to the range in which the color of methyl green is more stable than at the usually slightly alkaline range of tap water (Kurnick, 1950a); the glycerine serves no apparent useful purpose. Since the complex of polymerized DNA and methyl green is maximally colored up to pH 8.0 (Kurnick and Foster, 1950), and since the "leuco" dye is "regenerated" on combination with DNA throughout the pH range at which the complex is colored (permitting its use as a progressive stain), neither reagent contributed by Unna appears to be of value in the quantitative staining procedure (Kurnick, 1950b).

Unna (1902) observed that methyl green stainability was lost from nuclei by treating the section with hot water, but not cold water. He concluded that hot water dissolved an albuminoid which was responsible for the green staining. Brachet's demonstration (1940, 1941, 1942) that methyl green localized DNA (while pyronin localized RNA) contributed importantly to our knowledge through the use of the Pappenheim mixture. It did not, however, account for Unna's experiment with hot water treatment. Pollister and Ris (1947) reported, at the Cold Spring Harbor Symposium on nucleic acids, on the determination of the ratio of DNA to RNA in nuclei by measuring the intensities of pyronin and methyl green staining in accordance with Brachet's hypothesis. At the same conference, Kurnick (1947) reported that the selectivity of the two basic dyes for the two nucleic acids was a function of their states of polymerization: methyl green was readily bound by polymerized DNA, but only very slightly bound to heat or enzyme depolymerized DNA. This observation has been confirmed by Vercauteren (1950b), Vendrely and Vendrely (1949), Devreux, Johanson, and Errera (1951), Chayen (1952), Sibatani (1952), and others. Pollister and Leuchtenberger (1949b) repeated Unna's experiment and demonstrated that the loss of methyl green stainability after hot water treatment of sections was not due to the loss of nucleic acid

or protein. They concluded that the result was in agreement with our findings that DNA lost stainability with methyl green upon depolymerization. Kurnick (1950a), Errera (1951), and others have suggested that the stable binding of methyl green to DNA requires binding at two sites (two amino groups of the dye to two phosphoric acid groups) and that, therefore, a matching configuration must exist to permit approximation of the combining groups. The affinity of DNA for methyl green is influenced both by the degree of depolymerization and the extent to which internal configuration is altered by different depolymerizing agents (Kurnick, 1954). Thus, depolymerization to a given level of viscosity by X-ray (Errera, 1951) reduces methyl green binding more markedly than does heat, which is more disruptive to the configuration of the molecule than the enzyme deoxyribonuclease (Kurnick, 1954). It may be noted that both specific viscosity of DNA solutions and methyl green stainability become negligible long before the DNA is depolymerized to the point where it is dialyzable through a cellophane membrane or is acid soluble (Kurnick, 1954). The configuration of the molecule and the degree of polymerization requisite for maximal methyl green affinity have not yet been defined. However, preparation of polymerized DNAs from several tissues according to the methods of Mirsky and Pollister (1946) and of Kay, Simmons, and Dounce (1952) yielded preparations which bind methyl green to the same extent (Kurnick and Mirsky, 1950). This is not surprising in view of the fact that initial 50% reduction in viscosity of a solution of highly polymerized DNA by DNase is associated with only 3–4% reduction in methyl green affinity (Kurnick, 1954). The stoichiometry of this reaction has been studied by Kurnick and Mirsky (1950). Under conditions defined by Kurnick (1950b) this stoichiometry appears to hold for intranuclear DNA in a number of tissues studied, thus permitting quantitative histochemical analysis. This should not be construed to mean that DNA in all normal tissues is of the same molecular weight and configuration; as noted above, among high polymers, affinity for methyl green is similar over a fairly wide range of molecular weights. The prescribed conditions for quantitative analysis of DNA in nuclei with methyl green include preparation of the nuclei in an optically homogeneous state, removal of competing proteins, aqueous staining and rinsing, and the avoidance of depolymerizing treatments (heat, certain fixatives, such as Zenker-picric acid). It was pointed out (Kurnick, 1950b) that on theoretical grounds, it must be borne in mind that tissues may be encountered in which a significant number of reactive groups on the DNA molecule are bound to proteins not removed by water, dilute HCl or buffer. This did not appear to be the case in our studies, since the histochemical and biochemical data

were in reasonable agreement within the limits of error imposed by photometry *per se*. It appears probable that proteins, other than the basic histones and protamines which are extracted by our technique, are not bound to DNA by salt linkages with the phosphoric acid groups (i.e., they are bound at other sites) and therefore do not compete with methyl green.

Taft (1951a) failed to detect loss of methyl green stainability after treatment of tissues by some procedures thought to depolymerize DNA. However, as we have discussed elsewhere (Kurnick, 1952b), it is doubtful that DNA is actually depolymerized by some of his techniques; those which have been established to produce depolymerization (such as heat) did reduce methyl green staining in his hands also.

Recently, Alfert (1952) has reported data purporting to demonstrate that methyl green staining is not dependent upon polymerization of the DNA in tissues. However, Alfert's experiments were performed after dismissing all of the above conditions which we had reported to be essential. Alfert states, without supporting evidence, that the procedure followed by him gives equally specific results and more intense staining. He then demonstrated that his procedure is not specific. This finding, which is in agreement with our published data, leads him to conclude that our technique is also nonspecific. Alfert made no attempt to obtain homogeneous nuclei, which probably does not affect specificity, but renders photometry invalid. He did not extract with dilute HCl to remove histone which we had found (Kurnick, 1950a, b) competes with methyl green for DNA. It is not surprising, therefore, that he noted increase in methyl green stainability after removal of protein. Finally, he rinsed his stained slides in tertiary butyl alcohol rather than water (or dilute buffer). We had already reported that, whereas methyl green staining of cytoplasm was not observed after water rinsing, it was apparent after tertiary butyl alcohol rinsing (Kurnick, 1952a). Alfert noted that cytoplasmic staining with methyl green was increased after fixation in strong formalin or after deamination of cytoplasmic proteins. Both procedures render the proteins acidic. It is probable that weak linkages between only one amino group of methyl green and one acid group (phosphate of nucleic acid or carboxyl group of acidified protein) are preserved in nonaqueous solvents, whereas they are disrupted in a polar solvent which favors ionization (such as water or dilute buffer). As a result, rinsing in tertiary butyl alcohol, as performed by Alfert, permits staining of acidified proteins and RNA, whereas only the double linkage with DNA is preserved in water. We have observed slight cytoplasmic staining with methyl green after *n*-butyl alcohol rinsing, also, if the 4% formaldehyde used in fixation was not carefully neutralized before use. Commercial "neutral" formalin is quite

acid; and even formalin neutralized with dilute NaOH becomes acid on standing. At an acid pH, the reaction between formaldehyde and amino groups of proteins is favored. The proteins thus become stronger acids. Staining in a slightly acid buffer (pH 4.1) (Kurnick, 1950b) probably reduces this loose binding of methyl green to proteins by maintaining the pH on the acid side of the isoelectric point of tissue proteins (cf. the diffuse staining with methyl green obtained by Gerola and Vannini, 1948 at an alkaline pH). Under the conditions used, therefore, Alfert's observations on protein interference and staining with methyl green are not surprising, nor do they provide evidence against the theory of the role of polymerization in methyl green staining. A further demonstration of this theory may be found in the application of methyl green to the assay of deoxyribonuclease activity (Kurnick, 1950c, 1953).

A few substances other than polymerized DNA do stain with methyl green. Lignin (Chamberlain, 1932) and cartilage (Taft, 1951b; Kurnick, 1952a) stain vividly green. However, these are not likely to introduce difficulties in intracellular analyses.

The affinity of extensively depolymerized DNA and of RNA for methyl green are so slight (Kurnick, 1947; Kurnick and Mirsky, 1950) that they present no problem in histochemical analysis even in the presence of considerable concentrations, as in Nissl substance (although if tertiary butyl alcohol is used for rinsing, Nissl substance has been reported to stain green).

The fact that polymerized DNA stains with methyl green whereas depolymerized DNA does not, and the fact that both states of DNA are Feulgen positive, provides the basis for the analysis of the state of DNA in nuclei as performed by Leuchtenberger (1950), Klemperer, Gueft, and Lee (1949), Klemperer *et al.* (1950) in pathological states and by Harrington and Koza (1951) in irradiated nuclei. The fact that the inclusions of L.E. cells are Feulgen positive and methyl green negative, thus permitted the conclusion (Klemperer *et al.*, 1950) that they contain depolymerized DNA. This observation led to the investigations on the role of deoxyribonuclease in the pathogenesis of the phenomenon, also using the relationship between methyl green affinity and state of polymerization (Kurnick *et al.*, 1952, 1953).

The usefulness of methyl green for the detection of polymerized DNA may be enhanced by the depolymerization (or removal) of the DNA with heat, or, more specifically, deoxyribonuclease (Kurnick, 1952a). Thoma (1950) and Laves, Thoma, and Oberdorfer (1952) have reported loss of methyl green stainability after the application of crude ribonuclease to some blood cells. However, Thoma has more recently found that this

observation may be attributed to contamination of the enzyme preparation with deoxyribonuclease and proteolytic enzymes (personal communication).

It may be noted that commercial powders of methyl green require removal of contaminating purple dyes with chloroform. This is best performed by extraction of the aqueous solution (Kurnick, 1952b). Some further observations on methyl green are included in the next section, on pyronin with which the former dye is often mixed for histochemical application.

Malachite green has the same selectivity as methyl green (Kurnick, 1950a). Like methyl green it is a triphenylmethane dye with two tertiary amino groups. Both dyes, therefore, have only a single spacing of a pair of amino groups available to bind in a stable double linkage with DNA. Crystal violet, on the other hand, has three amino groups. It therefore provides three suitable spacings, which may account for its greater versatility and hence lack of specificity. It has been suggested (Kurnick, 1950a) that the stains specific for polymerized DNA find suitable phosphorus spacing for their amino-group spacing sufficiently frequently for detectable staining only in polymerized DNA. A mixture of malachite green and acridine red was used by Hitchcock and Ehrich (1930) and Ehrich, Drabkin, and Forman (1949) to distinguish DNA and RNA in tissues. Like Balbiani's stain, this mixture probably depends upon the selectivity of malachite green and a nonselective contrast stain. Malachite green has no advantage over methyl green, and gives less intense staining.

Korson (1951b) has used a mixture of methyl green and toluidine blue. The latter stain has no specificity, and its blue color gives less contrast with the green-stained polymerized DNA than does the methyl green-pyronin mixture.

4. Pyronin

As mentioned above, methyl green has usually been used in conjunction with pyronin. This stain, applied before and after ribonuclease extraction, has been extensively used by Brachet (1940, 1941, 1942, 1947, 1950, 1953) for the detection of ribonucleic acid. Kurnick (1947, 1950a) observed that pyronin behaved oppositely to methyl green in that it stained low polymers of nucleic acids preferentially. The degree of preference is not as marked as for methyl green. However, in the mixture of the stains, the preference is sufficiently well marked to permit effective competition of the methyl green for polymerized DNA to the exclusion of pyronin. Thus, even with a selective pyronin (see below), nuclei are stained red when the dye is used alone, but green when it is used in a mixture with methyl green (Kurnick, 1952a, 1955b).

Attempts to establish the stoichiometry of pyronin binding by RNA and depolymerized DNA yielded no constant results (Kurnick and Mirsky, 1950). Furthermore, protein staining by pyronin was noted. It was concluded that pyronin was not useful for quantitative analysis. However, it must be pointed out that the pyronin used in these studies was found to be a nonspecific stain in tissue sections because of the marked staining of protein (Kurnick, 1952a). After the trial of numerous pyronins (both B and Y), only the pyronin Y preparations of the British firms of George T. Gurr and Edward Gurr (Kurnick, 1955b) proved to be selective. Possible application of these dyes to quantitative histochemistry has not yet been investigated. Used alone, areas containing a variety of acid substrates (DNA, RNA, polysaccharides) stain red. Treatment with ribonuclease or warm buffer (Kurnick, 1952a) removes the stain from areas where the stain is due to RNA, but not from other areas. Protein does not stain with these pyronins, unlike pyronin B and a number of American pyronin Y preparations (including some which were only slightly soluble in chloroform). When the Gurr pyronin Y's are incorporated with methyl green in aqueous solution, followed by rinsing with *n*-butyl alcohol (Kurnick, 1952a), areas containing polymerized DNA stain green as mentioned above. The extraction of RNA by buffer or specific enzyme is still necessary, however, to differentiate acidic substrates (such as in eosinophil granules) which are stained by pyronin both before and after the extraction of RNA.

For qualitative use, the quantitative staining technique (Kurnick, 1950b) for methyl green is often objectionably faint (Frazer and Davidson, 1953). A vivid methyl green-pyronin stain with suitable specificity may be obtained by rinsing with *n*-butyl alcohol instead of water (Kurnick, 1952a). The stain is much more intense than with aqueous rinsing, and therefore more satisfactory for routine examination. Whether the results are quantitative remains to be examined. Unlike *tert*-butyl alcohol, *n*-butyl alcohol adequately rinses methyl green from cytoplasmic particles; whereas *tert*-butyl alcohol yields purple cytoplasmic granules in the liver and blue Nissl substance in neurones, both are bright red after *n*-butyl alcohol. As mentioned above, it is important that the formalin used for fixation be accurately adjusted to pH 7.0. If this is not done, faint green cytoplasmic staining may occur. The probable cause for this phenomenon has been discussed above.

The fact that pyronin stains depolymerized DNA, from which it is not displaced by methyl green, accounts for the pyroninophilia of the L.E. inclusions (Klemperer *et al.*, 1949, 1950), for the pyroninophilia of nuclei after excessive heat or certain fixatives (Kurnick, 1952a), and for the

pyroninophilia of necrotic nuclei (Harris and Harris, 1950, and personal communication). Of course, if depolymerization is permitted to proceed to the point where the DNA is solubilized and removed from the section, no pyroninophilia results. It should be emphasized that there are many possible degrees of depolymerization extending from a very slight reduction in the size of the macromolecule down to mononucleotides. Failure to precipitate with proteins (Kurnick, 1950c), dialyzability, and acid solubility (Kurnick, 1954) occur near the latter end of the scale. Methyl green affinity and specific viscosity are lost and pyroninophilia is acquired long before this level of depolymerization. This is stressed because of the apparent confusion about the meaning of depolymerization indicated by statements such as Swift's (1953): "In sections Leuchtenberger (1950) found no measurable pyronin increase. When tissue sections are treated with deoxyribonuclease, the DNA is depolymerized and is removed from the slide, so that nuclei become Feulgen negative. Kunitz (1950) found that polymerized, but not depolymerized DNA forms precipitates with proteins *in vitro*. Since hot water treatment of sections does not make them Feulgen negative, unless prolonged, this change is clearly different from that produced by the enzyme." It should be stressed that it is a matter of degree. Deoxyribonuclease depolymerization can be interrupted *in vitro* after a significant degree of depolymerization, but before protein precipitability is lost (Kurnick, 1950c). Temperatures of 80–100°C. cause pyroninophilia and loss of methyl green affinity in tissue sections, and *in vitro* they produce significant depolymerization which is, however, far from complete (i.e., to the point of acid solubility, dialyzability, and loss of protein precipitability) (Kurnick, 1954). The acquisition of pyroninophilia by depolymerized DNA in tissue sections, denied by Swift (1953), has been confirmed by a number of authors (Klemperer *et al.*, 1949, 1950; Harris and Harris, 1950; White, 1950a; Kurnick, 1952a; Yakar, 1952; Sibatani and Matsuda, 1952; Thomas, 1952; Pearse, 1953; Gossner, 1953). The differences in results depend, probably, upon whether depolymerization is carried to the point of dissolving the DNA, in which pyroninophilia does not occur, or is less extensive, in which case it is observed.

Pyronin has been used by Sanders (1946) and Davidson (1948) with celestine blue to obtain a differential stain similar to the mixture with methyl green. The observations of these authors suggest that celestine blue may be a specific stain for DNA. Proescher and Arkush (1928) used celestine blue as the iron lake prepared by boiling with ferric ammonium sulfate [cf. the chromalum lake of gallocyanin used by Einarson (1949)]

as a nuclear stain. The specificity of celestine blue remains to be investigated.

5. *Other Stains "Specific" for Nucleic Acids*

Turchini, Castel, and van Kien (1944, 1948) introduced staining with fluorones into histochemistry. These compounds, of which 9-phenyl-2,3,7-trihydroxy-6-fluorone (or, in more usual terminology, xanthenone) is said to be the best, depend upon their reaction with the sugar moiety of the nucleic acids. Hydrolysis, as with the Feulgen reaction, is, therefore, required to uncover the sugars. Unlike the Feulgen reaction, both ribose and deoxyribose react, the former to give yellow to red colors and the latter a blue to violet color. To prevent the extraction of RNA by the acid hydrolysis, the authors recommend fixation in chromic or picric fixatives. The required duration of hydrolysis varies with different fixatives as in the Feulgen reaction. Furthermore, Turchini (1949) notes that fixatives suitable for many tissues (such as Bouin's) give inconsistent results with others. For bacteria, he recommends osmic acid followed by cobalt nitrate and sublimate in acetic acid-formalin. The authors state that the reaction is quantitative, although the same reservations as apply to the effects of hydrolysis for the Feulgen reaction would apply here. In addition, it would appear difficult to quantitate the results photometrically in view of the wide range of colors produced with a single substrate (as, for example, violet to blue-black for DNA). The determining factors in this variability are not known. Glucosamines produce reddish violet colors which permit their distinction from RNA. Their refractiveness to extraction by ribonuclease is also used in their recognition by the authors. According to Turchini *et al.* (1948) saccharides which do not contain amino groups do not stain in tissue sections, although they do stain red or violet *in vitro* (Turchini, 1949).

The fluorone compounds recommended by the authors are not readily available. As a result, reports on their application are sparse. A number of papers by the original authors have appeared (see Turchini *et al.*, 1948 for references), and by Alexander and Backler (1951) and Backler and Alexander (1952). Both groups have reported satisfactory results. Pearse (1953), however, reports that in animal tissues the results have been variable and have at best yielded a pale rose-pink color in the nuclei instead of the blue reported by the original authors. We have had no personal experience with the method.

Novelli (1954) has recently reported a new staining reaction for nucleic acid with sambucyanin. The author states that both types of nucleic acid are stained red, while other components do not stain. At high concentra-

tion, the stain may be used supravitally. The report is too recent to have provided opportunity for the application of this method and its evaluation by others. The author provides no evidence on the mechanism of the reaction.

Among basic stains which do not distinguish between nucleic acids nor, even, reliably between nucleic acids and other acidic components of tissues, toluidine blue O, crystal violet, methylene blue, azure A (Flax and Pollister, 1949), and gallocyanin have had great popularity because of the intense and sharp staining in a color to which the human eye is particularly sensitive (blue). Kelly (1939) has studied the role of protein and nucleic acids in toluidine blue staining and demonstrated the principal role of nucleic acids. Einarson (1949) introduced a modification of the gallocyanin stain containing a chromalum mordant. By suitable selection of pH, greater specificity for nucleic acids and reproducibility is claimed. Differential extraction is still requisite for the differentiation of the nucleic acids, and staining of protein and acid polysaccharides is not completely avoided (Stenram, 1953, 1954). The stain has the advantage of being progressive (cf. leuco-methyl green, Kurnick and Foster 1950) so that removal of excess adsorbed stain is not necessary. Lagerstedt (1948) has reported high specificity in acid solutions, and stated that the stain may be suitable for quantitative analysis. Sandritter, Diefenbach, and Krantz (1954) have investigated the stoichiometry of the reaction between RNA and gallocyanin-chromalum. They reported that *in vitro* 1 gallocyanin molecule was bound per 15 P atoms of "polymerized" RNA and per 23 P atoms of heated RNA. Protein did not interfere. The stoichiometry of the reaction with DNA, which also stains intensely, was not investigated, nor was the application of these findings to tissue staining reported.

Aceto-orcein (La Cour, 1941) and aceto-carmine (Belling, 1926) have been popular chromatin stains for some materials. Their chemical specificity has not been extensively investigated. However, aceto-orcein staining probably depends upon DNA (Kurnick and Ris, 1948). On the other hand, aceto-carmine staining has been attributed to proteins (Rattenbury, 1952).

Hematoxylin appears to stain structures with and without nucleic acid depending upon the mordant used. With an alum mordant, as used in histological staining, chromatin is stained more or less specifically. Not only is selectivity for DNA suggested by this observation (Brenner, 1953), but there is evidence to indicate that the absorption spectrum of the stain may be a function of the state of polymerization of the DNA. Thus, normal nuclei stain blue-black, whereas the hematoxylin bodies of the L.E. cell, which contain depolymerized DNA (Klemperer *et al.*, 1949,

1950), stain purple or reddish purple. This possibility deserves further exploration. It has been suggested that Gomori's (1941) chromic hematoxylin has specificities similar to leuco-basic fuchsin, and therefore is specific for DNA after HCl hydrolysis, as in the Feulgen reaction (Melander and Wingstrand, 1953). It seems doubtful that the HCl treatment of the tissue would modify the reaction of hematoxylin with DNA since the stain is probably not a carbohydrate reagent like the Schiff reagent (cf. DeLamater's (1953) use of thionine and azure A and Choudhuri's (1943) use of basic fuchsin without decolorization after HCl hydrolysis). The hydrolysis may improve the specificity of these basic stains by removing RNA from the tissue.

Flax and Himes (1951, 1952) have introduced azure B as a differential stain for nucleic acids. These authors obtained blue-green staining of DNA and metachromatic purple staining of RNA. The stain has been used for the staining of Negri bodies (Jordan and Heather, 1929) and in Giemsa's stain (Roe, Lillie, and Wilcox, 1940). Confirmatory reports on its usefulness as a differential nucleic acid stain have not appeared. Roe *et al.* (1940) noted clear blue staining of lymphocyte cytoplasm and purple staining of chromatin with azure B alone, results which are at variance with those of Flax and Himes.

Acriflavine has been described as a supravital chromatin stain (De Bruyn, Robertson, and Farr, 1951). Whether this represents DNA staining by the basic dye or protein staining (Oster and Grimsson, 1949) is unsettled.

Haenel (1950) claims that the isoelectric point of DNA is higher than that of RNA. Therefore, basic dyes utilized at pH 4.0 stain only the nucleolus, nuclear membrane, and cytoplasm; chromatin is unstained. Our experience is not consistent with this observation, nor is evidence adduced for the contention of different isoelectric points for the two nucleic acids. Gerola and Vannini (1948) stained both nucleic acids over a wide range of pH, and Flax and Himes (1952) used azure B in their staining of both nucleic acids (DNA orthochromatically and RNA metachromatically) at pH 4.0, at which pH Haenel's hypothesis would not have provided for the staining of DNA. We are unable to account for the discrepancy between Haenel's results and those of others.

Reaction with diazonium (and tetrazonium) salts following destructive benzoylation of tryptophane, tyrosine, and histidine (which react with these reagents before benzoylation) was used by Mitchell (1942) to detect purines and pyrimidines. Danielli (1947) has reported irregular results with this procedure. He has pointed out, furthermore, that nucleosides do not couple with diazonium salts, although the free nitrogenous bases

do (Burian, 1904; Johnson and Clapp, 1908), and that the alkaline hydrolysis during benzoylation is certain to remove some nucleic acid from the sections. Danielli's own experiments (1947) indicate that the former objection is not valid, since he has obtained staining of nucleic acid with tetrazotized benzidine. In order to avoid the effect of concentrated NaOH, Danielli has performed benzoylation in dry pyridine. Danielli utilizes the fact that both ends of the molecule are diazotized in his compound to intensify the color obtained in the reaction with the tissue by forming an azo linkage with phenols (as β-naphthol). Pearse (1953), however, concludes that the tetrazonium reactions are not tests for nucleic acids, since the reaction in chromatin was positive both before and after benzoylation, following hot trichloracetic acid extraction so that the material was Feulgen negative. He suggests that in certain spatial configurations of polypeptides, the three reactive amino acids are protected from benzoylation, and are therefore still responsible for the tetrazonium reaction.

Dustin (1947) has used neutral red supravitally. He has concluded that it is sequestrated in vacuoles containing RNA. However, it is not claimed that all RNA in the cell is detected. Thus, this stain resembles the diamidines, such as stilbamidine. Snapper, Schneid, and Kurnick (1950) have demonstrated that this compound becomes sequestrated *in vivo* in cytoplasmic particles containing RNA and may be detected by fluorescence microscopy. Kurnick, Klein, and Klein (1950) have demonstrated the detection of these particles by ultraviolet absorption at the stilbamidine peak (3100 Å) as well as by fluorescence. Snapper *et al.* (1951) have found that hydroxystilbamidine is localized predominantly in the nucleus, suggesting a preference for DNA. It also is detected by fluorescence microscopy. Thus, it is possible that the diamidines, which react with both nucleic acids to form precipitates in solution, may prove to be useful as specific histochemical fluorescent stains. Stilbamidine, however, like neutral red, does not "stain" all the cytoplasmic nucleic acid supravitally, but forms discrete granules with some of the RNA. Thus, these stains do not provide accurate localization, since the previously diffuse cytoplasmic RNA is gathered into discrete granules by reaction to the stain (Larionov and Brumberg, 1948). Additional cytoplasmic RNA, not combined with the stain, is also demonstrable (Kurnick *et al.*, 1950).

According to Henry and Stacey (1943) and Stacey (1947), the Gram stain depends upon ribonucleic acid on the surface of bacteria. According to these authors, the nucleic acid may be extracted by bile salts, whereupon the organism is rendered gram-negative. Magnesium ribonucleate in the extract (or from other bacterial species) may be "replated" onto normally gram-positive, but not gram-negative organisms, re-establishing Gram

staining. Mitchell and Moyle (1950) contended that the Gram stain was not dependent upon RNA but upon an ester phosphorus compound present in gram-positive but not gram-negative bacteria. Jones, Muggleton, and Stacey (1950) have refuted their evidence, however, and maintained the role of magnesium ribonucleate. Mittwer, Bartholomew, and Kallman (1950) have provided evidence from the use of ribonuclease in support of the function of RNA; but they have also implicated the permeability of the cell membrane to the iodine-dye complex, since, unlike Henry and Stacey (1943), they were unable to "replate" the nucleate on crushed cells. In histological sections the Gram stain gives inconstant results. Pearse (1953) considers it to be of little value histochemically.

Finally, among staining techniques, may be considered colorimetric procedures for the detection of phosphorus. These techniques are somewhat less specific than those which depend upon the detection of the sugar moieties. However, when combined with specific extraction procedures (see discussion on reservations on their use above), localization and identification of nucleic acids is possible, as with the nonspecific basic dyes. Serra and Lopes (1945) have used the phosphomolybdate (reduced by benzidine to molybdenum blue) color reaction for the detection of phosphates liberated by nitric acid digestion. It is probable that some nucleic acid phosphate is lost during the hydrolysis and that diffusion of the ammonium phosphomolybdate occurs so that localization is inexact. A variant of the color techniques for the detection of phosphorus after wet ashing, is the detection of phosphorus after dry ashing (microincineration) (Uber and Goodspeed, 1935; Barigozzi, 1937; Caspersson and Schultz, 1939; Norberg, 1943). However, this more laborious technique is more suitable for the detection of cations than for the purpose under discussion.

V. Physical Methods

1. General Introduction

Whereas the distinction between chemical and physical methods is admittedly an inadequate classification, the procedures we shall now consider are usually considered to fall into the latter group. They include procedures dependent upon the natural absorption of nucleic acids in the ultraviolet, X-ray, and infrared portions of the energy spectrum, electron microscopy, interferometry, photofluorometry, and detection of radioactivity following the incorporation of radioisotopes into the nucleic acids. A brief resume of the problems in these approaches is given by Randall (1950). Most of these biophysical methods, including the potentiality of applying several to a single specimen, have been reviewed by Fitzgerald and Engstrom (1952).

2. *Ultraviolet Spectroscopy*

The technique of ultraviolet spectroscopy of tissues for the detection of nucleic acids was explored briefly by Wyckoff, Ebeling, and Terlaw (1932) and extensively developed and applied by Caspersson and coworkers (Caspersson, 1936, 1939, 1950). Kohler (1904) introduced this tool into microscopy for the purpose of improving resolution through the use of shorter wavelengths. In this regard, it has proved of negligible advantage. For cytochemical analysis, however, it has proved a powerful instrument. The problems discussed above in regard to photometry in general, apply here. Correction for nonspecific light losses is even more difficult in this part of the spectrum than in the visible range because light dispersion is so much greater at shorter wavelengths (Lord Rayleigh, 1871) so as often to be a major part of the measured absorption. As a result, the absorption curve is greatly distorted. The nonspecific light loss is usually assumed to be 50% reflection-refraction, which is only slightly influenced by wavelength, and 50% dispersion, which is assumed to follow Rayleigh's fourth-power formula. The correction for this loss is made from the measured absorption at a wavelength (300–330 mμ) at which there is assumed to be no specific light loss. Minor errors in the assumptions made may introduce large errors into the derived curve, particularly in regions where the specific absorption is low (as in the 280 mμ region). As a result, conclusions about the protein content may be more doubtful than about nucleic acids (since the 260 mμ absorption due to the purine and pyrimidine bases is usually much more pronounced). Nucleohistone in solution gives a curve which differs from that of pure DNA only in that the 260 mμ peak is slightly broadened and the extinction at the minimum near 238 mμ is increased (Mirsky and Pollister, 1942). In the derived curves for chromatin, a fairly prominent hump in the vicinity of 280 mμ is interpreted as due to protein (see Caspersson, 1950 for references). That this may be due to artifacts from inhomogeneity which are not adequately corrected in the calculations is suggested by our observation (Kurnick, 1950b, 1952c) that thymus nuclei prepared in an optically homogeneous state by sucrose and neutral formalin fixation give an absorption curve identical with that of nucleohistone in solution. The same nuclei fixed in Carnoy's solution, so as to be optically inhomogeneous, give a curve with a depressed maximum at 260 mμ (so that the calculated nucleic acid content is too low) and with a prominent hump in the vicinity of 280 mμ. With the nuclei prepared so as to be homogeneous, quantitative ultraviolet analysis appears to be feasible in isolated nuclei (Kurnick, 1950b; Leuchtenberger *et al.*, 1952).

Correction for nonspecific light losses may be possible by the preparation of a "blank" after extraction of nucleic acids (Pollister and Ris, 1947). The problems inherent in extraction techniques have already been discussed. Considerable alterations in nonspecific light losses due to acid treatments (as in hot trichloracetic acid extraction) would obviously vitiate the value of the technique.

The optical requirements for photometry in this part of the spectrum have been extensively examined by Caspersson (1950). The problems in this sphere have been somewhat simplified by the introduction of achromatic reflecting optics (Brumberg and Larionov, 1946; Burch, 1947; Grey, 1950, 1952; Johnson, 1952), high resolution grating monochromators, more intense light sources, and sensitive photomultiplier detectors. Caspersson has utilized some of these improvements in the design of a recording instrument (Caspersson, Jacobsson and Lomakka, 1951).

The higher energy and biological effects of ultraviolet radiation have been considered as possible sources of error in altering the absorption characteristics of the nucleic acids in cells during examination. The photometry in the ultraviolet of living cells has been reviewed by Walker and Yates (1952a) and Davies and Walker (1953). Brumberg and Larionov (1946) have reported that living cells do not absorb in the vicinity of 260 mμ, but do so only after cell death. Indeed, an apparent increase in absorption does occur upon cell death in most materials; but this is probably due to an increase in nucleic acid concentration due to shrinkage of the nucleus and increase in nonspecific light losses due to aggregation within the nucleus (Ris and Mirsky, 1949a; Ely and Ross, 1949; Klein, Kurnick and Klein, 1950; Walker and Davies, 1950). It is doubtful that there is actual induction of absorption by the purines and pyrimidines due to cell death. However, a very real problem is photolysis of absorbing groups during examination, particularly of unfixed material (Ludford, Smiles, and Welsh, 1948; Bradfield and Errera, 1949; Klein, Kurnick and Klein, 1950; Errera, 1953). Gersh and Catchpole (1950) have observed a decrease in ultraviolet absorption in fixed material during ultraviolet irradiation. Between exposures no fall in ultraviolet absorption was noted in the fixed material. This probably is due to the opening of the rings which are responsible for the absorption (Rapport and Canzanelli, 1950; Canzanelli, Guild, and Rapport, 1951) or to the formation of diffusible products which are lost (Bradfield, 1950). That this may be at least in part enzymatic is suggested by the fact that the effect is delayed in living cells and is much more severe in fresh than in fixed material. Davies and Walker (1953) have estimated that only one or two photographs of living cells may be taken without apparent change in absorption.

Since increased concentration due to changes in volume, increased light scattering due to changes in particle aggregation, and photolysis may all occur, changes in absorption may occur in any direction (Bradfield, 1950). These effects are independent of the effects of fixatives considered above, which may cause redistribution of nucleic acids between cytoplasm and nucleus (Chayen and Norris, 1953) and between mitochondria and cytoplasmic "ground substance" (Mellors, 1950; Mellors, Berger and Streim, 1950). Another source of error arises in the increase in extinction coefficient with depolymerization, both by nucleases (Kunitz, 1946, 1950) and heat (Kurnick, 1950c), which may amount to 30%. Since the ultraviolet absorption in tissues does not differ qualitatively in polymerized or depolymerized nucleic acids, thus providing no clue as to the state of polymerization, an error of 30% may arise in the calculation of content from this source alone. It may be possible to make suitable correction, however, by performing a methyl green analysis on the same material (Kurnick, 1950b). Ultraviolet irradiation itself probably produces depolymerization of DNA (Errera, 1953), thus increasing the extinction coefficient during the investigation. This mechanism may contribute to the increase in ultraviolet absorption during irradiation observed in some material (see preceding discussion).

The absorption of ultraviolet energy by compounds other than nucleic acids at the same wavelength as the nucleic acid maximum absorption may introduce error. Mononucleotides have absorption spectra nearly identical to those of the nucleic acids. However, in most materials these are removed by fixation during preparation for microscopy. Such precursors of nucleic acids probably exist in the living nucleus however (Walker and Yates, 1952b), and purine mononucleotides (such as ATP), abundant in the cytoplasm of some cells, would contribute to the absorption of unfixed material. Chayen (1952) has suggested that ascorbic acid and catechol at a neutral pH may be present in sufficient concentration in some materials to produce significant absorption at 260 mμ.

A more complete consideration of this technique, which has proved most productive for the development of current theory of nucleic acid localization and function through its power as a qualitative tool, would be out of place here in view of the availability of extensive discussions by Caspersson (1950), Davies and Walker (1953), and others. The color-translating ultraviolet microscope (Shurcliff, 1952) originally suggested by Brumberg (1939) deserves mention as an electronic and mechanical *tour de force*. In view of the problem of nonspecific light loss, it is improbable that the simultaneous rendition of three ultraviolet wavelengths in visible color can be interpreted chemically from a simultaneous three-color print. Our per-

sonal reaction to the blended colors is one of confusion. However, the rapid automatic production of a series of black and white photomicrographs at different monochromatic radiations may greatly facilitate the derivation of absorption spectra.

3. *Historadiography*

The technique of X-ray absorption analysis as applied to tissues has been developed by Engstrom (1950). The application of X-ray absorption for the photography of tissues was introduced by Dauvillier (1930) and extended by Lamargene (1936), who introduced the term "Historadiography," and Turchini (1937). The method has been reviewed recently by Clemmons, Lalich, and Angevine (1954). The technical requirements of the method have limited its use to a few laboratories. Thus far, the technique has been limited to photography of the absorbing image without magnification. The photograph is then enlarged. Resolution is thus limited by the fineness of available emulsions. Analysis of structures below one square micron in area is not at present feasible. However, the development of the X-ray microscope (Kirkpatrick, 1950) promises considerable widening of the scope of this technique. Whereas absorption due to specific elements is very sharp (as pertains to wavelength), the extinction is of such low order in most cases as to require very thick absorbing sections for elemental analysis. Thus, the analysis of iron in muscle would require a section approximately 1 mm. thick (Engstrom, 1946, 1952). It is apparent that this renders the technique useless for specific intracellular localization and analysis for most elements. The specific analysis of phosphorus for nucleic acid investigation is similarly virtually excluded.

Engstrom and Lindstrom (1949) have developed the use of soft X-rays for mass analysis. Absorption of X-rays of approximately 8–10 Å in wavelength by the elements of tissues may be readily measured in sections of 10μ thickness. Engstrom and Lindstrom (1950) (see also Fitzgerald and Engstrom, 1952, for review), have calculated the concentration of absorbing material, assuming a uniform composition of tissue in terms of carbon, oxygen, nitrogen, and hydrogen (as in protein). They have calculated the probable range of errors inherent in the assumptions (i.e., due to the presence of other elements, or compounds with different proportions of the elements). An accuracy of 10–30% is claimed for structures, other than bone, in animal tissues. The procedure is simplified by the inclusion of a reference system with similar absorption characteristics to that of tissue and with known mass per unit surface area. Nitrocellulose films have been used by Engstrom and Lindstrom (1950) and Brattgard and Hyden (1952). The preparation of the films and their calibration by interferom-

etry, which improves their accuracy over gravimetry by eliminating errors due to variations in thickness of the films, is described by Hallen (1953). The determination of thickness is essential to the calculation of mass per unit volume (or of a sectioned structure). The problems of thickness determination have already been considered above. Clemmons (personal communication) has attempted the determination of thickness, by X-ray absorption, of paraffin at the edge of the section. However, this provides no advantage over other techniques in view of the irregularity of cut sections (Glimstedt and Hakansson, 1951), which is likely to be most marked between paraffin alone and tissue-containing portions. For the analysis of nucleic acids, the method requires specific extraction of the nucleic acids and remeasurement of absorption. The problems of extraction have already been considered. Furthermore, the extracted nucleic acid would have to represent more than 10–20% of the total dry mass to be detected reliably, in view of the magnitude of the error of the method as reported by Engstrom and Lindstrom (1950). For nucleic acid analysis, therefore, the method is severely limited. However, Brattgard, Hallen, and Hyden (1953) have shown that the principal error is due to the photographic enlargement of the X-ray image. They have eliminated this step by using a microphotometer for the analysis of the original exposure. An error of ± 1.5% is claimed. Gomirato (1954), Brattgard and Hallen (1952), and Brattgard and Hyden (1952) have succeeded in applying the method to the quantitative analysis of RNA in motor neurons.

The technique, although having errors of its own, escapes many of the errors of nonspecific absorption characteristic of analysis in the ultraviolet, visible, and infrared portions of the spectrum. However, in its present state of development, without direct magnification, we are dubious of its providing any advantage over existing procedures for histochemical analysis of nucleic acids. Its greatest value, at present, appears to us to be in the determination of total dry mass.

4. Infrared Histospectroscopy

The infrared portion of the spectrum is limited in histochemical application because of the poor resolution obtainable at these long wavelengths, which approach the dimensions of the cell. The recognition of absorbing groups as specific for a given constituent (such as nucleic acid) is limited and difficult, particularly in low concentrations and in the presence of many different compounds. Barer, Cole, and Thompson (1949) and Blout and Mellors (1949) have utilized this part of the spectrum in microscopy (see Fraser, 1953 for review), but did not attempt to interpret infrared absorption bands. Fraser and Chayen (1952) were

able to demonstrate characteristic absorption bands of RNA and DNA in cells, and their disappearance after extraction with nucleases. The possibility of semiquantitative analysis is suggested. At present, however, it appears to us that this tool will prove more fruitful in the investigation of the structure of DNA than in cellular localization. We believe that this is the case, also, for the study of dichroism in the various parts of the spectrum, which can provide information about the orientation of nucleic acids in cells and in isolated material (Wilkins, 1950; Seeds, 1953; Fraser, 1953). These aspects are, therefore, considered outside the scope of this review.

5. *Electron Microscopy*

It has been suggested that electron microscopy may be capable of determining mass in sections (Ornstein, 1952) since absorption is a function of the density of transversed molecules (cf. historadiography). The indirect measurement of nucleic acids following extraction is, then, theoretically possible. No reports of this application have met our eye, however. The method awaits the investigation of the practical feasibility of calculating mass from electron absorption data and the limits of error (i.e., whether differential extraction of nucleic acids will produce detectable changes).

6. *Interferometry*

Davies and Wilkins (1952) and Barer (1952) have introduced interferometry as a method for mass determination in tissues, and have suggested phase contrast microscopy for the same purpose. The method is subject to the same errors introduced into colorimetric photometry on cells by inhomogeneity and light dispersion. On homogeneous structures it has proved a useful tool. Davies, Engstrom, and Lindstrom (1953) have compared mass determination by X-ray absorption and interferometry on the same material. The results are in reasonably good agreement. The authors concluded that the interferometric method has the advantage of greater resolving power (the same as that of light, as compared with 1μ for the X-ray method). The sensitivity of the X-ray method is approximately 1×10^{-10} mg./μ^2; by interferometry the sensitivity is increased by an order. Finally, the latter technique has the advantage that living material can be analyzed, thus circumventing errors from fixation. The determination of nucleic acid content would depend upon mass determination before and after specific extraction, however. Therefore, neither interferometry nor X-ray absorption would be applicable to living material for this purpose. If sections are used, the problem of thickness determination is present in both techniques. Mellors, Kupfer, and Holl-

ender (1953) have recently measured thickness, volume, and hydrous and anhydrous mass in living cells by interferometry. Nucleic acid content was determined on the same material by ultraviolet absorption measurement.

7. *Photofluorometry*

As mentioned previously, diamidines react with nucleic acids *in vivo* to form fluorescent compounds. Stilbamidine is localized in the cytoplasm (Snapper *et al.*, 1950; Kurnick, Klein, and Klein, 1950; Snapper *et al.*, 1951) in combination with RNA, whereas hydroxystilbamidine reacts preferentially with DNA in the nucleus and to a lesser extent with RNA in the cytoplasm (Snapper *et al.*, 1951). However, the reactions are certainly not quantitative, although tissue concentration of stilbamidine roughly parallels RNA concentration (Kurnick, unpublished data); indeed, only in cells with high RNA concentration is stilbamidine detectable histochemically. Thus, no fluorescence is detectable in leucocytes other than myeloma cells (Snapper *et al.*, 1951). We have followed Wenderoth (1953) in including the diamidines as nucleic acid histochemical detectors, but we do not believe that they are properly so classed notwithstanding their intriguing reactions with nucleic acids *in vivo*.

Mellors (1950) has utilized emission photofluorometry by staining cells with a basic fluorochrome such as berberine sulfate, which favors nuclear localization. The method is based on Friedman's (1950) qualitative use of fluorochromes. Friedman observed a yellowish white fluorescence of nuclei stained with berberine sulfate, a red-purple fluorescence of cytoplasm stained with acid fuchsin, and greenish gray nuclei and orange-red cytoplasm with acridine at pH 8. He used mixtures of the three dyes to obtain differential stains. In Mellors' application of the fluorochromes for nucleic acid detection, acid components other than nucleic acid are excluded by preliminary binding of acid polysaccharides with methylene blue and by binding free amino groups of protein with fast green to quench the fluorescence of carboxyl-bound fluorochrome. Staining is performed at pH 6.3, since both primary and secondary phosphoryl groups of the nucleic acids are said to be dissociated and to bind dye at this pH. The stained material is irradiated in the ultraviolet at 365 mμ and fluorescence is detected with a photomultiplier. It is claimed that "the fluorescence intensity is a measure of the chromatin content." It seems more reasonable that if the procedure is specific at all, intensity is a function of concentration, not content (see discussion above). Mellors, Glassman, and Papanicolaou (1952) have used the method to detect malignant cells in smears by their stronger fluorescence. In this procedure, blocking of possible interfering

compounds was omitted. The failure to detect cytoplasmic fluorescence is not explained. Presumably this is not due to specificity of the fluorochrome for DNA, since total nuclear nucleic acid is presumed to be detected (the discussion is somewhat confused by the comparison of chemical analysis of the *total cell* nucleic acid of ascites tumors, which is approximately 50% nuclear DNA and 50% cytoplasmic RNA (Klein *et al.*, 1950), with the photofluorometric data and with the DNA content of lymphocytes). The authors have not attempted to calculate nucleic acid content from fluorescence intensity, but have compared ratios of the fluorescence intensities of different cells.

Loeser and Berkley (1954) have introduced a similar technique in living cells with acriflavine hydrochloride as the fluorochrome. Television methods were applied for detection of cells rapidly traversing the field (as in the circulation).

8. *Autoradiography*

The detection of nucleic acids by labeling them with C^{14} (Lajtha, 1954) or P^{32} followed by autoradiography has been applied (Leblond, Stevens, and Bogoroch, 1948; Howard and Pelc, 1951). Of course, tissue components other than nucleic acids are certain to be tagged by both. Detection, then, depends upon extraction of interfering substances before radiography (e.g. the extraction of acid-soluble and lipid phosphorus, as in the phosphorus analysis of Norberg, 1943), and the removal of the nucleic acid under investigation followed by repeat radiography (Plaut, 1953). Separation of the nucleic acids depends upon differential extraction (Lajtha, 1954). The quantitative analysis is not well developed, depends upon densitometry, grain counting, or nuclear track counting, and is, in any event, limited by the necessity of determining the degree of labeling (i.e., relative number of radioactive and stable atoms per molecule). Resolution is approximately 1 to 1.5 μ. The technique has, for these reasons, found little favor for the purpose under discussion. It is of greater merit in detecting sites of turnover and relative rates of synthesis of new nucleic acid, than for its detection and localization. It may be noted, among potential sources of error in autoradiography, that some cells contain reducing substances (Fitzgerald and Engstrom, 1952) which may blacken the photographic film and be misinterpreted as radioactivity if suitable controls are not provided.

VI. Summary

Qualitative analysis for the intracellular localization of nucleic acids may be performed with reasonable assurance by staining techniques, and confirmed by the use of specific nucleolytic enzymes on fixed tissues. Ultra-

violet microscopy serves similarly, but can contribute little, if any, further information in this sphere. The newer methods of X-ray absorption, infrared microscopy, etc., at present find greater application in aspects of histochemistry other than nucleic acid analysis.

Quantitative analysis is beset with errors because of uncertainties about specificity, stoichiometry, diffusion, and optical factors. Reproducibility has been demonstrated for a number of quantitative procedures. Although essential, it is not in itself an adequate test of the reliability of the method. Thus, repeated analysis of nuclei of the same tissue, fixed in the same manner, hydrolyzed at constant temperature and constant time, and otherwise treated identically in the Feulgen procedure may be expected to give reproducible absorption values. Nevertheless, one may not conclude that extension of the procedure to other nuclei will give results which may be validly compared (see discussion of effect of heterogeneity above).

It is, therefore, necessary to evaluate the multitude of quantitative data in the literature with an awareness of the possible errors. It is our opinion that for a procedure to qualify as a quantitative method it must be possible to state the results in absolute values so that they may be tested (in suitable material) by independent methods (such as macrochemical analysis). This has been done for ultraviolet analysis of nucleic acid in whole thymus nuclei (Kurnick and Thorell in Kurnick, 1950b; Leuchtenberger *et al.*, 1952). We have also carried out such studies for methyl green staining in a number of tissues. However a wider variety of tissues should probably be studied before the method is extended to tissues in which confirmation of the result by chemical means is not feasible. The potential error in the assumptions as to uniformity in degree of polymerization and absence of competing substances (as proteins bound to phosphoric acid groups and not extracted by dilute HCl) must be borne in mind in attempting to extend the observations on ultraviolet absorption and methyl green staining. It is hoped that independent tests of other quantitative histochemical methods will be reported to assist in the evaluation of a large and increasing bulk of quantitative histochemical analyses by methods not yet adequately examined. Until this has been done, histochemical analyses for nucleic acids should be regarded as primarily qualitative, and interpretations based upon quantitation must be viewed with caution.

Acknowledgments

This work was aided by grants from the American Cancer Society, the Life Insurance Medical Research Fund, and the National Heart Institute, U. S. Public Health Service.

VII. References

Alexander, W. F., and Backler, B. (1951) *Proc. Soc. Exptl. Biol. Med.* **78**, 181.
Alfert, M. (1952) *Biol. Bull.* **103**, 145.
Atkinson, W. B. (1952) *Science* **116**, 303.
Avery, O. T., MacLeod, C. M., and McCarty, M. (1944) *J. Exptl. Med.* **79**, 137.
Backler, B. S., and Alexander, W. F. (1952) *Stain Technol.* **27**, 147.
Balbiani, E. G. (1881) *Zool. Anz.* **4**, 662.
Barber, H. N., and Callan, H. G. (1944) *Nature* **153**, 109.
Barber, H. N., and Price, J. P. (1940) *Nature* **146**, 335.
Barer, R. (1952) *Nature* **169**, 366.
Barer, R., Cole, A. R. H., and Thompson, H. W. (1949) *Nature* **163**, 198.
Barigozzi, C. (1937) *Boll. Soc. ital. biol. sper.* **12**, 583.
Bauer, H. (1932) *Z. Zellforsch. u. mikroskop. anat.* **15**, 225.
Bell, L. G. E. (1952) *Intern. Rev. Cytol.* **1**, 35.
Belling, J. (1926) *Biol. Bull.* **50**, 160.
Blout, E. R., and Mellors, R. C. (1949) *Science* **110**, 137.
Boivin, A., Delauney, A., Vendrely, R., and Lehoult, Y. (1945) *Experientia* **1**, 334.
Boivin, A., Tulasne, R., Vendrely, R., and Minck, R. (1947) *Arch. sci. physiol.* **1**, 307.
Boivin, A., Vendrely, R., and Vendrely, C. (1948) *Compt. rend.* **226**, 1061.
Brachet, J. (1940) *Compt. rend. soc. biol.* **133**, 88.
Brachet, J. (1941) *Enzymologia* **10**, 87.
Brachet, J. (1942) *Arch. Biol. (Liege)* **53**, 207.
Brachet, J. (1946) *Experientia* **2**, 142.
Brachet, J. (1947) *Symposia Soc. Exptl. Biol.* **1**, 207.
Brachet, J. (1950) "Chemical Embryology" Interscience, New York.
Brachet, J. (1953) *Quart. J. Microscop. Sci.* **94**, 1.
Brachet, J., and Shaver, J. R. (1948) *Stain Technol.* **23**, 177.
Bradfield, J. R. G. (1950) *Discussions Faraday Soc.* **9**, 481.
Bradfield, J. R. G., and Errera, M. (1949) *Nature* **164**, 532.
Brattgard, S. O., and Hallen, O (1952) *Biochim. et Biophys. Acta* **5**, 473.
Brattgard, S. O., Hallen, O., and Hyden, H. (1953) *Acta Radiol.* **39**, 494.
Brattgard, S. O., and Hyden, H. (1952) *Acta Radiol.* Suppl. 94.
Brenner, S. (1953) *Exptl. Cell Research* **5**, 257.
Brumberg, E. M. (1939) *Compt. rend. acad. sci. U.R.S.S.* **25**, 473.
Brumberg, E. M., and Larionov, L. T. (1946) *Nature* **158**, 663.
Bryan, J. H. D. (1951) *Chromosoma* **4**, 369.
Buck, J. B. (1939) *Genetics* **24**, 96.
Burch, C. R. (1947) *Proc. Phys. Soc. (London)* **B59**, 41.
Burian, R. (1904) *Ber.* **37**, 708.
Callan, H. G. (1943) *Nature* **152**, 503.
Campbell, R. M., and Kosterlitz, H. W. (1952) *Science* **115**, 84.
Canzanelli, A., Guild, R., and Rapport, D. (1951) *Am. J. Physiol.* **167**, 364.
Carr, J. G. (1945) *Nature* **156**, 143.
Caspersson, T. (1932) *Biochem. Z.* **253**, 97.
Caspersson, T. (1936) *Skand. Arch. Physiol.* **73**, Suppl. 8, 1.
Caspersson, T. (1939) *Chromosoma* **1**, 147.
Caspersson, T. (1940) *Chromosoma* **1**, 605.
Caspersson, T. (1944) *Nature* **153**, 499.

Caspersson, T. (1950) "Cell Growth and Cell Function." Norton, New York.
Caspersson, T., Jacobsson, F., and Lomakka, G. (1951) *Exptl. Cell Research* **2**, 301.
Caspersson, T., and Schultz, J. (1939) *Nature* **143**, 602.
Chamberlain, C. J. (1932) "Methods in Plant Histology," 5th ed. Univ. Chicago Press, Chicago.
Chargaff, E. (1951) *J. Cellular Comp. Physiol.* **38**, Suppl. 1, 41.
Chayen, J. (1952) *Exptl. Cell Research* **3**, 652.
Chayen, J., and Norris, K. P. (1953) *Nature* **171**, 472.
Choudhuri, H. C. (1943) *Nature* **152**, 475.
Clemmons, J. J., Lalich, J. J., and Angevine, D. M. (1954) *Lab. Invest.* **3**, 19.
Commoner, B. (1949) *Science* **110**, 31.
Danielli, J. F. (1947) *Symposia Soc. Exptl. Biol.* **1**, 101.
Danielli, J. F. (1953) "Cytochemistry—A Critical Approach." Wiley, New York.
Dauvillier, A. (1930) *Compt. rend.* **190**, 1287.
Davidson, J. N. (1948) *Cold Spring Harbor Symposia Quant. Biol.* **12**, 50.
Davidson, J. N., Leslie, J., and White, J. C. (1948) *J. Pathol. Bacteriol.* **60**, 1.
Davidson, J. N., and McIndoe, W M. (1949) *J. Biochem.* **45**, xvi.
Davies, H G, and Walker, P M. B. (1953) *Progr. Biophys. and Biophys. Chem.* **3**, 195.
Davies, H. G., and Wilkins, M. H. F. (1952) *Nature* **169**, 541.
Davies, H. G., Engstrom, A., and Lindstrom, B. (1953) *Nature* **172**, 1041.
Davies, H. G., Wilkins, M. H. F., and Boddy, R. H. G. (1954) *Exptl. Cell Research* **6**, 550.
DeBruyn, P. P. H., Robertson, R. C., and Farr, P. S. (1951) *Anat. Record* **108**, 279.
Deeley, E. M., Richards, B. M., Walker, P. M. B., and Davies, H. G. (1954) *Exptl. Cell Research* **6**, 569.
DeLamater, E D. (1953) *Intern. Rev. Cytol.* **2**, 158.
Dempsey, E. W., and Wislocki, G. B. (1946) *Physiol. Revs.* **26**, 1.
Dempsey, E. W, Singer, M., and Wislocki, G. B. (1950) *Stain Technol.* **25**, 73.
DeSmul, A., and Lecomte, C. (1953) *Arch. Biol.* **64**, 259.
Devreux, S., Johannson, M., and Errera, M. (1951) *Bull. soc. chim. biol.* **33**, 800.
Di Stefano, H. S. (1948a) *Chromosoma* **3**, 282.
Di Stefano, H. S. (1948b) *Proc. Natl. Acad. Sci. (U.S.)* **34**, 75.
Di Stefano, H. S. (1952) *Stain Technol.* **27**, 171.
Dobson, E. O. (1946) *Stain Technol.* **21**, 103.
Doyle, W. L., and Metz, C. W. (1935) *Biol. Bull.* **69**, 126.
Dustin, P. (1947) *Symposia Soc. Exptl. Biol.* **1**, 114.
Ehrich, W. E., Drabkin, D. L., and Forman, C. (1949) *J. Exptl. Med.* **90**, 157.
Einarson, L. (1949) *Exptl. Cell Research* Suppl. 1, 359.
Ely, J. O., and Ross, M. H. (1949) *Anat. Record* **104**, 103.
Engstrom, A. (1946) *Acta Radiol.* Suppl. 63.
Engstrom, A. (1950) *Progr. Biophys. and Biophys. Chem.* **1**, 164.
Engstrom, A. (1952) *Lab. Invest.* **1**, 278.
Engstrom, A., and Lindstrom, B. (1949) *Nature* **163**, 563.
Engstrom, A., and Lindstrom, B. (1950) *Biochim. et Biophys. Acta* **4**, 351.
Erickson, R. O., Sax, K. B., and Ogur, M. (1949) *Science* **110**, 472.
Errera, M. (1951) *Biochim. et Biophys. Acta* **7**, 605.
Errera, M. (1953) *Progr. Biophys. and Biophys. Chem.* **3**, 88.
Feulgen, R., and Rossenbeck, H. (1924) *Z. physiol. Chem.* **135**, 203.

Fitzgerald, P. J., and Engstrom, A. (1952) *Cancer* **5**, 643.
Flax, M. H., and Himes, M. H. (1951) *J. Natl. Cancer Inst.* **12**, 240.
Flax, M. H., and Himes, M. H. (1952) *Physiol. Zool.* **25**, 297.
Flax, M. H., and Pollister, A. W. (1949) *Anat. Record* **105**, 535.
Foster, C. L., and Wilson, R. R. (1952) *Quart. J. Microscop. Sci.* **93**, 147.
Franz, F., Weiden, V., and Meyer-Arendt, J. (1954) *Naturwissenschaften* **7**, 165.
Fraser, R. D. B. (1953) *Progr. Biophys. and Biophys. Chem.* **3**, 47.
Fraser, R. D. B., and Chayen, J. (1952) *Exptl. Cell Research* **3**, 492.
Frazer, S. C., and Davidson, J. N. (1953) *Exptl. Cell Research* **4**, 316.
Friedman, H. P., Jr. (1950) *Am. J. Obstet. Gynecol.* **59**, 282.
Gerola, F. M., and Vannini, E. (1948) *Atti accad. nazl. Lincei Rend. Classe sci. fis. mat. e nat.* **5**, 27.
Gersh, I., and Catchpole, H. (1950) *Discussions Faraday Soc.* **9**, 471.
Glick, D. (1953) *Intern. Rev. Cytol.* **2**, 447.
Glick, D., Engstrom, A., and Malmstrom, B. G. (1951) *Science* **114**, 252.
Glimstedt, G., and Hakansson, R. (1951) *Nature* **167**, 397.
Gomirato, G. (1954) *J. Neuropathol. Exptl. Neurol.* **13**, 359.
Gomori, G. (1941) *Am. J. Pathol.* **17**, 395.
Gomori, G. (1952) "Microscopic Histochemistry." Univ. Chicago Press, Chicago.
Gossner, W. (1953) *Zentr. allgem. Pathol. u. pathol. Anat.* **89**, 173.
Grey, D. S. (1950) *J. Opt. Soc. Amer.* **40**, 283.
Grey, D. S. (1952) *Lab. Invest.* **1**, 85.
Haenel, U. (1950) *Helv. Med. Acta* **17**, 627.
Hallen, O. (1953) *Exptl. Cell Research* **4**, 494.
Hammarsten, E., Hammarsten, G., and Teorell, T. (1928) *Acta Med. Scand.* **68**, 219.
Harrington, N. J., and Koza, R. W. (1951) *Biol. Bull.* **101**, 138.
Harris, S., and Harris, T. N. (1950) *Proc. Soc. Exptl. Biol. Med.* **74**, 142.
Hashim, S. A. (1953) *Stain Technol.* **28**, 27.
Heisenberg, W. (1930) "The Physical Principles of the Quantum Theory." Univ. Chicago Press, Chicago.
Henry, H., and Stacey, M. (1943) *Nature* **151**, 671.
Herrmann, H., Nicholas, J. S., and Boricious, J. K. (1950) *J Biol. Chem.* **184**, 321.
Hillary, B. B. (1939) *Botan. Gaz.* **101**, 276.
Hillary, B. B. (1940) *Nature* **146**, 335.
Hitchcock, C. H., and Ehrich, W. E. (1930) *Arch. Pathol.* **9**, 625.
Hogeboom, G. H. (1951) *Cancer Research* **11**, 1.
Howard, A., and Pelc, S. R. (1951) *Exptl. Cell Research* **2**, 178.
Itikawa, O., and Ogura, Y. (1954a) *Stain Technol.* **29**, 9.
Itikawa, O., and Ogura, Y. (1954b) *Stain Technol.* **29**, 13.
Johnson, B. K. (1952) *J. Quekett Microscop. Club* **3**, 392.
Johnson, T. B., and Clapp, S. H. (1908) *J. Biol. Chem.* **5**, 163.
Jones, A. S., Muggleton, P. M., and Stacey, M. (1950) *Nature* **166**, 650.
Jordan, J. H., and Heather, A. H. (1929) *Stain Technol.* **4**, 121.
Kaufmann, B. P., Gay, H., and McDonald, M. R. (1951) *Am. J. Botany* **38**, 268.
Kaufmann, B. P., McDonald, M. R., and Gay, H. (1951) *J. Cellular Comp. Physiol.* **38**, Suppl. 1, 171.
Kay, E. R. M., Simmons, H. S., and Dounce, A. L. (1952) *J. Am. Chem. Soc.* **74**, 1724.
Kelly, E G. (1939) *J. Biol. Chem.* **127**, 155.

Kirkpatrick, P. (1950) *Nature* **166**, 251.
Klein, E., Kurnick, N. B., and Klein, G. (1950) *Exptl. Cell Research* **1**, 127.
Klemperer, P., Gueft, B., and Lee, S. L. (1949) *J. Mt. Sinai Hosp. N.Y.* **6**, 61.
Klemperer, P., Gueft, B., Lee, S. L., Leuchtenberger, C., and Pollister, A. W. (1950) *Arch. Pathol.* **49**, 503.
Knaysi, G. (1946) *J. Bacteriol.* **51**, 113.
Koenig, H., and Stahlecker, H. (1952) *Proc. Soc. Exptl. Biol. Med.* **79**, 159.
Kohler, A. (1904) *Z. wiss. Mikroskop.* **21**, 129, 275.
Korson, R. (1951a) *J. Exptl. Med.* **93**, 121.
Korson, R. (1951b) *Stain Technol.* **26**, 265.
Kunitz, M. (1940) *J. Gen. Physiol.* **24**, 15.
Kunitz, M. (1946) *J. Biol. Chem.* **164**, 563.
Kunitz, M. (1950) *J. Gen. Physiol.* **33**, 349.
Kurnick, N. B. (1947) *Cold Spring Harbor Symposia Quant. Biol.* **12**, 141.
Kurnick, N. B. (1949) *1st Intern. Biochem. Congr., Cambridge, Engl., Abstr. Communs.* p. 264.
Kurnick, N. B. (1950a) *J. Gen. Physiol.* **33**, 243.
Kurnick, N. B. (1950b) *Exptl. Cell Research* **1**, 151.
Kurnick, N. B. (1950c) *Arch. Biochem.* **29**, 41.
Kurnick, N. B. (1952a) *Stain Technol.* **27**, 233.
Kurnick, N. B. (1952b) *Exptl. Cell Research* **3**, 649.
Kurnick, N. B. (1952c) *J. Natl. Cancer Inst.* **13**, 265.
Kurnick, N. B. (1953) *Arch. Biochem. and Biophys.* **43**, 97.
Kurnick, N. B. (1954) *J. Am. Chem. Soc.* **76**, 417.
Kurnick, N. B. (1955a) *J. Histochem. Cytochem.* **3**, # 4, in press.
Kurnick, N. B. (1955b) *Stain Technol.* **30**, in press.
Kurnick, N. B., and Foster, M. (1950) *J. Gen. Physiol.* **34**, 147.
Kurnick, N. B., and Herskowitz, I. H. (1952) *J. Cellular Comp. Physiol.* **39**, 281.
Kurnick, N. B., and Mirsky, A. E. (1947) Unpublished: quoted in Mirsky and Ris, 1951.
Kurnick, N. B., and Mirsky, A. E. (1950) *J. Gen. Physiol.* **33**, 243.
Kurnick, N B, Klein, E., and Klein, G. (1950) *Experientia* **6**, 152.
Kurnick, N. B., and Ris, H. (1948) *Stain Technol.* **23**, 17.
Kurnick, N. B., Pariser, S., Schwartz, L. I., Lee, S. L., and Irvine, W. (1952) *J. Clin. Invest.* **31**, 1036.
Kurnick, N. B., Schwartz, L. I., Pariser, S., and Lee, S. L. (1953) *J. Clin. Invest.* **32**, 193.
La Cour, L. (1941) *Stain Technol.* **16**, 169.
Lagerstedt, S. (1948) *Acta Haematol.* **5**, 212.
Lajtha, L. G. (1954) *Nature* **173**, 587.
Lamargene, P. (1936) *J. belge radiol.* **27**, 563.
Lange, P. W., and Engstrom, A. (1954) *Lab. Invest.* **3**, 116.
Larionov, L. T., and Brumberg, E. M. (1948) *Am. Rev. Soviet Med.* **5**, 111.
Laves, W., Thoma, K., and Oberdorfer, W. (1952) *Wien. klin. Wochschr.* **64**, 4.
Leblond, C. P., Stevens, C. E., and Bogoroch, R. (1948) *Science* **108**, 531.
Lessler, M. A. (1953) *Intern. Rev. Cytol.* **2**, 231.
Leuchtenberger, C. (1950) *Chromosoma* **3**, 449.
Leuchtenberger, C., Leuchtenberger, R., Vendrely, C., and Vendrely, R. (1952) *Exptl. Cell Research* **3**, 240.

Levene, D. (1901) *J. Med. Research* **6**, 135.
Lewis, W. H. (1951) *Ann. N.Y. Acad. Sci.* **51**, 1287.
Lhotka, J. F., and Davenport, H. A. (1951) *Stain Technol.* **26**, 35.
Li, C. F., and Stacey, M. (1949) *Nature* **163**, 538.
Linderstrøm-Lang, K., and Mogensen, K. R. (1938) *Compt. rend. trav. lab. Carlsberg, Sér. chim.* **23**, 27.
Loeser, C. N., and Berkley, C. (1954) *Science* **119**, 410.
Longley, J., and Lillie, R. D. (1951) *J. Natl. Cancer Inst.* **12**, 230.
Loring, H. S. (1942) *J. Gen. Physiol.* **25**, 497.
Loring, H. S., Carpenter, F. H., and Roll, P. M. (1947) *J. Biol. Chem.* **169**, 601.
Lowe, C. U., Williams, W. L., and Thomas, L. (1951) *Proc. Soc. Exptl. Biol. Med.* **78**, 818.
Ludford, R. J., Smiles, J., and Welsh, F. V. (1948) *Nature* **162**, 650.
Lumb, E. S. (1950) *Quart. Rev. Biol.* **25**, 278.
McDonald, M. R. (1948) *J. Gen. Physiol.* **32**, 39.
Mandel, P., Jacob, M., and Mandel, L. (1949) *Compt. rend.* **229**, 1370.
Marinone, G. (1953) Bibliotica Haematologica No. 16, Pavia.
Melander, Y., and Wingstrand, K. G. (1953) *Stain Technol.* **28**, 217.
Mellors, R. C. (1950) *Discussions Faraday Soc.* **9**, 398.
Mellors, R. C., Berger, R. E., and Streim, H. G. (1950) *Science* **111**, 627.
Mellors, R. C., Glassman, A., and Papanicolaou, G. M. (1952) *Cancer* **5**, 458.
Mellors, R. C., Kupfer, A., and Hollender, A. (1953) *Cancer Research* **6**, 372.
Meyer-Arendt, J., and Meyer-Arendt, E. (1953) *Z. wiss Mikroskop.* **61**, 354.
Michaelis, L. (1947) *Cold Spring Harbor Symposia Quant. Biol.* **12**, 131.
Micklewright, H. L., Kurnick, N. B., and Hodes, R. (1953) *Exptl. Cell Research* **4**, 151.
Mirsky, A. E., and Pollister, A. W. (1942) *Proc. Natl. Acad. Sci. (U.S.)* **28**, 344.
Mirsky, A. E., and Pollister, A. W. (1946) *J. Gen. Physiol.* **30**, 117.
Mirsky, A. E., and Ris, H. (1949) *Nature* **163**, 666.
Mirsky, A. E., and Ris, H. (1951) *J. Gen. Physiol.* **34**, 451.
Mitchell, J. S. (1942) *Brit. J. Exptl. Pathol.* **23**, 296.
Mitchell, P., and Moyle, J. (1950) *Nature* **166**, 218.
Mittwer, T., Bartholomew, J. W., and Kallman, B. J. (1950) *Stain Technol.* **25**, 169.
Moses, M. J. (1950) *J. Morphol.* **87**, 493.
Moses, M. J. (1951) *J. Natl. Cancer Inst.* **12**, 205.
Muntwyler, E., Seifter, S., and Harkins, D. M. (1950) *J. Biol. Chem.* **184**, 181.
Naora, H. (1951) *Science* **114**, 279.
Naora, H., and Sibatani, A. (1952) *Biochim. et Biophys. Acta* **9**, 582.
Norberg, B. (1943) *Acta Physiol. Scand.* **5**, Suppl. 14.
Novelli, A. (1954) *Nature* **173**, 691.
Ogur, M., and Rosen, G. (1949) *Federation Proc.* **8**, 234.
Opie, E. L., and Lavin, G. I. (1946) *J. Exptl. Med.* **84**, 107.
Ornstein, L. (1952) *Lab. Invest.* **1**, 250.
Oster, G., and Grimson, H. (1949) *Arch. Biochem.* **24**, 119.
Overend, W. G. (1950) *J. Chem. Soc.* p. 2769.
Overend, W. G., and Stacey, M. (1949) *Nature* **163**, 538.
Panijel, J. (1948) *Bull. soc. chim. biol.* **30**, 116.
Pappenheim, A. (1899) *Virchow's Arch. pathol. Anat. u. Physiol.* **157**, 18.
Patau, K. (1952) *Chromosoma* **5**, 341.

Pearse, A. G. E. (1951) *J. Clin. Pathol.* **4**, 1.
Pearse, A. G. E. (1953) "Histochemistry: Theoretical and Applied." Little, Brown, Boston.
Pease, D. C., and Baker, R. F. (1949) *Am. J. Anat.* **84**, 175.
Plaut, W. S. (1953) *Hereditas* **39**, 438.
Pollister, A. W. (1952) *Lab. Invest.* **1**, 231.
Pollister, A. W., and Leuchtenberger, C. (1949a) *Proc. Natl. Acad. Sci. (U.S.)* **35**, 66.
Pollister, A. W., and Leuchtenberger, C. (1949b) *Proc. Natl. Acad. Sci. (U.S.)* **35**, 111.
Pollister, A. W., and Ris, H. (1947) *Cold Spring Harbor Symposia Quant. Biol.* **12**, 147.
Pollister, A. W., and Swift, H. H. (1950) *Science* **111**, 68.
Proescher, F., and Arkush, A. S. (1928) *Stain Technol.* **3**, 28.
Rafalko, J. S. (1946) *Stain Technol.* **21**, 91.
Randall, J. T. (1950) *Discussions Faraday Soc.* **9**, 353.
Rapport, D., and Canzanelli, A. (1950) *Science* **112**, 469.
Rattenbury, J. A. (1952) *Stain Technol.* **27**, 113.
Lord Rayleigh (1871) *Phil. Mag.* **41**, 274.
Ris, H., and Mirsky, A. E. (1949a) *J. Gen. Physiol.* **32**, 489.
Ris, H., and Mirsky, A. E. (1949b) *J. Gen. Physiol.* **33**, 125.
Roe, M. A., Lillie, R. D., and Wilcox, H. (1940) *Public Health Repts. (U.S.)* **55**, 1272.
Sanders, F. K. (1946) *Quart. J. Microscop. Sci.* **87**, 203.
Sandritter, W., Diefenbach, H., and Krantz, F. (1954) *Experientia* **10**, 210.
Schneider, W. C. (1945) *J. Biol. Chem* **161**, 293.
Seeds, W. E. (1953) *Progr. Biophys. and Biophys. Chem.* **3**, 27.
Semmens, C. S. (1940) *Nature* **146**, 130.
Serra, J. A., and Queirez Lopes, A. (1945) *Portugaliae Acta Biol.* **1**, 111.
Seshachar, B. R. (1952) Presidential Address "The Biological Importance of the Nucleic Acids," 39th Indian Science Congress, Calcutta. Bangalore Press, Bangalore City.
Seshachar, B. R., and Flick, E. W. (1949) *Science* **110**, 659.
Setterfield, G., Schreiber, R., and Woodard, J. (1954) *Stain Technol.* **29**, 113.
Sharma, A. K. (1951) *Nature* **167**, 441.
Sharma, A. K. (1952) *Acta Biol.* **3**, 289.
Shurcliff, W. A. (1952) *Lab. Invest.* **1**, 123.
Sibatani, A. (1950) *Nature* **166**, 355.
Sibatani, A. (1952) *Cytologia (Tokyo)* **16**, 315.
Sibatani, A. (1953) *J. Biochem.* **40**, 119.
Sibatani, A., and Fukuda, M. (1953) *Biochim. et Biophys. Acta* **10**, 93.
Sibatani, A., and Matsuda, H. (1952) *Med. J. Osaka Univ.* **3**, 325.
Sibatani, A., and Naora, H. (1952) *Experientia* **8**, 268.
Snapper, I., Schneid, B., and Kurnick, N. B. (1950) *Acta Hematol.* **3**, 129.
Snapper, I., Schneid, B., Lieben, F., Gerber, I., and Greenspan, E. (1951) *J. Lab. Clin. Med.* **37**, 562.
Stacey, M. (1947) *Symposia Soc. Exptl. Biol.* **1**, 86.
Stearn, A. E., and Stearn, E. W. (1929) *Stain Technol.* **4**, 111.
Stedman, Ellen, and Stedman, E. (1943) *Nature* **152**, 267.

Stedman, Ellen, and Stedman, E. (1947) *Cold Spring Harbor Symposia Quant. Biol.* **12**, 224.
Stedman, Ellen, and Stedman, E. (1950) *Biochem. J.* **43**, 111.
Stenram, U. (1953) *Exptl. Cell Research* **4**, 383.
Stenram, U. (1954) *Acta Anat.* **20**, 36.
Stowell, R. E. (1945) *Stain Technol.* **20**, 45.
Stowell, R. E. (1946) *Stain Technol.* **21**, 137.
Stowell, R. E. (1948) *Arch. Pathol.* **46**, 164.
Stowell, R. E., and Zorzoli, A. (1947) *Stain Technol.* **22**, 151.
Sulkin, N. M. (1951) *Proc. Soc. Exptl. Biol. Med.* **78**, 32.
Sulkin, N. M., and Kuntz, A. (1950) *Proc. Soc. Exptl. Biol. Med.* **73**, 413.
Svensson, G. (1954) *Exptl. Cell Research* **6**, 529.
Swift, H. (1950a) *Physiol. Zool.* **23**, 169.
Swift, H. (1950b) *Proc. Natl. Acad. Sci. (U.S.)* **36**, 643.
Swift, H. (1953) *Intern. Rev. Cytol.* **2**, 1.
Taft, E. B. (1951a) *Exptl. Cell Research* **2**, 312.
Taft, E. B. (1951b) *Stain Technol.* **26**, 205.
Tamm, C., Hodges, M. E., and Chargaff, E. (1952) *J. Biol. Chem.* **195**, 49.
Thoma, K. (1950) *Acta Haematol.* **4**, 81.
Thomas, R. (1950) *Bull. soc. chim. biol.* **32**, 469.
Thomas, R. (1952) *Bull. soc. chim. biol.* **34**, 47.
Thomson, R. Y., and Frazer, S. C. (1954) *Exptl. Cell Research* **6**, 367.
Thorell, B., and Ruch, F. (1951) *Nature* **167**, 815.
Turchini, J. (1937) *Bull. histol. appl. physiol. et pathol. et tech. microscop.* **14**, 5.
Turchini, J. (1949) *Exptl. Cell Research* Suppl. 1, 105.
Turchini, J., Castel, P., and van Kien, L. K. (1944) *Bull. histol. appl. physiol. et pathol. et tech. microscop.* **21**, 124.
Turchini, J., Castel, P., and van Kien, L. K. (1948) *Compt. rend. soc. biol.* **142**, 1277.
Uber, F. M., and Goodspeed, T. H. (1935) *Botan. Gaz.* **97**, 418.
Unna, P. G. (1902) *Monatsh. prakt. Dermatol.* **35**, 76.
Vendrely-Randavel, C. (1949) *Compt. rend. soc. biol.* **143**, 228.
Vendrely, C., and Vendrely, R. (1949) *Compt. rend. soc. biol.* **143**, 1388.
Vercauteren, R. (1950a) *Bull. soc. chim. biol.* **32**, 343.
Vercauteren, R. (1950b) *Enzymologia* **14**, 134.
Vialli, M. (1953) *Biol. Revs.* **45**, 3.
Walker, P. M. B., and Davies, H. G. (1950) *Discussions Faraday Soc.* **9**, 461.
Walker, P. M. B., and Yates, H. B. (1952a) *Symposia Soc. Exptl. Biol.* **6**, 265.
Walker, P. M. B., and Yates, H. B. (1952b) *Proc. Roy. Soc. (London)* **B140**, 274.
Wenderoth, H. (1953) *Acta Haematol.* **9**, 47.
White, J. C. (1947) *J. Pathol. Bacteriol.* **59**, 223.
White, J. C. (1950a) *Biochem. J.* **46**, xxiv.
White, J. C. (1950b) *Biochem. J.* **47**, xvi.
Widstrom, G. (1928) *Biochem. Z.* **199**, 298.
Wieland, H., and Scheuing, G. (1921) *Ber.* **54**, 2527.
Wilkins, M. H. F. (1950) *Discussions Faraday Soc.* **9**, 363.
Wyckoff, R. W. G., Ebeling, H. H., and Terlaw, A. L. (1932) *J. Morphol.* **53**, 189.
Yakar, N. (1952) *Botan. Gaz.* **114**, 72.
Zamenhof, S., and Chargaff, E. (1949) *J. Biol. Chem.* **178**, 531.

Structure and Chemistry of Nucleoli

W. S. VINCENT

Department of Anatomy, Upstate Medical Center, State University of New York, Syracuse, New York

		Page
I.	Introduction	269
II.	The Cycle of the Nucleolus	271
III.	Interphase Morphology of the Nucleolus	272
	1. Inclusions	272
	2. Physical Nature of the Interphase Nucleolus	273
	3. The Structure of Telophase Nucleoli	274
	4. Nucleolar Changes during Metabolic Activity of Cells	275
	5. Experimental Modification of Nucleolar Morphology	276
	6. Theories of Nucleolar Structure	279
IV.	Chemistry of the Nucleolus	280
	1. The RNA Content of the Nucleolus	281
	2. Changes in Nucleolar RNA Content	283
	3. DNA Associated with Nucleoli	285
	4. Nucleolar Proteins	286
	5. Lipids	288
	6. Enzymes	288
	7. Mineral Constituents of Nucleoli	289
V.	The Origins of Nucleolar Materials	289
	1. Evidence from Studies of Telophase Reconstitution	289
	2. Evidence from Studies of Nucleolar Chemistry	290
	3. Genetic Evidence of Nucleolar Origin and Composition	291
	4. The Role of Nucleolonema	292
	5. The Role of Nuclear Sap in Nucleolar Origin	293
VI.	Nucleolar Function	293
VII.	References	295

I. Introduction

Since the time of its discovery the nucleolus has remained an enigmatic structure. Little has been added to our understanding of nucleolar function by the many observations of its morphology, its constant presence in the cell nucleus, the recognition of its cyclic disappearance and reappearance at mitosis, and its variations in size and staining characteristics. With the onset of the recent era of the application of qualitative and quantitative chemistry to tissue sections, details of nucleolar chemical morphology have been added to the earlier descriptions of structural anatomy of the nucleolus. The use of these techniques has added information regarding the presence of nucleic acids, proteins, enzymes, lipids, and minerals. It should be emphasized that the available information regarding the nucleolus

is essentially descriptive morphology, either structural or chemical, and that a "physiology" of the nucleolus has yet to be developed. Although conclusions as to function based strictly on morphological descriptions are valid, too often in histochemical literature descriptions of chemical morphology have been considered to be physiology and not anatomy, so that cause and effect have become either synonymous or confused.

According to Montgomery (1898) the nucleolus was first described as a nuclear structure by Fontana in 1781. From Montgomery's exhaustive review of the literature it is evident that the nucleolus was soon recognized as a typical cellular structure. Nucleoli are generally described as homogeneous, spheroid, intranuclear bodies which have an affinity for eosin in hematoxylin-eosin preparations, but are basophilic in basic dye solutions. However, the presence of vacuoles and inclusions of various kinds has been widely recognized. Studies of electron micrographs of thin sections of nucleoli have shown that the nucleolus contains two structural phases: one being essentially structureless, the other being a fine, highly coiled, stranded material. In addition the electron micrographs have revealed no indication of a limiting membrane around the nucleolus.

Heitz (1931a, b) pointed out that the nucleolus in plants was associated with a particular region of a given chromosome. This region was characterized by its lack of staining with the Feulgen reaction for desoxyribonucleic acid (DNA). McClintock (1934) refined this concept by pointing out that associated with the DNA-poor region was an organized body which was really responsible for the localization of the nucleolus at that particular point on the chromosome. She designated this structure to be the nucleolus organizer. In general, the concept of the nucleolus organizer has been extended to all tissues with little modification until recently, when the participation of all the chromosomes in the nucleus has been emphasized in the formation of the nucleolus, e.g. see De Robertis, Nowinski, and Saez (1954).

Observations on nucleolar size have led to the general conclusion that, in the interphase, the nucleolus becomes hypertrophied during anabolic activity of the cell, and conversely becomes smaller during drastic catabolic states. A number of experimental analyses have suggested that the nucleolus is composed of concentrated protein with variable amounts of ribonucleic acid (RNA). The nucleolus shows marked changes on exposure to hydrating ions, pH changes, and a number of organic drugs, as well as to ionizing radiations. All of these agents bring about swelling, fragmentation, and, finally, dissolution of the nucleolus.

A large portion of the information which will be reviewed below has been acquired by the application of histochemical techniques. The ease

and availability of techniques, as well as the popularity of certain compounds in current research endeavor often leads to the channeling of research effort into relatively narrow fields of exploitation. Also, there is the line of reasoning that because a nuclear structure contains a compound which is predominantly cytoplasmic in its distribution, then that nuclear structure must be functionally related to cytoplasmic activities. Both of these situations are exemplified in the literature on the nucleolus. The discovery of RNA in the nucleolus has led to a concentration of study and speculation on the role of this substance in nucleolar function.

Studies on the nucleolus during varying states of cellular activity have led to the general conclusion that its function is related in some manner to the synthetic processes of the cell. Because it contains RNA, which is widely found in the cytoplasm on the one hand and which resembles chromatin on the other, the nucleolus has usually been considered to act in some way as a mediator between genetic activity of the nucleus and the expression of those activities in the cytoplasm.

The following discussion will generally take the course of the brief outline of the nucleolus which has been given in the preceding paragraphs.

II. The Cycle of the Nucleolus

The sequence of nucleolar life history during a single mitotic cycle is as follows: Small bodies recognizable as nucleolar material appear between the chromosomes as the telophase nucleus begins to form. These bodies are generally described as forming adjacent to the specific nucleolar chromosome, although the positive identification of this chromosome is usually impossible in the telophase stage. With the reconstitution of the interphase nucleus, the typical number of nucleoli (i.e., the number which corresponds to the number of haploid chromosome sets) is generally evident. Throughout the interphase the nucleolus is typically spherical and, with normal function of the cell in which it is resident, it is reasonably constant in size and general morphology. During prophase, as the individual chromosomes become demonstrable again, the relationship of the nucleolus to a particular chromosome becomes evident. With the onset of metaphase, usually at the time of nuclear membrane breakdown, the nucleolus dissolves, or at least is no longer evident as a formed body. It then reappears following anaphase as described above.

Exceptions to this general picture have been noted and some are given below, but the above summary of the life cycle of the nucleolus can be considered as typical or classical.

III. Interphase Morphology of the Nucleolus

The nucleolus may vary considerably in morphology in different tissues as well as in different stages of cellular activity. The nucleoli of somatic cells are considered to be morphologically similar from tissue to tissue, whereas the nucleoli of egg cells, particularly, seem to differ widely. The large size of the nucleoli in egg cells may account for this variation, since the problems of observation become much simpler. Among the variations found in nucleoli are differences in size, shape, extent of associated heterochromatin, basophilia and acidophilia, and inclusions such as vacuoles, nucleolini, thread-like structures, and crystalloids.

1. Inclusions

The most prevalent inclusion is the vacuole. Such structures are characterized by their refractiveness to stains of all types. They are typical of oöcyte nucleoli (Montgomery, 1898; Jörgenson, 1913), and have been noticed in many somatic cells (e.g., maize, McClintock, 1934; bean root tips, Chayen, 1952; tissue culture nucleoli, Lewis, 1943; and many others). The reciprocal of vacuoles, which are inclusions of more intense staining capabilities and often more dense than the rest of the nucleolus, have also been described. These structures, which are called nucleolini or nucleololi, often remain visible in the cytoplasm after the rest of the nucleolus has disappeared at metaphase. No function can as yet be assigned to vacuoles or nucleolini.

A recently discovered nucleolar inclusion has been termed the nucleolonema (Estable and Sotelo, 1951, 1952). These authors have described strands of material which form a typical portion of the nucleolus and can be demonstrated with a special silver impregnation technique. At about the same time several investigators observed similar structures in electron micrographs of thin sections of nucleoli from mammalian cells. Bernhard, Hagenau, and Oberling (1952) described nucleoli of *apparance pelotonée* in a large number of mammalian tissues. Borysko and Bang (1951) have described coiled strands of material of greater electron opacity embedded in less opaque nucleolar material. Examination of published electron micrographs from many sources reveals a similar nucleolar structure (e.g., see Porter, 1954). Davies (1952) published ultraviolet photographs of living cells illustrating what he calls "grape-like structure" of the nucleolus which could be interpreted as nucleolonema. The nucleolonema does not appear to contain DNA, for it is not demonstrated by the Feulgen reaction. Estable and Sotelo (1951, 1952) have postulated a role for nucleolonema which will be discussed at length below.

Filamentous and granular material which is Feulgen-positive is also found in some instances in nucleoli. Mulnard (1949) and Panijel (1951) have described some typical occurrences of this type. The nature and role of such inclusions is not yet understood.

The nucleolus of animal cells usually possesses an associated body or layer of Feulgen-positive material, usually considered to be DNA. This DNA is supposedly homologous to the nucleolus organizer of plants and has been designated by Caspersson (1950) and co-workers as nucleolus associated heterochromatin. Caspersson considers it to be the actual site of synthesis of nucleolar materials. A more thorough discussion of the role of the nucleolus organizer and the nucleolus-associated heterochromatin will be given below.

Protein crystalloids in nucleoli have been described in both plant cells and in mammalian cells under certain abnormal conditions (see Gates, 1942; Hughes, 1952b). Their significance is not known at the present time.

2. *Physical Nature of the Interphase Nucleolus*

The interphase nucleolus is variously described as being a fluid or semi-solid body. In the giant salivary gland nuclei of *Chironomus,* for example, distortion of the nucleolus is often seen in the preparation of chromosome smears. Care should be used in the interpretation of the distortion of nucleoli in fixed preparations as evidence for the fluid nature of nucleoli. Duryee (1950) has reported on distortions of the nucleoli of amphibian oöcytes caused by some of the common fixing agents, although the very rigid nature of these nucleoli has been demonstrated by micromanipulative techniques. The author (unpublished data) has observed similar effects of fixing agents on starfish oöcyte nucleoli. However, the phenomenon of ameboid motion and nucleolar fusion has been reported many times (Montgomery, 1898; Gates, 1942), so that at times nucleoli must possess a fluid character.

The high density of the nucleolus is well known. Herrick (1895) reported that the nucleolus of lobster eggs would fall through the nuclear fluid under the influence of gravity. Later Gray (1927) and Harding (1949) studied this phenomenon more extensively, and the latter has calculated the viscosity of the nuclear fluid by measuring the rate of fall of the nucleolus through the nucleus of starfish oöcytes. When a wide variety of tissues are subjected to ultracentrifugation, the nucleolus comes to lie against the nuclear membrane at the centrifugal pole (Dornfeld, 1936; Lagerstedt, 1949). When starfish oöcytes are placed in sucrose solutions of appropriate density, the nucleolus may actually be centrifuged out through both the nuclear and cell membranes to form a satellite attached

by a fine, drawn-out strand of the membranes (Chambers, see comment by Lessler in discussion following paper by Pollister, 1952). The author has utilized the high density of the starfish oöcyte nucleolus in developing a technique for their isolation in quantity (Vincent, 1952). He found that the density of the isolated nucleoli was approximately matched by sucrose solutions having a density of 1.35. This value is somewhat misleading as the nucleoli are freely permeable to sucrose, and contain vacuoles which occupy up to 50% of the total nucleolar volume. The density value of 1.35 must therefore be that for the nonvacuolar portion of the nucleolus. Measurements of the dry matter content of isolated starfish nucleoli with the interference microscope of Dr. A. H. Huxley give values up to 90% dry matter (expressed as % protein) for nucleoli of 5 μ in diameter (Vincent and Huxley 1954). The values decrease linearly with increasing size to a value of 35% in the mature nucleolus of 20 μ in diameter. These values do not make allowance for the presence of vacuoles. As these vacuoles occupy up to 50% of the total nucleolar volume, and were found to contain negligible dry matter, the concentration in the nonvacuolar portions of the nucleolus must be of the order of 70% or greater. Pollister and Ris (1947), basing their conclusions on protein determinations with the cytophotometer, concluded that the nucleolus of maize contained in excess of 40% protein. Nurnberger, Engström, and Lindström (1952) reported the dry matter content of neuron nucleoli to be 70% and greater from their determinations with X-ray absorption and ultraviolet absorption. These data, based on a number of different techniques, and on several different tissues, suggest that the nucleolus is typically a solid or near-solid body containing proteins in a state of considerable dehydration.

3. *The Structure of Telophase Nucleoli*

The recent discovery of the nucleolonema necessitates a re-examination of the classical concepts of nucleolar origin. The persistence of nucleolar materials associated with metaphase and anaphase chromosomes was emphasized by Zirkle (1928). More recently Estable and Sotelo (1951), using a silver impregnation technique, and Rattenbury (1952), with a modification of the aceto-carmine procedure, have demonstrated what they consider to be nucleolar material in the metaphase and anaphase chromosomes. Estable and Sotelo have elaborated on their description of nucleolonema to develop a new concept of nucleolar formation which is explained fully in De Robertis *et al.* (1954, pp. 253-254). Essentially, these workers feel that the nucleolonema constitutes a permanent nucleolar structure associated with the chromosomes. At telophase, the nucleolonema loses its association with individual chromosomes and organizes into the basic nu-

cleolar structure. During interphase, the nucleolus accumulates additional materials, the *pars amorpha.* Serra and co-workers (Albuquerque and Serra, 1951; Rattenbury and Serra, 1952) have advanced a similar hypothesis of nucleolar formation except that they have not utilized the nucleolonema concept. Both of these groups ascribe to the nucleolus organizer region a "collecting" rather than a "synthesizing" function.

The origin of nucleolar materials on the surfaces of telophase chromosomes is well documented. Heitz (1931b) noticed small nucleoli in accessory nuclei which did not contain the organizer chromosome. Creighton (unpublished data, cited by McClintock, 1934) found that experimental removal of the organizer region from its chromosome resulted in the failure of a typical nucleolus to form, but she did find small nucleolar bodies on many of the other chromosomes of the complement. McClintock (1934) considered that her experiments demonstrated the origin of nucleolar materials from all the chromosomes of the complement. The recent elegant analyses of nucleolar formation in rat pronuclei by Austin (1951, 1952, 1953) have demonstrated that many (up to 20) nucleolar bodies form simultaneously in the pronucleus even though the chromosome complement is haploid and thus should have but a single organizer.

On the basis of these and other observations, the author considers that the following general statement of nucleolar formation in the recently divided cell can now be made: At the time of swelling of the chromosome vesicles in the telophase, nucleolar materials are found along the surface of the entire chromosome complement. These materials, with the enlarging and final formation of the interphase nucleus, aggregate and fuse at the site of the nucleolar organizer.

4. *Nucleolar Changes during Metabolic Activity of Cells*

A discussion of the changes in nucleolar morphology during metabolic activity of the cells is now pertinent. One morphological change which has often been cited is that nucleoli associated with cells in active synthesis become hypertrophied. Cells which are not actively synthetic have reduced nucleoli, or may appear to have no nucleoli whatsoever. For example, embryonic muscle cells have prominent nucleoli; the nucleoli of mature muscle cells are scarcely demonstrable. Starvation or feeding of protein-free diet may cause drastic reduction in nucleolar size in rat livers, with an immediate return to normal immediately after normal food intake is resumed (Lagerstedt, 1949; Stenram, 1953). Caspersson and Santesson (1947) found a significant increase in nucleolar size in epithelial cell tumors, although this hypertrophy is not necessarily a diagnostic criterion for malignancy. Oöcytes, which are producing large amounts of storage

protein, typically have very large or multiple nucleoli. On the other hand, the nucleoli of cleavage stages are small or absent (Brachet, 1950). [Exceptions to this statement are seen in the early cleavage stages of the rat and mouse (Austin, 1952; Dalcq, 1952)]. Nucleoli first became prominent in embryos at the time of tissue differentiation, presumably when the synthesis of new proteins is occurring. According to Ehrenburg (1946) extranormal environmental temperature ranges will cause changes in nucleolar size.

The effects of metabolic states of the tissue seem to have the following relationship to nucleolar size: Cells in *anabolic* activity usually possess or form large nucleoli, whereas the onset of *catabolic* activity results in the reversible diminution of nucleolar size.

A number of authors have described extrusions of nucleoli or nucleolar materials into the cytoplasm. The largest number of such observations have been made on fixed material, although Duryee (1950) has reported observing such phenomena in living amphibian oöcytes. Callan (1952), on the other hand, has not observed passage of nucleolar materials through the nuclear membrane of similar material. A detailed review of the literature on this phenomenon is given by Gates (1942). Recent reports on the extrusion of nucleolar material and its possible role in protein synthesis include those of Dodson (1953), and Srivastana (1952).

5. *Experimental Modification of Nucleolar Morphology*

The above changes in nucleolar morphology may be considered as normal or physiologic modifications of the nucleolus. A number of extraneous agents have been found to influence nucleolar morphology. Among these are hypo- and hypertonicity, pH, heat, numerous organic compounds, ionizing radiations, and various solutions of ions.

Duryee (1941, 1950) has described the effects of a number of ions on nucleoli of amphibian oöcytes. The primary effect of these agents is one of fragmentation and dissolution of the nucleoli. Gersch (1940), using isolated germinal vesicles of amphibians, studied the solubility of these nucleoli as a function of ionic concentration and of the state of oöcyte maturity. He found solubility of nucleoli in distilled water to increase with increasing age. The solubility of nucleoli in a large number of ionic solutions was also noted. The effect of distilled water differs from organism to organism. The nucleolus of the surf clam is immediately soluble in distilled water, whereas the contained nucleolinus is unaffected, (Vincent, unpublished data). The nucleolus of starfish oöcytes (*Asterias* sp.) is relatively unaffected by prolonged exposure to distilled water, as is the nucleolus of one of the spider crabs (Vincent, 1952).

Churney (1942) reported volume changes in the nucleoli of the oöcytes of *Arbacia* when exposed to hypotonic sea water, this change being reversible. Stockinger (1953) reported reversible swelling and alterations of nucleolar shape when living tissue culture cells were exposed to hypo- and and hypertonic media. Zollinger (1948) noted that nucleoli of tissue culture cells became dispersed when the culture was exposed to distilled water, whereas restoration to the normal medium brought about restitution of the nucleolus.

Anderson and Wilbur (1952) reported seemingly nonosmotic effects on nucleolar size when isolated nuclei were exposed to salt solutions of varying pH and tonicity. The author has studied the effect of controlled pH changes on isolated nucleoli of starfish (Vincent, 1952). The volume of these nucleoli is constant throughout the range of pH 0.1 to 7.6. At pH levels above this the nucleolus swells markedly, so that at pH 8.4 the volume had increased approximately 90%; pH levels higher than this result in the nucleolus becoming so hydrated as to be invisible. This swelling is reversible, and return to pH levels below 7.6 restored the normal size of the nucleolus. Actually, little morphological alteration was noted in nucleoli which were cycled three times through pH levels of 0.1 to 11.

A number of organic compounds affect the morphology of the nucleolus. Hughes (1952a) has found that a number of purine derivatives will cause fragmentation and dissolution of nucleoli in tissue culture cells. Adenosine, adenylic acid, ATP, benzimidazole, and guanosine have shown this effect. Again, restoration of the normal culture media brings about the return of apparently normal nucleoli. Acridine has been observed to cause abnormal vacuolization of chick culture cell nucleoli (Lasnitzki and Wilkinson, 1948). Some compounds have been found to cause hypertrophy of nucleoli (Rather, 1951), as does pilocarpine (Hisako and Stowell, 1951). Nitrogen mustards also cause nuclear and nucleolar hypertrophy (Bodenstein, 1947).

Physical agents which can affect nucleolar morphology are ionizing radiations and heat, with nucleoli seeming to be particularly susceptible to ultraviolet radiations. Carlson and McMaster (1951), in an extensive study of dose and wavelength effectiveness, have studied the ability of ultraviolet radiation to cause nucleolar fragmentation in grasshopper neuroblasts. The most effective wavelengths are in the 2537 to 2803 Å region; this is the area of maximum absorption of both nucleic acids and proteins. Harding (personal communication) has noted that the nucleoli of *Asterias* eggs are very sensitive to ultraviolet radiation and the author has confirmed this observation. 10 seconds irradiation from a G.E. germicidal lamp at 10 cm. will result in the dissolution of 99% of the nucleoli

of mature *Asterias* eggs. Intact nucleoli in egg brei, or isolated nucleoli in distilled water, are not affected by such doses of radiation. X-irradiation causes increased vacuolization of nucleoli. 1000 r. will cause vacuolization of amphibian oöcyte nucleoli according to Duryee (1949), and 50,000 r. brings about nucleolar swelling and fusion. Carlson and McMaster (1951) have found that the early stages of nucleolar formation are most susceptible to X-irradiation in grasshopper neuroblasts; 10,000 r. causes moderate changes in nucleolar morphology. The effect is not as extensive as ultraviolet irradiation. Carlson and McMaster have also shown that exposure of the grasshopper embryo to 44°C. for five minutes will cause immediate fragmentation of the nucleolus which spontaneously repairs after a few minutes. The actions of heat and ultraviolet are neither synergistic nor additive.

The effects of the various chemical and physical agents mentioned above can be divided into two general groups: those which effect changes in nucleolar morphology by direct action on the nucleolar structure, and those which affect the nucleolus through modifications of cell metabolism. Representative of the latter class are nitrogen mustards, thioacetamide, pilocarpine, changes in environmental temperature, and modification of dietary intake. Modification of ionic environment, through the addition of extraneous ions, hypo- or hypertonicity, distilled water, pH changes, and the purine derivatives, appear to have a more direct effect on the nucleolus.

Nitrogen mustards, purine derivatives, and ionizing radiations, all are agents which will cause physical damage to chromosomes and the spindle mechanism, as well as modifying nucleolar structure. The cellular effects of nitrogen mustards are quite extensive, but primarily they act by interfering with DNA synthesis (Bodenstein, 1947) and by blocking —SH groups of proteins (Barron, Nelson, and Ardao, 1948). Thus the effects of nitrogen mustards (i.e., hypertrophy) could be to halt cell division by interfering with DNA synthesis for chromosome multiplication, while other synthetic activities continue with the associated increase of nucleolar materials. The effect of purine derivatives on nucleoli is apparently quite different from their action on chromosomes; the nucleoli are affected extensively and immediately, whereas the delayed effect on chromosomes is normally considered to be due to interference with the normal nucleic acid metabolism (Duncan and Woods, 1953). The effects of ionizing radiations upon nucleoli are apparently both direct and indirect (e.g., Duryee, 1949). The observation that nucleoli are insensitive to ultraviolet light outside their normal milieu of nucleoplasm is added evidence for the assumption that the effect of ionizing radiations is mediated through the protoplasm.

The effect of the first group of agents given above seems to be one of modifying the ionic environment of the cell. Just how extensively these changes are reflected in the nucleus is not known. The experiments of Chambers (1949) and of Duryee (1941) have demonstrated that the nucleus has relatively little buffering capacity, and therefore slight changes in the ionic environment might bring about pH changes with a major effect on the nucleolus. Whether the modification of the ionic milieu acts by directly affecting nucleolar structure, or causes modifications indirectly by affecting nuclear pH, the response of the nucleolus is one of hydration, which results in fragmentation, swelling, and dissolution.

6. *Theories of Nucleolar Structure*

Although the stability of the nucleolar structure is precarious, the evidence given above allows the conclusion that the nucleolus is composed primarily of proteins in a state of relative dehydration. One of the principal questions of nucleolar morphology at this point then becomes: what is the nature of nucleolar organization which permits such a localized concentration of proteins?

One hypothesis of nucleolar structure is that it is of the nature of a complex coacervate. This was originally suggested by Bungenborg de Jong (1936). The phenomenon of complex coacervation is described as the mutual flocculation (in the form of droplets) of two oppositely charged colloidal solutions in such a manner that mutual discharge does not occur. Such systems are extremely sensitive to ionic concentration. Bungenborg de Jong has pictured spontaneously produced structures resembling nucleoli in coacervate systems consisting of colloidal mixtures of negatively charged gelatin, serum albumin, or egg albumin with positively charged colloidal solutions of gum arabic or nucleates. The concept of nucleoli as complex coacervates has been vigorously advanced by Duryee (1941, 1950), and Ehrenburg (1946) has concluded that the effects of temperature on the nucleoli of *Salix* confirm this concept. Hyden (1943) concluded that nucleoli were of a coacervate nature from his studies of the effects of cyanide on *Micrasterias* nucleoli.

One of the major objections to the coacervate hypothesis is the high protein content of the nucleolus. Dervichian (1949) concludes that coacervates rarely exist where the solid phases reach a concentration of greater than 15% and are stable down to concentrations of 3 to 4%. In addition he has demonstrated that an excess of one phase normally results in the failure of the coacervate to form. Thus, until it can be demonstrated that coacervates will form with solutions of protein up to 60 and 70% in the presence of less than 5% nucleic acid, the conditions in the nucleolus are not applicable to our current knowledge of complex coacervates.

The author feels that the following suggestion for nucleolar structure is more plausible. Danielli (1949, 1953) has advanced the hypothesis that one of the functions of RNA would be to trap products of protein synthesis. It seems not unlikely that the nucleolus could be formed by the precipitating action of nucleic acid on proteins. This action would be presumed to bring about a dehydration of the protein which would allow the nucleolus to accumulate through inter-protein bonds, as well as protein-nucleate bonds. The possibility that calcium acts as a binding agent in nucleoli has been suggested by the author (1952). Other intermolecular binding groups are suggested by the presence of extensive —SH and —SS— groups in nucleoli (Brachet, 1950; unpublished data of the author).

IV. Chemistry of the Nucleolus

The largest portion of the available information on the chemical morphology of the nucleolus has been gained from the application of histochemical techniques. Some of these techniques are well established and the results obtained from their application are reliable, whereas other procedures, unless proper precaution is taken in performing the technique and there is suitable caution taken in interpretation, can yield misleading information. (For discussions on evaluation of histochemical procedures see Danielli, 1953; Glick, 1949, 1953; Gomori, 1952; Pearse, 1953). As the largest number of observations on nucleoli have been made incidental to some other main line of investigation, the findings are mostly qualitative. Where quantitative measurements of the chemical constituents of the nucleolus have been made, usually with histochemical techniques, the results are subject to critical analysis on both theoretical and empirical bases. The most satisfactory approach to nucleolar chemistry would seem to be a combination of histochemical analysis of tissue sections with direct analysis of isolated nucleoli. An attempt at such a correlative attack on the nucleolus was begun by the author in the laboratory of Brachet in Brussels, Belgium in 1950, and is presently being continued both in Brussels and in the laboratory of the author. The results of such an approach are of course subject to the same criticisms as mentioned above, and in addition, to the uncertainty of the integrity of the preparations of isolated nucleoli.

The first generalized attempt to derive the chemical nature of the nucleolus is found in the work of Montgomery (1898) in which he used a sort of intuitive cytochemistry based on a cataloguing of the differential staining of nucleoli and chromatin. He concluded that "the true nucleolar substance probably has no chemical relation to the true chromatin (nuclein)", (p. 498). Recognizing that Montgomery's dye mixtures were staining essentially protein, the data to be discussed below prove him to be quite

accurate in his conclusion. Jörgenson in 1913 then performed the first scientific analysis of nucleolar chemistry, within, of course, the limits of the techniques and chemistry of the time. Actually, the considerations of these authors include nearly every concept of nucleolar function which has been advanced.

1. The RNA Content of the Nucleolus

The basophilic properties of the nucleolus were puzzling to early cytologists. The experiments of Miescher had led to the general recognition that chromatin was synonymous with deoxyribonucleic acid (DNA) and while the staining properties of nucleoli were different in many ways, the intimate association between nucleoli and chromosomes led to the suggestion that nucleoli were the source of the chromatin material (see Wilson, 1928, pp. 91 ff.). With the recognition that the Feulgen reagent was specific for DNA (see Shinke and Shigenaga, 1933; Bauer, 1933), the probability that nucleolus was the source of chromatin became less likely, and the nucleolus began to figure more prominently as a possible intermediary in the transfer of chromosomal influences to the cytoplasm.

In 1940 Caspersson and Schultz observed that whereas the nucleolus had a nucleic acid absorption spectrum, it did not give a positive Feulgen reaction and therefore concluded that the nucleolus and cytoplasm contained ribonucleic acid (RNA). Independently, Brachet (1940) reported that the pyroninophilia of oöcyte cytoplasm and nucleoli could be removed with a preparation of ribonuclease, and likewise concluded that nucleolar and cytoplasmic basophilia were due to the presence of RNA. Since ribonuclease from commercial sources has become generally available (about 1946) reports of RNA in nucleoli far too numerous to mention here have been published, and to the author's knowledge, no claims for the absence of RNA from nucleoli have been made. Complementary, but probably less specific, techniques for the demonstration of RNA in tissue sections have involved the use of perchloric acid (Erickson, Sax, and Ogur, 1949) and HCl (Vendrely and Lepardy, 1946). The author (Vincent, 1952) found ribose as well as uracil to be present in hydrolysates of RNA extracts of isolated starfish oöcyte nucleoli by chromatographic analysis, thus demonstrating that the nucleic acid of these nucleoli, at least, contains a ribose nucleic acid.

The quantity of RNA in nucleoli in terms of actual concentration or amount has been estimated in only a few instances. Caspersson and Schultz (1940) concluded that the absorption spectrum of female *Drosophila* salivary gland nucleoli was indicative of a nucleoprotein containing about 3.5% nucleic acid, whereas the male salivary gland nucleolus contained

"about twice the percentage of nucleic acid." Nurnberger, Engström and Lindström (1952) reported values of about 2% RNA for cat axon nucleoli. The author found values of 2.4 to 4.6% RNA in different preparations of isolated starfish nucleoli. Baltus (personal communication) has found slightly more RNA (about 5%) in starfish nucleoli isolated in sucrose solutions.

Measurements of the ultraviolet absorption of nucleotides in isolated and *in situ* starfish nucleoli by Errera and Vincent (unpublished data) gave nucleic acid values ranging from 8% in nucleoli of 10 μ in diameter (found in oöcytes of about ⅕ mature size) to 1.7% in nucleoli of 25 μ in diameter (from mature oöcytes). The discrepancy between the values obtained from microabsorption measurements and actual determinations on extracts of isolated nucleoli can be explained in part as follows: the mature nucleoli possess extensive vacuoles; measurements on a few suitable nucleoli indicate that the volume of the vacuoles of the mature nucleolus approaches 50% of the total nucleolar volume. As the ultraviolet absorption of these vacuoles was found to be negligble, the calculated concentration of nucleic acid in the mature nucleolus should be approximately doubled; this brings the value within the same range as that found by chemical analysis.

Litt, Monty, and Dounce (1952) have reported the analysis of preparations of rat liver nucleoli in which they find 15% DNA and a few per cent RNA. In as much as the DNA content of nucleoli is conspicuously low, as evidenced by the Feulgen reaction, the preparations of these workers may be contaminated with blocks of heterochromatin or chromosomes.

From these data, it is evident that the RNA content of the nucleolus is rarely greater than 5% and probably considerably less than that value in most cells. This value can be compared with reported values for RNA content of other cell organelles: average value for nuclei of mammalian tissues, 1% (Pollister, 1952); recent analyses of mammalian nuclei isolated by nonaqueous techniques, up to 40% (Swift, 1953); isolated chromosomes 1.2–2.4% (Mirsky and Ris, 1947); isolated mitochondria and microsomes, 9–16% (Barnum and Huseby, 1948). Whole tissue concentrations range from 0.9% in brain tissue to 9% in pancreas (Davidson, 1947). Thus, in spite of the general assertions the the nucleolus is rich in ribonucleotides, it appears to contain a proportion of RNA of the same order as other cell organelles, possibly even less than the nucleus as a whole, and certainly less than the microsomes.

A point regarding nuclear localization of RNA should be made here. That the nucleoli contain all of the nuclear RNA has been a general assumption (see, for example, Tyner, Heidelberger, and LePage, 1953;

Elson and Chargaff, 1951). The data of Stowell (1949) and Lagerstedt (1949) clearly demonstrate that in rat nuclei the volume of the nucleoli rarely exceeds 1% of the nuclear volume; thus even if the nucleoli were solid, dry RNA, they could not contain in excess of 2% dry weight of the nucleus. Obviously, RNA in the nucleus must be found in other parts of the nucleus besides the nucleolus (see Jacobson and Webb, 1952).

Although the value of 3.5% for RNA concentration in the nucleolus was given by Caspersson and Schultz in 1940, numerous statements that the nucleolus contains high concentrations of RNA have been made since that time. The basis for such claims has been primarily a subjective comparison of the intensity of staining of the nucleolus with another associated cellular structure or area. Such a comparison can lead to considerable error, for the properties of basophilic stains used in such comparisons are often not clearly defined, and unless such variables as pH, staining time, and fixation are adequately controlled, many other substances in addition to nucleic acid will be stained to a greater or lesser degree. In addition, the stoichiometry of the basic dye-phosphoric acid reaction has not been established. Thus basophilia of the same real intensity in nucleoli of a nerve cell and of a pancreas cell might be subjectively classified "high" and "low" respectively, although no real difference exists. Such statements, without a scale of reference, have led to subjective estimates of nucleolar RNA content as high as 20–30% (Serra, 1948), when the true value is probably one-tenth of that concentration (see above).

2. *Changes in Nucleolar RNA Content*

The results of Errera and Vincent mentioned above demonstrate that with increasing age and growth of the starfish oöcyte nucleolus the RNA concentration decreases (although the actual amount of RNA continues to increase). Actually, the RNA content (not concentration) is directly proportional to the surface area of the nucleolus. The import of these relationships to cell metabolism has not been determined. As mentioned, the *Drosophila* salivary gland nucleolus RNA content seems to be related to the sex of the animal. Schultz, Caspersson, and Acquilonius, (1940) have investigated other variations in genetic makeup of *Drosophila* in relation to nucleolar RNA and protein. They found that different strains, as well as differing chromosomal arrangements, would modify the nucleic acid-protein proportion in the salivary gland nucleolus. In addition they found that temperature of incubation would influence the nucleic acid-protein ratio. Thorell (1947) indicates that nucleolar RNA decreases with maturation of erythropoietic cells. This occurs with a similar decrease in cytoplasmic RNA. Caspersson and Santesson (1942) found an ex-

tensive increase in nucleolar volume and nucleic acid in tumor cells, and Caspersson (1950) has summarized a considerable body of evidence collected by him and his co-workers which he interprets as indicating that a positive correlation exists between nucleolar volume, RNA, and cytoplasmic protein synthesis. According to Caspersson, this correlation indicates an intimate association between the nucleolus, RNA, and protein synthesis.

Experimental modification of metabolic activity with relation to the nucleolus has not been studied extensively with quantitative techniques. Stowell (1949) found that prolonged starvation increased nucleolar volume considerably, but ultraviolet absorption analysis did not reveal any significant change in nucleic acid. Lagerstedt (1949) found that starvation for periods of up to 18 days, as well as low-protein diets, brought about an abrupt decrease in nucleolar volume which returned to normal within a few hours after normal feeding was resumed. He did not report changes in nucleic acid. A few studies have been made which suggest something of the nature of the RNA of the nucleolus. There is evidence that the nucleolus contains two types of RNA, one of which is fairly soluble in dilute acids or similar media, the other of which is more tightly bound into the structural framework of the nucleolus. Pollister and Leuchtenberger (1949) found that about 40% of the ultraviolet absorption due to nucleotides in maize nucleoli was removed by extraction with cold, dilute trichloracetic acid; the remainder of the nucleotide was resistant to this agent, but could be extracted in hot trichloracetic acid. They considered that the soluble material was a polynucleotide of undetermined size. The data of Errera and Vincent (unpublished) indicate that isolated starfish nucleoli contain less nucleotide than *in situ* ones of the same size and stage of development, the difference between absorption of isolated and *in situ* nucleoli being proportional to the total amount of absorbing material present during the first half of nucleolar enlargement. During the last half of the period of nucleolar growth, however, the difference between *in situ* and isolated nucleoli remained constant, although the total quantity of nucleotide increased markedly during this period. These data were interpreted as evidence for the presence of a soluble nucleotide which was lost during isolation of the nucleolus.

Several workers have recently investigated the incorporation of radioisotopes into nucleoli, primarily the RNA fraction. Stich and Hämmerling (1953) and Taylor (1953) have reported that the nucleoli of *Acetabularia* and *Drosophila,* respectively, accumulate P^{32} more rapidly in nucleolar RNA than in any other cellular component. Ficq (1953) reported similar results for the nucleoli of starfish oöcytes using C^{14}. This worker has

used semiquantitative grain counts on radioautograph preparations in her studies. The author has recently completed preliminary experiments with P^{32} on starfish oöcyte nucleoli which essentially confirms the work of Ficq. In addition the author (Vincent, 1954) has measured the P^{32} uptake of nucleolar fractions directly by isolating nucleoli from starfish ovaries exposed to P^{32} solutions. When the specific activity of several phosphorus fractions was then determined, the RNA of the nucleolus was found to have a specific activity of less than that of the cytoplasmic RNA, and only one half to one fourth the activity of the nucleolus as a whole. The discrepancy between these results and those obtained by radioautography could be explained by the loss of a highly active nucleotide component in the isolation procedure. Evidence for such loss has been presented previously, and the results from P^{32} studies tend to confirm the previous conclusions. In addition, these results substantiate the conclusion that the nucleolus contains two types of ribonucleotides: one a soluble, metabolically "labile" form; the other a more tightly bound "residual" fraction.

There are numerous data which indicate that the nuclear RNA has a greater "turnover" rate than other cellular fractions (Marshak and Calvet, 1949; Jeener and Szafarz, 1950; Barnum, Huseby, and Vermund, 1953). Incorporation into cytoplasmic nucleotides then occurs at a slower rate. The data gained from radioautograph studies of nucleoli are in line with the gross analyses. Thus one might conclude, as have Marshak and Calvet, that the data indicate that precursors of cytoplasmic RNA are formed in the nucleus (or nucleolus). Barnum *et al.*, have made extensive mathematical analyses of the time relationships involved and have concluded that in their material, cytoplasmic RNA could not have been formed from nuclear precursors. In addition, as the recent compilation by Elson and Chargaff (1954) indicates, there are very great differences in the purine and pyrimidine base relationships of nuclear and cytoplasmic RNA preparations. These data make it difficult to conceive of the nucleus supplying cytoplasmic ribonucleotides in very large polynucleotide fragments. The relationship of the labile RNA fraction of nucleoli needs to be investigated further in this regard, for as yet we have no knowledge of its composition.

3. DNA Associated with Nucleoli

As was mentioned in Section III, 1, DNA-containing materials are occasionally found in or associated with nucleoli. This has generally been interpreted as being chromosomal in origin. In addition Brachet (1950) had noted Feulgen-positive granules in amphibian oöcyte nucleoli, and Panijel (1951) has reported similar granules in the oöcyte nucleoli of *Ascaris* as well as amphibians. (For a discussion of other authors report-

ing Feulgen-positive granules in nucleoli, see Panijel, pp. 59-60). Most of these authors have considered such a demonstration as evidence for nucleolar conversion of RNA to DNA. Painter and Taylor (1942) and Painter (1945) came to similar conclusions in studies of oöcyte nuclei. The significance of these Feulgen-positive granules is not yet clearly understood. The author has never observed them in correctly prepared Feulgen preparations, but the workers who have reported such granules are fully aware of necessary controls in applying this technique so that their results can scarcely be criticized on this basis.

Other DNA associated with the nucleolus is that designated the "nucleolus—associated heterochromatin." This designation for an accumulation of DNA in the region of the nucleolus organizer is particularly evident in *Drosophila* and mammals. It has been considered as the site of nucleolar genes by Schultz *et. al.*, (1940), and in this role has assumed a place of considerable importance in the work of the Caspersson school. Rattenbury and Serra (1952) have argued strongly against the concept of nucleolar origin from the heterochromatin on the basis that heterochromatic areas are not typical of nucleolar organizing regions. Other objections to the concept of "nucleolar genes" in the associated heterochromatin are given below. In most mammalian cells the heterochromatin forms a dense "shell" about the nucleolus which may interfere with observations of basophilia as well as ultraviolet absorption measurements.

4. *Nucleolar Proteins*

A considerable portion of the quantitative data on nucleolar nucleic acids has been related to protein content. This relationship has been established by assuming that the ultraviolet absorption at 260 mμ and 280 mμ are due to nucleic acids and proteins, respectively; the reasoning behind such an assumption is adequately given by Caspersson (1950). In addition to ultraviolet absorption, nucleolar protein content has been determined by X-ray absorption (Nurnberger *et al.*, 1952); by interference microscopy (Vincent and Huxley, 1954); and by microphotometry of the Millon reaction (Pollister and Ris, 1948), of the bromophenol blue reaction (Vincent and Huxley), and of fast green (Schrader and Leuchtenberger, 1950). Differentiation of the types of protein present in nucleoli has been attempted by analysis of ultraviolet absorption spectra, acid dye binding, and specific reactions for amino acids.

We have already mentioned (section III, 2) that the density of the nucleolus is very high, and have given the reasoning for the assumption that the largest constituent of the nucleolus is protein. Actual values referable to protein in the nucleolus range from 40 to 90% and greater (See

Pollister and Ris, 1947; Nurnberger *et al.*, 1952; Vincent and Huxley, 1954; Vincent, 1952). Although agreement as to the quantity of protein in the nucleolus is satisfactory, there are contradictory claims as to the kinds of proteins present in the nucleolus. Caspersson (1950) has defined the nucleolus as "a body rich in diamino acids;" Serra and co-workers (Serra, 1948; Serra and Lopez, 1944; 1945a, b, c), have emphasized the presence of basic proteins, but have also considered that nonbasic proteins were present; Pollister and Ris (1947), and Vincent (1952) could not find basic proteins in the nucleoli of maize and starfish respectively. Caspersson and his co-workers (Caspersson, 1950) have considered that the nucleolus is rich in diamino acids because of a shift in the nucleic acid peak which is found in their microabsorption curves. The physical reasons for such a shift have not been adequately explained (Caspersson, 1947), but in cases where tissues were rich in diamino acids, the shift was consistently found. A confirming procedure for the demonstration of basic proteins has been the use of acid dyes at very low pH, the assumption being that nearly all amino groups become available for dye binding. In addition, the specific arginine reaction of Sakaguchi has been applied to nucleoli by Serra and Lopez (1944) with the conclusion that the nucleolus is rich in basic protein.

These workers have concluded that because many amino groups can be demonstrated, this is evidence for the presence of a protein containing an excess of diamino acids, therefore a basic protein. This need not be so in the case of the nucleolus. For as is shown above, the nucleolus is concentrated protein, and therefore the number of amino groups present *per unit volume* will be great regardless of the type of protein involved. For example, one would expect that same intensity of color from the Sakaguchi reaction applied to a 5% solution of protein containing 50% arginine (as is found in some histones) as from a 50% solution of a protein containing 5% arginine (as casein or egg albumin). The problem of protein concentration has been largely ignored in interpreting the staining reactions for amino acids, particularly where large variations in concentration of protein as well as the constituent amino acids may occur. Whether protein concentration is in any way related to the spectral shift noted by the Stockholm group is not known, but at present the evidence for the presence of more than a few per cent of the total mass of the nucleolus as basic protein is not convincing.

A number of workers have concluded that nucleoli contain largely "acid" proteins. The author found that the major structural component of starfish oöcyte nucleoli was a protein rich in glutamic acid, poor in basic proteins and containing about 1% of tightly bound phosphorus. Panijel (1950,

1951) concluded that *Ascaris* nucleoli had a similar composition. Stockinger (1953) concludes that the nucleoli of his tissue culture cells contain albumin-like protein. Caspersson and Schultz (1940) conclude that the ultraviolet absorpion spectrum of female *Drosophila* salivary gland nucleoli is best fitted by RNA and an albumin type protein. It may be that nucleoli contain varying amounts of basic proteins, depending on the metabolic activities of the cell at the moment. A competitive role for histone and RNA in this regard has been suggested by Danielli (1953). It is evident that further study of the nucleolar proteins is necessary before the presence and role of basic proteins is satisfactorily settled.

5. *Lipids*

The presence of lipoidal materials in the nucleolus has been inferred from the action of certain lipid solvents on nucleolar structure (Gates, 1942). However, very few instances of positive identification of lipoidal material in nucleoli have been reported (Gates, 1942; Serra, 1948; Albuquerque and Serra, 1951; Rattenbury, 1952). Serra and co-workers have reported the successful application of the Smith-Dietrick test, and BZL blue to nucleoli after appropriate pretreatment. They conclude that the nucleolar lipids are masked as phospholipids. Although the author was unable to demonstrate significant amounts of lipid in isolated nucleoli, the extreme refractiveness of phospholipid to extraction has been established. The results of Immers (1954) could be due to the presence of phospholipid, as well as other phosphate esters, in sea urchin nucleoli. Nuclei are known to contain considerable quantities of lipoidal material but whether this is typical of nucleoli is not yet established.

6. *Enzymes*

Relatively few studies of the enzymes in nucleoli have been reported. Alkaline phosphatase has been widely accepted as a nucleolar constituent (Danielli, 1953; Bradfield, 1951), and in general the nucleolus is one of the most intensely stained areas in a phosphatase preparation. The ability of the nucleolus to split a number of phosphate esters has been demonstrated by this technique. However, the problem of nuclear specificity for phosphatase reactions is of major concern (see Gomori, 1952). With respect to the nucleolus, the following facts are available: Tandler (1953) found that the nucleoli of several tissues have an affinity for the divalent ions used in the phosphatase reactions, and thus will give a positive reaction irrespective of the amount of enzyme present. The author has found that starfish oöcyte nucleoli give intense alkaline phosphatase reactions in tissue sections, but that no activity could be detected for this enzyme on

direct analysis of isolated nucleoli. Obviously, the problem of nucleolar phosphatase is by no means exhausted. Baltus (personal communication) has found nucleotide phosphorylase activity in preparations of isolated starfish nucleoli, but beyond this, other than the doubtful occurrence of acid phosphatase, no other enzymes of nucleoli are known. Vincent (1952) was unable to demonstrate dipeptidase, DPN reductase, or methylene blue reduction in starfish oöcyte nucleoli preparations. Recently Roth and Vincent (unpublished data) could not detect ribonuclease activity in similar preparations. Of course, the absence of enzymatic activity in preparations of isolated cellular components is by no means proof of their absence from the structure in the intact cell. At this time, however, the above studies constitute the only available evidence on nucleolar activities with regard to enzymes.

7. *Mineral Constituents of Nucleoli*

The presence of phosphate in nucleoli has been already indicated as a component of nucleic acids. In addition, phosphate may be present as a component of protein and as phospholipid (Vincent, 1952; Immers, 1954). As mentioned above, the presence of sulfur in sulfhydryl and disulfide groups has also been reported. Sulfate, in the form of acid polysaccharide ester, has been reported in clam nucleoli by Thomas (1954), but this has not been confirmed by the author on the same form, nor by Immers on the sea urchin nucleoli. Gersch (1940) applied tests for a number of minerals to amphibian oöcyte nucleoli, but could not demonstrate potassium or iron. The effects of certain chelating agents on nucleoli have led the author to suggest that calcium is present in nucleoli, although Shanklin (unpublished data) was unable to detect any calcium in a preparation of isolated starfish nucleoli even through his analytical procedure was sensitive to a few hundredths of 1% of this element. Marza, Marza, and Guthrie (1937) found calcium and potassium but no iron in *Fundulus* nucleoli.

V. The Origins of Nucleolar Materials

1. *Evidence from Studies on Telophase Reconstitution*

An examination of the data reviewed above suggests that the nucleolar materials may have a different origin during the time of telophase reconstitution of the nucleolus than during interphase. The observations on nucleolar formation at telophase seem to be extensive enough to permit the generalization that nucleolar materials appear on the surfaces of the entire chromosome complement and then are collected or accumulate at the site of the nucleolus organizer. At the present time there are few observations

pertaining to the chemical nature of the newly formed nucleolar material, although the available evidence (e.g. Austin, 1953) suggests that it is not much different from the composition of interphase nucleoli. The accumulation of nucleolar materials at the organizer site appears to be due to a process similar to that found when interphasic nucleoli recover after being dispersed by a number of different physical agents. It is therefore likely that the telophase and interphase nucleoli are of similar composition.

A considerable body of information on the quantitative relations of the nucleolus and nucleus of the fertilized rat egg has been accumulated by Austin and his co-workers (Austin, 1951, 1952, 1953; Austin and Braden, 1953; Braden and Austin, 1953). Of great interest is some of their information on the rate of formation of nucleolar materials in the pronucleus. For example, Austin has found that the volume of the nucleolus attains a volume as great as, or greater than the original pronucleus in less than six hours. Nucleolar volume exceeds 10% of the pronuclear volume in the early stages of pronuclear growth, and during the later stages maintains approximately that level. Fusion of the many nucleoli in the pronucleus occurs for the simple reason that the volume increase of the nucleoli in early stages is so great that multiple nucleoli cannot exist without touching each other. Obviously this system is one of very rapid accumulation of nucleolar materials.

As the photographs published by Austin (1953) indicate that in comparison with the associated heterochromatin the nucleoli have a very low ultraviolet absorption at 260 mμ, it seems likely that the nucleic acid content is not great. On the other hand, the accompanying phase contrast photographs indicate a very high density of the nucleoli in contrast with other cellular components. On this basis it seems safe to assume that the structure of the rat pronucleus nucleoli is the same as other nucleoli and predominantly contains proteins. Thus the size increase noted by Austin must be due to an extremely rapid accumulation of proteins in the nucleolus. These proteins must certainly have come from the egg cytoplasm, for within six hours after fertilization the nucleolus has attained a volume some tens of times greater than that of the original sperm. The advantages in the use of this material for the study of the origin of nucleolar materials are obvious.

2. *Evidence from Studies of Nucleolar Chemistry*

It is obvious, of course, that at least some of the nucleolar materials must have at some time come through or from the cytoplasm, so the problem of the extent of nuclear modifications before incorporation into the nucleolus becomes the important one. That at least one of the nucleolar

components differs from the cytoplasm has been shown by the author in the analysis of the relative base content of RNA from isolated nucleoli and from egg cytoplasmic granules. In the case of the starfish the nucleolar RNA was shown to contain significantly more guanine and significantly less uracil than the cytoplasmic RNA. That nuclear and cytoplasmic RNA differ has been shown numerous times (Elson and Chargaff, 1954), but whether nucleolar and nuclear RNA differ has not yet been established. As pointed out above, the data do not favor the concept of direct precursor activity for nuclear ribonucleic acid, so neither does the reverse situation hold in this case. One must assume that nucleolar nucleic acids are manufactured in the nucleus and not in the cytoplasm. As yet there is no information on the comparative nature of nucleolar and cytoplasmic proteins, so that no conclusions can be drawn as to whether they likewise are manufactured or at least modified in the nucleus.

3. *Genetic Evidence of Nucleolar Origin and Composition*

Schultz, Caspersson, and Acquilonius (1940) present evidence which they have interpreted as demonstrating that the chemical composition of the nucleolus is under the control of "nucleolar genes" localized in the heterochromatic regions of the chromosome associated with the nucleolus. They have based their conclusions on the analysis of absorption curves of the salivary gland nucleoli of 9 *Drosophila* larvae, each of which was from a different culture and of different genetic constitution. In addition, 4 of the larvae were raised at 25° C., 2 at 18° C., 1 at 16° C., and 1 at 20–22°C., the temperature at which the remaining larva was grown cannot be determined from the data given. According to the authors the data were collected from preparations which had been made for other purposes. There are a number of variables in the experiment that have been shown to affect nucleic acid content and nucleolar size, for which controls are not given. Among these are: (1) age and stage of maturity of cells and larvae measured, (2) dietary intake of the larvae; and (3) environmental temperature. Since each of the 9 larvae measured had differing genetic constitution they must have been reared in different cultures, and as no measurements on culture mates are reported, adequate controls as to dietary intake and larval age are not found in the data cited. The authors state that the animals reared at lower temperatures had increased nucleic acid to protein ratio; this is the result that one would expect from immature or metabolically less active cells. Both of these conditions could derive from lowered environmental temperature. Lowered temperature would be expected to complicate or to be involved in the other two variables mentioned as well. In addition, the ultraviolet absorption curves given by the authors

show no correction for nonspecific light loss. This is apparent in that nearly every curve which shows a high absorption at 260 mμ also shows a high extinction at 310 mμ. According to Caspersson (1950) the extinction at 310 mμ is due to nonspecific causes and is directly related to the nonspecific light loss at 260 mμ. It is questionable whether such a correction would change appreciably the data of these authors, but this is a variable which should have been taken into consideration. Although the authors state that the paper is of the nature of a preliminary report, the conclusions therein that the nucleolus is under the direct control of nucleolar genes located in the associated heterochromatin, form the basis for a widely disseminated hypothesis of nuclear control of cytoplasmic protein synthesis through nucleolar mediation (Caspersson, 1950). Until more complete analyses of genetic control of nucleolar composition appear, the conclusion that the nucleolus is the nuclear agent in expressing genetic control of the cytoplasmic activities seems to be unwarranted.

4. The Role of Nucleolonema

The implications of the nucleolonema in the origins of nucleolar materials are not yet fully evaluated. If, as Estable and Sotelo (1951, 1952) have suggested, the nucleolonema is associated with the chromosomes during mitosis and, therefore, form a continuing part of the cellular genetic structure, it may be of considerable importance in the formation of nucleolar materials. The chemical nature of the nucleolonema has not yet been determined, although it appears not to contain DNA. The combination of the nucleolonema and amorphous material then allows speculation as to the nature of each nucleolar component. According to Borysko (personal communication), the organization of the nucleolonema and amorphous materials seems to be dependent on cellular type and activity. Thus, in line with the normal cycle of the nucleolus, one might assume that the amorphous material has to do primarily with the metabolic activities of the cell, whereas the nucleolonema is of importance in longer term aspects of cellular function, such as the maintenance of nucleoli in future cell generations. The author's suggestion of two types of RNA is of interest here, in that the "labile" fraction would logically be associated with the amorphous material, and the "residual" fraction could be considered as part of the nucleolonema.

The general similarity of the loops of the lampbrush chromosomes of oöcyte germinal vesicles to the nucleolonema is striking. Recent studies on the chemistry and morphology of these structures include the papers by Dodson, 1948; Duryee, 1950; Callan, 1952; and Gall, 1954. These authors have suggested that the loops may either transform into nucleoli

or may be instrumental in forming them. In 1952 Gall described a structure on his chromosome I of *Triturus* oöcytes which he has now called (1954) accumulated loop material. He had formerly noted the striking resemblance of this material to nucleoli in other forms, and in 1952 had designated it the "true or plasmasome nucleolus." In the 1954 paper, Gall has found on the same chromosome that bears the nucleolus organizer in somatic cells a location which produces the typical free nucleoli of the amphibian oöcyte. The strong resemblance of the loops of these chromosomes to the nucleolonema, the hypertrophy of large masses of loop material, and the accumulation of large numbers of the refractile nucleoli which resemble the *pars amorpha* nucleolar material—these all suggest to the author that there is present in some oöcytes a more elaborate and refined mechanism for nucleolus formation which is still within the nucleolonema concept.

5. *The Role of Nuclear Sap in Nucleolar Origin*

Another suggestion for the origin of nucleolar material is that it is formed from nuclear sap. This is suggested by Frey-Wyssling (1953), and strongly supported by Rattenbury and Serra (1952) in a slightly modified form (periplasm). Basic to this suggestion is the physical state of the interphase nucleus. The papers of Ris and Mirsky, 1949; D'Angelo, 1950; and Anderson, 1953 strongly support the concept that the interphase nucleus is filled with hydrated chromosomes, with little or no "nuclear sap." On the other hand, the presence of nuclear sap in the germinal vesicle is obvious. In the latter the chromosomes can often be demonstrated in the intact cell, occupying only a portion of the nuclear volume. The possibility is raised here that the only nuclear sap of somatic cells is that contained in the nucleolar material. The analyses of Brown, Callan, and Leaf (1950) are of interest in this regard. These authors have made chromatograms of hydrolysates of the contents of isolated germinal vesicles in which they found amino acids typical of an acid protein, with no RNA or basic protein. In line with the previous suggestion that RNA can act as a protein precipitant in the formation of nucleoli, the precipitation of this nuclear sap by RNA would result in a structure of similar composition to that known for nucleoli. This suggestion would require of whatever area that produced visible amounts of nucleolar material either the production of RNA or a specific attraction for ribonucleoproteins.

VI. Nucleolar Function

In 1898 Montgomery suggested three hypotheses of nucleolar function which contain the major premises of more recent hypotheses. His first choice is that the nucleolus represents cytoplasmic material taken into the

nucleus in relation to the nutritive and growth processes of the latter. His second suggestion of nucleolar function is taken from Häcker, and states that the amount of nucleolar substance stands in direct proportion to the intensity of the functional changes which take place between the cytoplasm and the nucleus. A third possibility which is mentioned by Montgomery is that the nucleolus represents reserve supplies of nutritive substances retained in the nucleus.

To these suggestions might be added the conclusion by plant cytologists that the nucleolus contributes to the chromosome matrix during mitosis (e.g. see McClintock, 1934). The association of the nucleolonema with the chromosomes which has been described by Estable and Sotelo might be considered a special case of this hypothesis.

Most modern hypotheses of nucleolar function can be derived from the hypothesis of Häcker, and indeed, the circumstantial evidence pointing toward some relationship between nucleolus and cytoplasmic synthesis is extensive. The most detailed modern hypothesis of nucleolar function which relates the nucleolus to the activities of the cytoplasm is that of Caspersson and co-workers (Caspersson, 1950). As has been mentioned above, in this hypothesis the nucleolus is considered to act as a mediator in the expression of chromosomal control of cytoplasmic synthesis. Very recently, the concept of the nucleolus as a "controlling center" for cellular activities has been advanced. (See Stockinger, 1953; Ranzoli, 1953).

From a large number of suggested functions of the nucleolus, the following list has been compiled because, with the possible exception of the first, each of them is reasonably consistent with our available knowledge of nucleolar morphology, chemistry, and dynamics. Actually most of the suggestions are found in, or derived from the older literature.

(1) The shielding of the chromosomes from the cytoplasm during mitosis.

(2) The transfer of chromosomal influences to the cytoplasm.

(3) A reservoir of materials produced at a limited rate by the chromosomes.

(4) The site for a limiting rate synthetic reaction necessary for the maintenance of cytoplasmic synthesis.

(5) An accumulation of unused chromosomal or intranuclear products.

(6) An accumulation of unutilized and/or unusable materials of cytoplasmic origin which enter the nucleus but cannot return to the cytoplasm.

(7) A reservoir of energy source for nuclear activities.

Certainly any role of the nucleolus must relate to, and be contained in, hypotheses of nuclear function as a whole. The recent reviews of nuclear function by Brachet (1952) and Mazia (1953) have tended to emphasize

a dual role of the nucleus. One of these roles is the classical concept of hereditary function; the other suggests that the nucleus has a maintenance or replacement function in the normal synthetic activity of the cell. This latter role has been illuminated by studies on enucleate cells. Phosphorus metabolism, protein synthesis, respiration, nucleic acids, and other manifestations of cellular activity have been measured after enucleation, and after replacement of a nucleus in an enucleate cell. (See: Brachet, 1952; Hammerling, 1953; Lorch and Danielli, 1953; Mazia, 1953). Although no clear picture of nuclear activity has emerged from these studies, there is good evidence for what Mazia has designated the "replacement concept" of nuclear function.

The evidence discussed in this review points toward the conclusion that if the nucleolus has any active function in the cell it will play a part in the shorter-term, nongenetic function of the nucleus. For this reason the author prefers for one function of the nucleolus, at least, the suggestion that the nucleolus may be the site of a limiting rate reaction necessary for the maintenance of cytoplasmic synthesis. This preference is based on the evidence for nucleotide liability in terms of "turnover" and solubility in the nucleolus which is discussed in section IV, 2 above. As this concept of a function for the nucleolus is largely inferential, the possibility of other functions in addition to, or in place of, this one or any of the others listed above is indeed great.

It should be kept in mind, however, that the basic premise of all of the hypotheses of nucleolar function to date has been that the nucleolus is involved in nuclear-cytoplasmic interchange. This relationship has never been experimentally established.

VII. References

Albuquerque, R. M., and Serra, J. A. (1951) *Portugal. Acta Biol.* **3**, 187.
Anderson, N. G. (1953) *Exptl. Cell Research* **5**, 361.
Anderson, N. G., and Wilbur, K. M. (1952) *J. Gen. Physiol.* **35**, 781.
Austin, C. R. (1951) *J. Roy. Microscop. Soc.* **71**, 295.
Austin, C. R. (1952) *Australian J. Sci. Research* **B5**, 354.
Austin, C. R. (1953) *Exptl. Cell Research* **4**, 249.
Austin, C. R., and Braden, A. W. H. (1953) *Australian J. Biol. Sci.* **6**, 324.
Barnum, C. P., and Huseby, R. A. (1948) *Arch. Biochem.* **19**, 17.
Barnum, C. P., Huseby, R. A., and Vermund, H. (1953) *Cancer Research* **13**, 880.
Barron, E. S. G., Nelson, L., and Ardao, M. I. (1948) *J. Gen. Physiol.* **32**, 179.
Bauer, H. (1933) *Z. Zellforsch. u. mikroskop. anat.* **18**, 254.
Bernhard, W., Hagenau, F., and Oberling, C. (1952) *Experientia* **8**, 58.
Bodenstein, D. (1947) *J. Exptl. Zool.* **104**, 311.
Borysko, E., and Bang, F. B. (1951) *Bull. Johns Hopkins Hosp.* **89**, 468.
Brachet, J. (1940) *Compt. rend. soc. biol.* **133**, 88.

Brachet, J. (1950) "Chemical Embryology." Interscience, New York.
Brachet, J. (1952) *Symposia Soc. Exptl. Biol.* **6**, 173.
Braden, A. W., and Austin, C. R. (1953) *Australian J. Biol. Sci.* **6**, 665.
Bradfield, J. R. G. (1951) *Quart. J. Microscop. Sci.* **92**, 87.
Brown, G. L., Callan, H. G., and Leaf, G. (1950) *Nature* **165**, 600.
Bungenborg de Jong, P. J. (1936) "La Coacervation." Hermann et Cie, Paris.
Callan, H. G. (1952) *Symposia Soc. Exptl. Biol.* **6**, 243.
Carlson, J. G., and McMaster, R. D. (1951) *Exptl. Cell Research* **2**, 434.
Caspersson, T. (1947) *Symposia Soc. Exptl. Biol.* **1**, 127.
Caspersson, T. (1950) "Cell Growth and Cell Function." Norton, New York.
Caspersson, T., and Santesson, L. (1942) *Acta Radiol.* Suppl 66.
Caspersson, T, and Schultz, J. (1940) *Proc. Natl. Acad. Sci. (U.S.)* **26**, 507.
Chambers, R. (1949) *Biol. Revs.* **24**, 246.
Chayen, J. (1952) *Symposia Soc. Exptl. Biol.* **6**, 290.
Churney, L. (1942) *Biol. Bull.* **82**, 52.
Dalcq, A. (1952) *Bull. acad. med. Belg.* **17**, 236.
Danielli, J. F. (1949) *Cold Spring Harbor Symposia Quant. Biol.* **14**, 32.
Danielli, J. F. (1953) "Cytochemistry." Wiley, New York
Davidson, J. N. (1947) *Cold Spring Harbor Symposia Quant. Biol.* **12**, 50.
Davies, H. G. (1952) *Exptl. Cell. Research* **3**, 453.
D'Angelo, E. G. (1950) *Ann. N.Y. Acad. Sci.* **50**, 910.
De Robertis, E, Nowinski, W. W., and Saez, F (1954) "General Cytology," 2nd ed. Saunders, Philadelphia.
Dervichian, D. G. (1949) *Research* **2**, 210.
Dodson, O. E. (1948) *Univ Calif. (Berkeley) Publs. Zool.* **53**, 281.
Dodson, O. E. (1953) *J. Roy. Microscop. Soc.* **72**, 177.
Dornfeld, E. J. (1936) *Anat. Record* **65**, 403.
Duncan, R. E., and Woods, P. S. (1953) *Chromosoma* **6**, 45
Duryee, W. R. (1941) "Cytology, Genetics, and Evolution" Univ of Pennsylvania Press, Philadelphia.
Duryee, W. R. (1949) *J. Natl. Cancer Inst.* **10**, 735.
Duryee, W. R. (1950) *Ann. N.Y. Acad. Sci.* **50**, 920.
Ehrenberg, L. (1946) *Hereditas* **32**, 407
Elson, D., and Chargaff, E. (1951) *Federation Proc.* **10**, 180.
Elson, D., and Chargaff, E. (1954) *Nature* **173**, 1037
Erickson, R. O., Sax, K. B., and Ogur, M. (1949) *Science* **110**, 472.
Estable, C., and Sotelo, J. R. (1951) *Publs. inst. invest. cien. biol.* **1**, 105.
Estable, C., and Sotelo, J. R. (1952) *Stain Technol.* **27**, 307.
Ficq, A. (1953) *Experientia* **9**, 377.
Frey-Wyssling, A. (1953) "Submicroscopic Morphology of Protoplasm," 2nd ed. Elsevier, Houston.
Gall, J. G. (1952) *Exptl. Cell Research* Suppl. 2, 95.
Gall, J. G. (1954) *J. Morphol.* **94**, 283.
Gates, R. R. (1941) *Biol. Bull.* **81**, 298.
Gates, R. R. (1942) *Botan. Revs.* **8**, 337.
Gersch, M. (1940) *Z. Zellforsch. u. mikroskop. anat.* **30**, 483.
Glick, D. (1949) "Techniques of Histo- and Cytochemistry." Interscience, New York.
Glick, D. (1953) *Intern. Rev. Cytol.* **2**, 447.
Gomori, G. (1952) "Microscopic Histochemistry." Univ. of Chicago Press, Chicago.

Gray, J. (1927) *Brit. J. Exptl. Biol.* **5**, 102.
Hammerling, J. (1953) *Intern. Rev. Cytol.* **2**, 475.
Harding, C. (1949) *Proc. Soc. Exptl. Biol. Med.* **70**, 705.
Heitz, E. (1931a) *Planta* **12**, 775.
Heitz, E. (1931b) *Planta* **15**, 495.
Herrick, F. H. (1895) *Anat. Anz.* **10**, 55.
Hisako, O., and Stowell, R. E. (1951) *J. Natl. Cancer Inst.* **11**, 939.
Hughes, A. (1952a) *Exptl. Cell Research* **3**, 108.
Hughes, A. (1952b) "The Mitotic Cycle." Academic Press, New York.
Hyden, H. (1943) *Acta Physiol. Scand.* **6**, Suppl. 17.
Immers, J. (1954) *Exptl. Cell Research* **6**, 127.
Jacobson, W., and Webb, M. (1952) *Exptl. Cell Research* **3**, 163.
Jeener, R., and Szafarz, D. (1950) *Arch. Biochem.* **26**, 54.
Jorgenson, M. (1913) *Arch. Zellforsch.* **10**, 1.
Lagerstedt, S. (1949) *Acta Anat.* Suppl. 7.
Laznitski, I., and Wilkinson, J. H. (1948) *Brit. J. Cancer* **2**, 369.
Lewis, W. H. (1943) *Cancer Research* **3**, 531.
Litt, M., Monty, K., and Dounce, A. (1952) *Cancer Research* **12**, 279.
Lorch, I. J., and Danielli, J. F. (1953) *Quart. J. Microscop. Sci.* **94**, 445.
Marshak, A., and Calvet, F. (1949) *J. Cellular Comp. Physiol.* **34**, 451.
Marza, V. D., Marza, E. V., and Guthrie, M. J. (1937) *Biol. Bull.* **73**, 67.
Mazia, D. (1953) *in* "Modern Trends in Physiology and Biochemistry" (Barron, ed.), p. 77. Academic Press, New York.
McClintock, B. (1934) *Z. Zellforsch. u. mikroskop. anat.* **21**, 294.
Mirsky, A E, and Ris, H. (1947) *J. Gen Physiol* **31**, 1.
Montgomery, T. H. (1898) *J. Morphol.* **15**, 265.
Mulnard, J. (1949) *Compt. rend. Assoc. Anat. 36th Reunion* **36**, 519.
Nurnberger, J, Engstrom, A., and Lindström, B. (1952) *J. Cellular Comp. Physiol.* **39**, 215.
Painter, T. S. (1945) *J. Exptl. Zool* **100**, 523.
Painter, T. S., and Taylor, A. N. (1942) *Proc. Natl. Acad. Sci. (U.S.)* **28**, 311.
Panijel, J. (1950) *Biochim. et Biophys. Acta* **6**, 79.
Panijel, J. (1951) "Les Problèmes de l'Histochemie et la Biologie Cellulaire." Hermann et Cie, Paris.
Pearse, A. G. E. (1953) "Histochemistry." Little, Brown, Boston.
Pollister, A. W. (1952) *Exptl. Cell Research* Suppl. 2, 59.
Pollister, A. W., and Leuchtenberger, C. (1949) *Nature* **163**, 360.
Pollister, A. W., and Ris, H. (1947) *Cold Spring Harbor Symposia Quant. Biol.* **12**, 147.
Porter, K. E. (1954) *J. Histochem. Cytochem.* **2**, 346.
Ranzoli, F. (1953) *Caryologia* **5**, 137.
Rather, L. J. (1951) *Bull. Johns Hopkins Hosp.* **88**, 38.
Rattenbury, J. A. (1952) *Stain Technol.* **27**, 113.
Rattenbury, J. A., and Serra, J. A. (1952) *Portugal. Acta Biol.* **3**, 187.
Ris, H., and Mirsky, A. E. (1949) *J. Gen. Physiol.* **32**, 489.
Schrader, H., and Leuchtenberger, C. (1950) *Exptl. Cell Research* **1**, 421.
Schultz, J., Caspersson, T., and Aquilonius, L. (1940) *Proc. Natl. Acad. Sci. (U.S.)* **26**, 515.
Serra, J. A. (1948) *Cold Spring Harbor Symposia Quant. Biol.* **13**, 192.

Serra, J. A., and Queirez Lopes, A. (1944) *Naturwissenschaften* **32**, 47.
Serra, J. A., and Queirez Lopes, A. (1945a) *Nature* **155**, 792.
Serra, J. A., and Queirez Lopes, A. (1945b) *Portugal. Acta Biol. Ser. A* **1**, 51.
Serra, J. A., and Queirez Lopes, A. (1945c) *Portugal. Acta Biol. Ser. A* **1**, 111.
Shinke, N., and Shigenaga, M. (1933) *Cytologia* **4**, 189.
Srivastava, D. S. (1952) *La Cellule* **55**, 132.
Stenram, U. (1953) *Exptl. Cell Research* **5**, 539.
Stich, H., and Hammerling, S. (1953) *Z. Naturforsch.* **8b**, 329.
Stockinger, L. (1953) *Protoplasma* **42**, 365.
Stowell, R. E. (1949) *Cancer* **2**, 121.
Swift, H. (1953) *Intern. Rev. Cytol.* **2**, 1.
Tandler, C. J. (1953) *J. Histochem Cytochem.* **1**, 151.
Taylor, J. H. (1953) *Science* **118**, 555
Thomas, L. J. (1954) *Biol. Bull.* **106**, 129.
Thorell, B. (1947) *Cold Spring Harbor Symposia Quant. Biol.* **12**, 247.
Tyner, E. P., Heidelberger, C., and LePage, G. A. (1953) *Cancer Research* **13**, 186.
Vendrely, R., and Lepardy, J. (1946) *Compt rend.* **223**, 342.
Vincent, W. S. (1952) *Proc. Natl. Acad. Sci. (U.S.)* **38**, 139.
Vincent, W. S. (1954) *Biol. Bull.* **107**, 327.
Vincent, W. S., and Huxley, A. H. (1954) *Biol. Bull.* **107**, 290.
Wilson, E. B. (1928) "The Cell." Macmillan, New York.
Zirkle, C. (1928) *Botan. Gaz.* **86**, 402.
Zollinger, N. V. (1948) *Mikroskopie* **3**, 1.

On Goblet Cells, Especially of the Intestine of Some Mammalian Species

HARALD MOE

Department of Anatomy, University of Copenhagen, Denmark

		Page
I.	Introduction	299
II.	Goblet Cells in General	300
III.	Goblet Cells in the Intestine	304
	1. Intestinal Epithelium	304
	2. Secretory Mechanism of the Intestinal Goblet Cells	307
	3. Formation, Secretory Cycle and Later History of the Goblet Cells	310
	4. Quantity and Distribution of Goblet Cells	319
	5. Functional and Pharmacological Aspects of Goblet Cells	329
IV.	References	333

I. Introduction

Mucus-secreting surface epithelia and glands are extremely widespread in the animal kingdom. Their mucous secretions fulfill a number of fundamental functions, some of which are concerned with the defense and cleansing of the body. Mucus is often an important factor in transport mechanisms, not merely as an excellent lubricant, but also in regulating the transport by elastic cohesion of suspended and adherent particles. Mucus is capable of binding water and, as a result, swelling. Its characteristic properties, the physico-chemical basis of which is still partly unknown (Keiser-Nielsen, 1953), depend largely on the degree of hydration. During recent years, the chemistry of mucus, which is known only in its broad features, has been attracting increasing attention. Surveys have been published by Meyer (1945) and Stacey (1946). Quite recently Werner (1953) has been studying mucus of epithelial origin in greater detail.

Throughout the animal kingdom, mucus-producing surface epithelia present a large number of structural varieties (cf. Schaffer, 1927). In some sites, every cell of the epithelium is mucus-secreting. This applies for instance to the stomach and the uterine cervix. Usually, however, the functions have been divided between the cells, some being entrusted with mucus-production, whereas others are concerned with other tasks. Such mucus-secreting cells are often termed goblet cells (Schulze, 1867) because of their characteristic shape.

This paper does not purport to deal with all mucus-secreting cells in surface epithelia throughout the animal kingdom. Despite common

features, they make up a group so diverse that an extensive review dealing with all types would be tightrope-walking between unjustified generalization and detailed enumeration. Instead, the writer will first touch upon a few facts of general interest and then proceed to deal in greater detail with the intestinal goblet cells which have been the subject of most intensive research.

II. Goblet Cells in General

In the higher vertebrates, goblet cells occur primarily in the respiratory tract, in the intestine, and in a number of excretory ducts. In amphibia, reptiles, and fishes, they are more widespread, occurring also in the oral cavity, pharynx, and esophagus. In the epidermis also, goblet cells may occur, e.g. in certain fishes. The varieties of epithelium containing mucin-producing cells are, with a few exceptions, simple or pseudostratified columnar epithelium, or stratified squamous epithelium. As a rule, the goblet cells are spread fairly evenly over the epithelium.

A well-developed goblet cell is usually oblong and oriented at a right angle to the surface. Its basal cytoplasmic portion holds the nucleus, whereas the luminal, broader portion contains stored mucus. The mucus and the cytoplasm in which it is lodged was called the theca by Schulze (1867). [Several authors have misunderstood this or failed to follow Schulze's definition (possibly for linguistic reasons), considering the theca to be the layer of cytoplasm surrounding the mucus]. The aperture through which the cell discharges its secretion is called the stoma.

The shape of the goblet cells is to a large extent adapted to the thickness of the epithelium to which they belong. In low epithelium, such as in the intestinal crypts of mammals, a well-developed goblet cell is barrel-shaped with a round or piriform theca, attaching broadly to the basement membrane. Such goblet cells are often called unpedunculated. In thicker epithelium, the goblet cells increase in length, stretching, with a few exceptions, through the entire epithelium. Thus, the theca assumes an ovoid shape and the cytoplasm between the theca and the basement membrane that holds the nucleus becomes longer and more slender. Such goblet cells are called pedunculated. In simple columnar epithelium, e.g. in intestinal villi and in small bronchi, the stalk is short and thick. In thick stratified epithelium, e.g. in the nasal cavity and the throat of man, it is very long and slender, the length of the theca changing far less than the length of the basal part. Pedunculated goblet cells may be furnished with a flat or branched "foot" (cf. Schaffer, 1927).

Another factor that influences the shape of the goblet cell is the amount of mucus in the theca, i.e. the degree of maturation and the functional

phase of the cell. Within the same organ and within the same epithelial type, there may be moreover minor species differences (cf. Martin, 1910; Clara, 1926). Thus a complete list of appropriate comparisons would have to include almost every type of drinking glass!

The nucleus of the goblet cell is basal to the theca. Generally, it is smaller than that of other epithelial cells, but it stains more deeply. Its shape varies considerably, harmonizing with the shape of the cells and the size of the theca. In unpedunculated goblet cells it is usually round or more or less flattened. In pedunculated goblet cells it is sometimes ovoid, and oriented longitudinally in the cell, whereas in other instances it may be cone-shaped or heart-shaped, the base towards the theca. Divergences from round or ovoid shapes are claimed to be due to pressure by the theca (Martin, 1910; Clara, 1926). As a rule, the cytoplasm of goblet cells stains more darkly than that of other epithelial cells. This applies to the cytoplasm between the theca and the basement membrane as well as to the thin layer that surrounds the theca. According to Cajal (1915) goblet cells contain a Golgi apparatus, situated between the nucleus and the theca. The appearance seems to vary according to approximately the same conditions that apply to the nucleus. In well-developed, pedunculated goblet cells it presents a network around the upper nuclear pole and extending up around the basal part of the theca (Kopsch, 1926; Yokochi, 1951), so that, according to Kopsch, the total apparatus may present a biconcave shape. The relationship of the Golgi apparatus to the secretory granules will be discussed in the section on the secretory mechanism of the intestinal goblet cells.

Mitochondria seem to be few (Eklöf, 1914). There is no evidence that they take direct part in the production of mucin (Nassonow, 1923; Florey, 1932), and there seem to be none in the theca (Eklöf, 1914; Yokochi, 1951). Centrioles have been demonstrated by Zipkin (1903), Tschassownikow (1914), and others.

There is hardly any doubt that normally the mucus is present in the theca in the form of granules or droplets. This is indicated partly by the finding of a granular structure in living tissue *in situ* or immediately after removal (Schulze, 1867; Paneth, 1888; Bizzozero, 1892; Bensley, 1908; and others) and partly by the fact that specimens prepared by freeze-drying and, following deparaffinization, coagulated by alcohol show a dense mass of fine, round granules in the theca, staining deeply with the periodic acid-Schiff method (Gersh, 1949; Moe, unpublished data). The granules are often named mucigen or premucin granules, terms that with our incomplete knowledge can only be taken to mean substances that form mucus when combined with water. Whether the thecal mucus occurs only in the

form of granules is still an open question. *In vivo,* some thecae have been found to display a homogeneous appearance (cf. Paneth, 1888; Florey, 1931). In the opinion of Clara (1926) and others the mucous granules may swell and coalesce before being discharged from the cell.

Ordinary fixation of the specimens alters the normal thecal structure more or less, depending on the fixative (Schulze, 1867; Paneth, 1888; von Seillers, 1891; Bizzozero, 1892; Martin, 1910; Clara, 1926; and others). In order to be able to determine which agents are suitable for fixing the mucus, it is important to be familiar with the structural varieties seen in the stained sections. Conclusions regarding the functional role of the goblet cells can only be made with reserve when the fixation is inadequate. The structural varieties may briefly be divided into the following groups that may be assumed to represent progressive destruction: (1) densely arranged, stained granules in an unstained or nearly unstained intergranular substance, (2) stained granules with a paler inner and deeply staining outer zone (vacuoles?), (3) a fine or coarse, stained meshwork with unstained or faintly stained meshes, and (4) scattered, irregular, stained trabecular structures in a theca of a "washed-out" appearance.

With a given fixation, the same structure is often consistently repeated in most goblet cells of the same organ. At other times, it may vary, differing e.g. in young and old cells, in deep and luminal parts of the same theca, or from animal to animal.

It is worth noting that in most fixed specimens, the normal granular structure of the theca has been "reversed," the mucus-stained material presenting itself not as granules, but as a net. The explanation has been given that the swollen mucigen granules coalesce, forming fibrils, or that they are precipitated as fibrils on the remains of the cytoplasmic strands of the theca (Clara, 1926).

Theoretically, there is a risk that the swelling of the mucus which precedes the precipitation may involve such a distention of the thecal walls that the nucleus and other cellular elements are exposed to compression, the stoma opens, and the mucus is discharged. In fact, we do not know to what extent this happens. Even a slight flow of secretion through the stoma (whether or not it is already open) as an artifact will seriously interfere with the determination of the functional phase. Frequently, histological sections, e.g. of the villi (Plate Ia, b) or of the colonic crypts will show practically all the goblet cells in the process of discharging mucus into the lumen through an occasionally almost gaping stoma. It seems scarcely possible to accept such appearances as an evidence of real activity.

Thus, the successful study of the thecal content and the functional con-

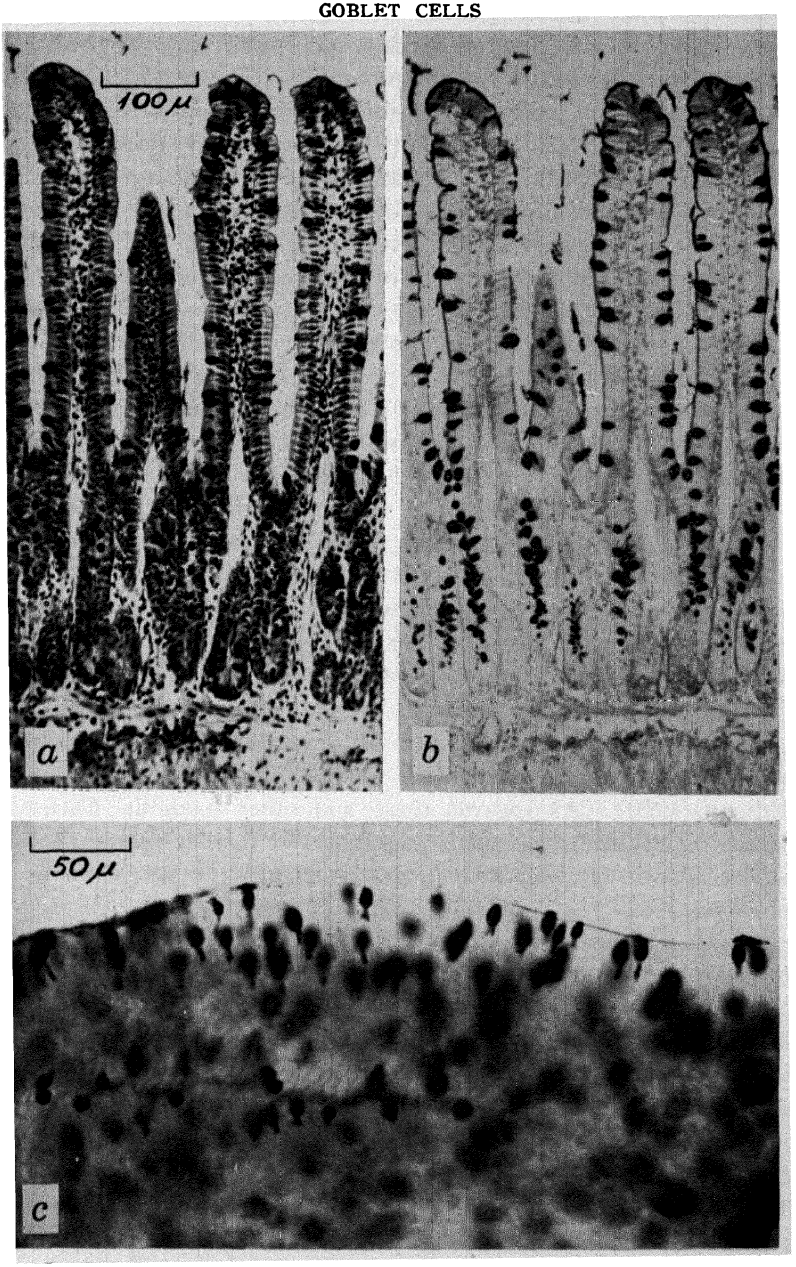


PLATE I. Longitudinal sections of the jejunum of a rat. (a) Periodic acid-Schiff and haematoxylin staining. (b) Periodic acid-Schiff staining. A number of the goblet cells appear to be in the process of discharging their secretion. (c) Lateral edge of a villus from a rat. Whole mount. Periodic acid-Schiff staining. The thecae of the goblet cells continue in a stalk, ending in the Golgi area.

dition of the goblet cells depends largely upon preserving the normal structure of the theca in the course of preparation. The fixatives usually recommended are formol-alcohol, potassium bichromate-formol, and saturated aqueous picric acid [cf. Romeis (1948) for further references]. It is not reasonable to emphasize the value of either of these agents at the cost of the other. Mucigen granules of different occurrence and cellular localization do not behave in the same way during fixation, and one must feel one's way in each individual case. Not infrequently, the best result obtainable is actually of mediocre quality. The main obstacle in obtaining reliable preparations is indubitably the swelling of mucin with water. The mucigen granules must, therefore, be precipitated before the imbibition with water has taken place, at any rate, to a disturbing extent. This is probably more easily fulfilled by the precipitation with alcohol or other mucus-precipitating agents of freeze-dried deparaffinized tissue sections that have not yet been in contact with water than by the usual method of immersing the tissue sections in the fixative. This entire aspect calls for a thorough study.

The mucin content of the theca may be stained by a number of different methods. Good results are often obtained by the well-known agents, such as mucicarmine, Delafield's hematoxylin, toluidine blue, and thionine, and by Bauer's polysaccharide reaction. Recent years have brought several, new, excellent staining methods, primarily the periodic acid-Schiff method (Hotchkiss, 1948; McManus, 1948) and staining with Alcian blue 8 GS (Steedman, 1950). As regards these and other methods, discussion of histochemical problems in mucus staining, and further references, the interested reader is referred to Romeis, 1948; Glick, 1949; Gomori, 1952; and Pearse, 1953.

III. Goblet Cells in the Intestine

1. Intestinal Epithelium

The following survey of the intestinal epithelium deals exclusively with higher vertebrates the goblet cells of which have been most thoroughly studied. For further information and for data regarding the structure of the intestinal mucosa in lower animals, the reader is referred to Patzelt (1936).

The epithelium of the small intestine does not line a smooth surface, but, as is well-known, a surface with an extremely complicated relief due to numerous villi and Lieberkühn's crypts (Plate Ia, b).

The villi usually range in height from about 200 μ to 1 mm.; their shape is often likened to that of fingers. Many other shapes are seen, however, and there may be extremely marked differences between the

various species, differences that may be ascertained by viewing the intestinal surface through a stereomicroscope. In the rat they are usually flat, resembling a hand without fingers (cf. Plate IId showing a whole villus viewed towards the surface). In the cat they are often very tall, varying from tongue to finger shape (Plates IV and V). In nearly all species, the villi change in appearance down through the intestine, the most slender varieties being encountered in the ileum.

From the grooves between the villi, the intestinal crypts extend like fine epithelial tubes down into the mucosal wall, all ending at the same level. Their shape suggests that of test tubes, but they may be branched. In the distal part of the small intestine of the cat there are, for instance, numerous branched crypts with up to three successive divisions, whereas unbranched crypts predominate in the proximal and middle portions.

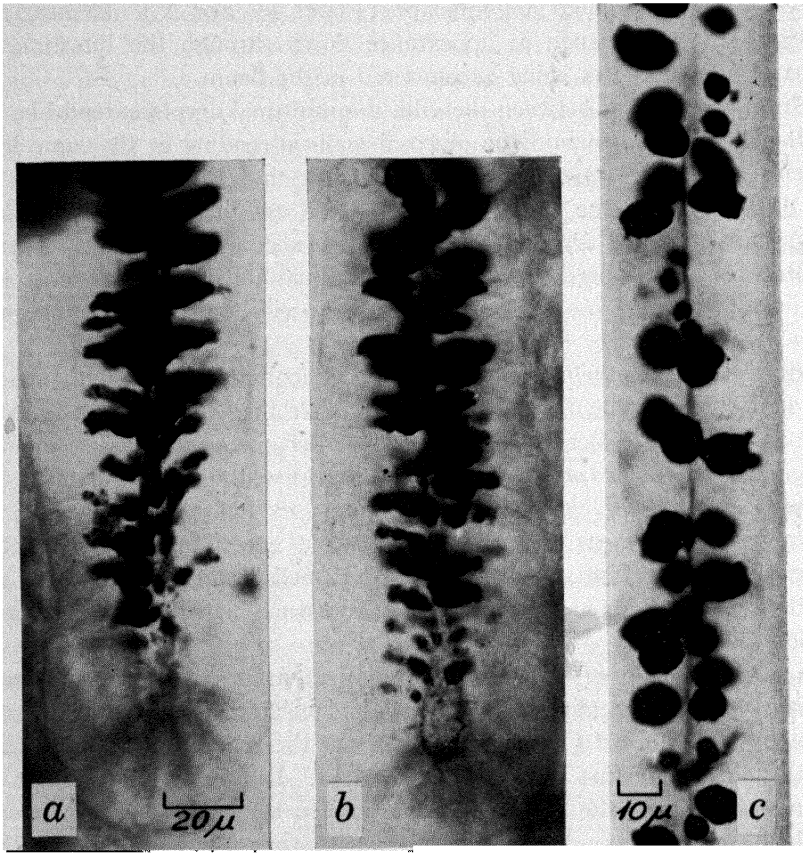
In the large intestine there are generally no villi, but the crypts are well-developed.

Most of the epithelial cells are so-called columnar cells with striated borders. On the villi they are generally taller than in the crypts. In addition, there are goblet cells which occur in crypts as well as on the villi, argentaffin (and/or argyrophil) cells that usually predominate in the crypts, and Paneth cells which are almost exclusively localized in the bottoms of the crypts. Paneth cells are not encountered in all species. Finally, there are the so-called "small dark cells" and, in the deeper part of the crypts, there are indifferent or immature cells with a capacity to divide.

A characteristic of the intestinal epithelium is a lively regenerative capacity. Counting mitoses in normal and colchicine-treated albino rats, Leblond and Stevens (1948) calculated that the renewal of the epithelium of the small intestine took about 1.5 days. Dividing cells are not encountered all over the epithelium, but only in the crypts and particularly in their deeper parts. As early as the past century, Bizzozero (1888, 1889, 1892, 1893) advanced the theory that the regeneration of the intestinal epithelium takes place in the crypts and that then the new-formed cells migrate up through the crypts towards the tops of the villi, where they are shed or destroyed. In the case of the individual epithelial cell this means that, in the course of its remarkably short existence, it moves as on a band conveyor from the crypt to the top of the villus while first developing from a primitive to a mature cell and then executing one or more functions which are perhaps interrupted by periodical metamorphotic processes.

Nothing has yet been found to disprove Bizzozero's 60-year-old theory. On the contrary, there have been found in the epithelium from the regenerative zone to the top of the villus (or the surface of the colon) de-

velopmental and functional gradients that exactly fit in with this theory. The exact mechanism of the migration and the desquamation or destruction of the epithelial cells remains to be elucidated.

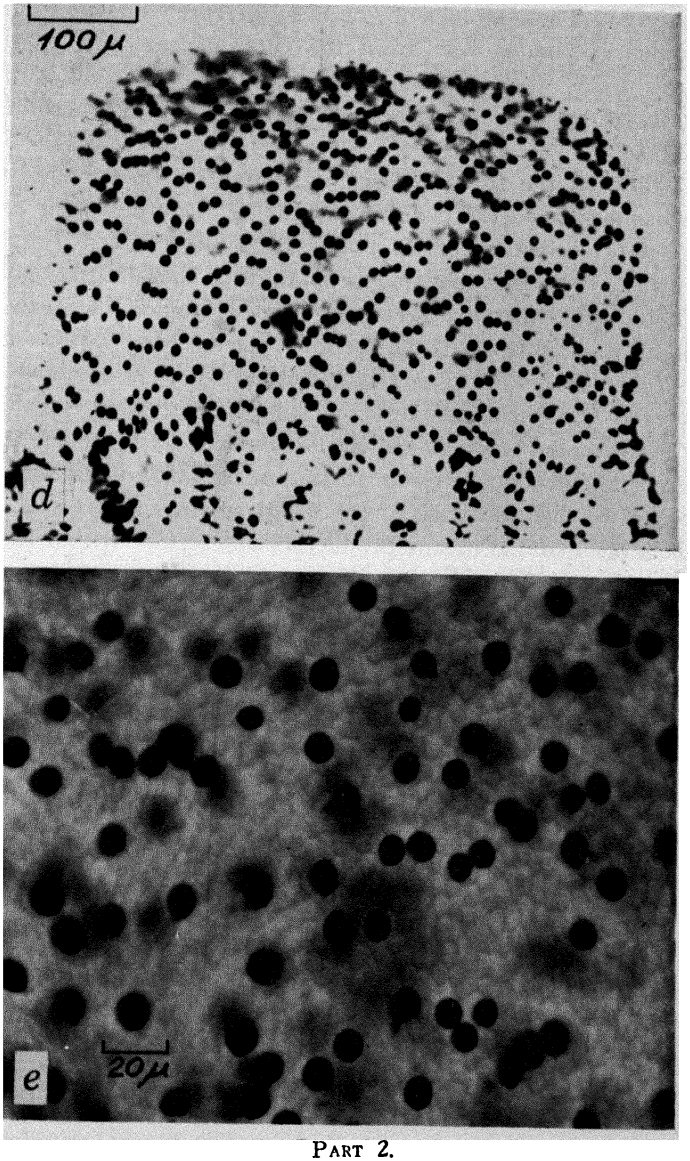


PART 1.

PLATE II. (a) and (b). Whole mounts of the basal two-thirds of crypts from the small intestine of a rat. Periodic acid-Schiff staining. The outer contours of the crypts are discernible. The size of the thecae increases with the distance from the base of the crypt. (c) Middle part of intact crypt from the duodenum of a pig. In top right-hand corner twin cells with supranuclear staining. Several twin cells of varying size. (d) Goblet cells on one surface of an intact villus from a rat. Periodic acid-Schiff staining. The thecae of the other surface may be discerned superiorly and to the right as small, ill-defined patches. Only the thecae in the focal plane are reproduced in the correct size. (e) Part of villus surface from a whole mount. Periodic acid-Schiff staining. In areas where the intensely stained goblet-cell thecae are in the focal plane, the polygonal ends of the columnar cells are seen.

2. *Secretory Mechanism of the Intestinal Goblet Cells*

The secretory mechanism of the goblet cells comprises the production of mucin, its storage in the cell, and its discharge. Whereas previously it was believed that the mucin was produced in the thecal area (Paneth, 1888 and others), investigations of the past decades indicate that the cytoplasmic



PART 2.

area immediately above the nucleus is the probable site of its formation (Nassonov, 1923; Clara, 1926; Dornesco and Valverde, 1930; Florey and Webb, 1931; Florey, 1932; Schumann, 1936; Yokochi, 1951). This opinion is based on the finding that in mucin-stained preparations the theca often tapers down, being connected by streaks of small intensely stained granules with a somewhat larger group of granules at the upper nuclear pole (Plate Ic). These streaks of granules, interpreted as a sign that new-formed mucus is under way from the supranuclear zone to the theca (Nassonov, 1923; Clara, 1926), and supranuclear granules that take mucin stain, afford the only direct criteria that mucinogenesis is taking place.

Quite naturally, the supranuclear localization of the youngest granules of mucin has suggested the Golgi apparatus as the site of their formation, but the exact mechanism still remains to be elucidated. According to Nassonov (1923) the net-like Golgi apparatus in the intestinal goblet cells of the *Triton* opens distally when new-formed droplets of mucus become detached and pass up towards the theca. After studying various mammals, Florey (1932) concluded that no evidence had been obtained that the Golgi material was "used up" in elaborating the secretion; he thinks that rather it is dislocated towards the base of the cell owing to the accumulation of secretion products. Even an intense stimulation of the secretion does not lead to disintegration of the Golgi apparatus. In man, Yokochi (1951) believes that the mucus production takes place within the threads or in the meshes of the Golgi net.

A somewhat divergent opinion has been offered by Duthie (1933), basing his conclusions on vital staining of the rat and mouse colon with neutral red. He maintains that, whereas the origin of stainable mucin is in the Golgi network, the primary origin of the secretory granules is at the periphery of the cell in the mitochondrial zone; the granules then pass into the Golgi area to undergo transformation into mature mucin granules. Huber (1945), studying the mouse colon, also assumes that the Golgi apparatus is concerned with preparing and transforming precursors produced by other cellular components into the finished secretion. Following azan staining, the pre-substance presents itself as fine, blue granules in the thin layer of cytoplasm between the nucleus and the Golgi apparatus and around the lateral aspects of the nucleus. It is believed to originate partly from the mitochondria and partly from the nucleoli that discharge their contents through the nuclear membrane. Galeotti (1895) also attributed a function in the synthesis of mucin to the nucleus.

Of course, the different views regarding mucinogenesis depend on whether the observed structures really represent mucin granules or their

preliminary stages. Especially the secretory pre-stages call for investigation by methods other than those used so far.

In new-formed goblet cells, the secretion is stored right below the surface. In certain species, the new depot of mucus and the supranuclear mucin reaction which is particularly striking in these cells, lend them a characteristic, bizonal appearance (Plate IIc). Gradually, as the mucous depot grows, the cell swells and the theca outmeasures the area of supranuclear reaction like a balloon its gondola. In mature goblet cells it is sometimes difficult to distinguish a supranuclear reaction from the theca which may extend as far as the nucleus, which it apparently deforms. This complicates the possibility of deciding whether any signs of mucinosynthesis are present. A further difficulty is that in some species the supranuclear mucin reaction obtained by staining is, on the whole, faint.

The mechanism of the formation of the stoma through which the mucus is discharged is not quite clear. According to Clara (1926) the young goblet cell has a striated border that gradually becomes thinner in order to burst or disappear when the cell lets out its secretion. Chlopkow (1928) has described the development of a complicated mechanism in great detail. In the finished state, it is a funnel-shaped apparatus enveloping the luminal part of the theca like a hood. The narrow end of the funnel faces the surface and is formed by the terminal bars constricting into a more or less closed ring about the distal end of the cell. Active regulation of the emptying takes place by opening and closing this ring. The present author has not succeeded in observing Chlopkow's apparatus by his technique (unpublished data). By electron microscopy, Dalton *et al.* (1951) found an undifferentiated cell membrane covering the free surface, but they do not state whether this applies to goblet cells at all ages and phases.

From a functional point of view it would be important to know whether the appearance of the luminal end of the goblet cells makes it possible to distinguish cells that are (or have been) active in discharging secretion from cells that have not yet become active.

How does an intestinal goblet cell give off its secretion? Whether it is by explosive discharge, or by a more or less slow, continuous or periodical oozing, we do not know with certainty. Fixed histological sections sometimes show in nearly all goblet cells a lively secretory activity through widely opened stomata. It is, however, difficult to take such exaggerated activity seriously. The surprising thing about such preparations is perhaps not the large number of cells that are discharging their secretions at the same time, but rather the violence with which they do so, a violence which would seem to exhaust them completely in a short time. Perhaps

in vivo studies may solve the problem regarding the mechanism of the discharge.

The secretory or functional cycle of the goblet cells comprises the interplay between synthesis, storage, and discharge of the mucus, starting from the first trace to the final emptying of the theca or the death of the cell. (The term "secretory cycle", which strictly speaking should be used only when the secretory process can be repeated, will be used here as is customary in dealing with the goblet cells, even though the question regarding repetition of the secretory processes in these cells has not been finally solved).

Within a typical cycle, there are probably two or three phases. In the initial phase (first phase) the stored quantity of mucus increases because of mucinosynthesis; in the final phase (third phase) the theca decreases in size because the secretion is being discharged; in a possible intermediate phase (second phase) the size of the theca remains constant.

But is that all, or does a discharge also take place during the first phase, only at a slower rate than the synthesis? Is the thecal size in the intermediate phase a result of equilibrium between synthesis and discharge or is it a sign that the secretory mechanism is quiescent; and does synthesis take place in the third phase simultaneously with discharge?

The combined results of many studies indicate that synthesis, as well as discharge, may take place in all three phases (Bizzozero, 1893; Nassonov, 1923; Clara, 1926; Florey, 1932; Schumann, 1936; and others), but it is far from clear to what extent or how far the interplay between synthesis and discharge can be influenced by external factors. The reliability of what has been said and what will later be said in this respect depends primarily on the exclusion of disturbing artifacts, erroneously interpreted as discharge. Some of the problems relating to the secretory cycle will be discussed in the following section.

3. Formation, Secretory Cycle, and Later History of the Goblet Cells

The formation, secretory cycle, and subsequent fate of the goblet cells constitute in fact the entire story of their life. So large a chapter might appear better treated if each section were to be examined separately. In many theories of the life-span of the goblet cells, however, the three stages are so intimately interlinked that the total aspect of each theory might be lost if the subject were thus divided. For this reason, all three stages will be considered together.

On the basis of the early literature, the cytomorphosis and later history of the intestinal goblet cells has been admirably treated by Macklin and Macklin in Cowdry's "Special Cytology" (1932). Now, as then, there are

two conflicting main views. According to one, the goblet cells are specifically differentiated cells derived from primitive elements preserved in the cryptal zones of regeneration from the intestinal anlage. According to the other view, they are formed by the principal columnar cells or other special cells of the intestinal epithelium to which they can revert. Goblet cells are often described as modified columnar cells or as representatives of a temporary functional phase of columnar cells.

The problems which have been submitted to investigation and speculations are mainly: From which cells do the goblet cells develop? Do they pass through one or more secretory cycles? If the cycle is repeated, in what shape does the cell exist in the interval? What is the fate of the cell, when the secretory activity has been completed?

Bizzozero (1889, 1893) and Cloetta (1893) regard goblet cells as independent cells with a functional period (lasting throughout or almost throughout their lives) which starts while they are moving away from their sites of formation in the deeper parts of the crypts. The main arguments are that early stages are encountered in the mitotic area of the crypts, and that mature cells are localized to the necks of the crypts and the basal parts of the villi, whereas cells on the surface of the colon and on the tops of the villi, believed to be oldest, are characterized by a secretory discharge. This theory is supported by the studies of Zipkin (1903), Florey (1932), Karasek (1933), and Moe (1953).

Most authors, however, have found matters to be more complicated. In Clara's (1926) opinion goblet cells may develop from columnar cells all over the intestinal epithelium and the cycle may be repeated. In some cells, he observed the simultaneous presence of residual secretion in the theca and supranuclear granules. This he interprets as a sign that in some cases a new cycle has started before the preceding one has been finished. Frequently, however, there is a quiescent period between the two cycles and at this stage the cell is a "small dark cell". According to Clara, Huber (1945), and many others (cf. Patzelt, 1936), the cycle may also end in the way that the cell reverts to a columnar cell, directly or by way of an intermediate stage as a "small dark cell" (Paneth, 1888). Whereas some workers interpret the "small dark cells" as the ultimate phase in the cycle of the goblet cell (for a different origin of these cells, cf. Clara, 1926) Chlopkow (1928), studying the cat, rabbit, and dog by a special technique maintains that they represent an early stage in the cycle, being an intermediate link in the development of columnar cells into goblet cells.

Recently, the argentaffin cells have also been related to the goblet cell cycle. Popoff (1939), studying rabbit and human material, concludes that the secretory cycle in the large and small intestine manifests itself

repeatedly in the same goblet cell by complete or partial emptying of the theca in each cycle. When eventually the cell reaches a stage of functional exhaustion, it does not perish but is transformed into an argentaffin and then into an indifferent cell of the same appearance as the undifferentiated epithelial cells. The indifferent cells are capable of division and may again develop into goblet cells. This entire process Popoff calls "functional rejuvenation." In guinea pigs, mice, and human subjects Schofield (1951, 1952, 1953) found no signs that the argentaffin cells did enter into a goblet cell cycle in the large intestine, but only in the small intestine, in a way different from that described by Popoff. Schofield's cycle is as follows: In small, agranular cells situated basally between the columnar cells, argentaffin granules accumulate around the nucleus and also gradually in a cytoplasmic process growing towards the surface. Then, the granules of these "mature" argentaffin cells undergo a metamorphosis into granules which are mucous in nature, the process commencing with the apical granules and extending ultimately to involve the remainder of the supranuclear and the basal granulation, finally resulting in a cell which presents the characteristic form and staining reaction of a goblet cell. Following discharge of the mucinogen mass the goblet cell is presumed to revert to an agranular basal cell. Schofield does not believe that all mucous cells in the small intestine have argentaffin or argyrophil cells (which to Schofield are identical) as their precursors. With the techniques at present available, he states, it is not possible to distinguish between mucous cells which may have originated from argentaffin cells and those which have not, with the possible exception of the few mucous cells in which argentaffin remains may be found.

In a few experimental studies, the problem of the cycle is touched upon. Florey and Webb (1931), report that following application of mustard oil to the intestinal mucosa of the cat, the cells at the bases of the crypts undergo discharge and exhibit their cycle of changes before those nearer the surface. Whereas the lower half of the crypts may be completely devoid of mucus, fully formed goblet cells which have apparently discharged little mucin are present on the surface and at the mouths of the crypts. This applies to the small as well as to the large intestine. The young, completely emptied goblet cells in the basal parts of the crypts can continue the mucus formation and renew the theca. Huber (1945) arrived at a similar result following injection of delphinine into mice.

It seems impossible to bring into harmony the different theories regarding the life span of the goblet cells. The sum of these theories is that undifferentiated cells, columnar cells, argentaffin cells, and "small dark cells" may occur as precursors as well as descendants of goblet cells. In

other words, the functional, cytomorphotic phenomena would be more or less fortuitous.

Some of the incongruities are perhaps due to differences between the various species or between the large and the small intestine, whereas other causes of the kaleidoscopic picture may probably be put down to technical imperfections. The procedure of practically all the studies has quite naturally been to prepare sections of material fixed in various media and stained by more or less complicated methods, and on this basis to pick out various cell types and what are considered transitional varieties, making a classification into what are believed to be genetic series. Artifacts due to the fixation and the application of insufficiently specific staining methods involve a risk of misinterpretation. In addition, fragmented cells, overlapping cells, and unfavorable orientation will make the interpretation at best incomplete and at the worst erroneous. But unfortunately, these are risks that are inevitable in classical histology.

To these difficulties are added the problems regarding the migration of the intestinal epithelium. If Bizzozero's migration theory is correct, the localization of the different cell types has also to be taken into account in setting up genetic series and in analyzing cytomorphic and metamorphic processes in the intestinal epithelium, but this is often neglected.

The efforts to reduce some of the sources of error that complicate the study of goblet cells when using histological sections have resulted in a method (Moe, 1952) that in certain respects permits more exact observations, primarily because the cutting of villi, crypts, and cells is avoided. By microdissection, preparations are made that contain rows of intact villi and crypts. Following staining with the periodic acid-Schiff reagent, these preparations are rendered transparent by embedding in a medium with the same refractive index as the tissue. A suitable dilution of Schiff's reagent and grading of the staining period, that must be adjusted for each animal, gives intense staining of the theca and supranuclear areas, whereas other PAS positive structures still react so faintly as not to disturb the transparency. (All the histological illustrations in this paper, except Plate Ia, b are such whole mounts of villi and crypts).

The main advantages of this method are as follows: (1) it visualizes all the thecae in a crypt, or of a villus, (2) the size of the intact thecae affords an estimate of the phase of the goblet cells in the secretory cycle, (3) by locating initial and terminal stages of the cycle it is possible to determine at which site, ranging from the bottom of the crypt to the top of the villus, goblet cells arise and disappear, and (4) the number and distribution of the goblet cells in crypts and on villi can be recorded.

Whole mounts of crypts and villi from rats have shown that goblet

cells are not common at the bottoms of the crypts. Somewhat higher up, there are densely arranged, small thecae increasing in size with the distance from the bottoms and reaching a maximum in the upper third of the crypts (Plate IIa, b). Thereafter, the size of the thecae remains practically constant in the basal and intermediate portions of the villi, finally decreasing towards the top (Plate IId).

In the cat (partly published data, Moe, 1953) thecae are seen right from the bottoms of the crypts (Plate IIIa). In the deep part all sizes are seen, up to about 10μ in diameter. Towards the middle of the crypt, the number of small thecae rapidly decreases, the upper half showing only well-developed thecae, 6–12μ in diameter, in practically all crypts. In the diagram in Fig. 1 the shaded columns indicate the number and distribution

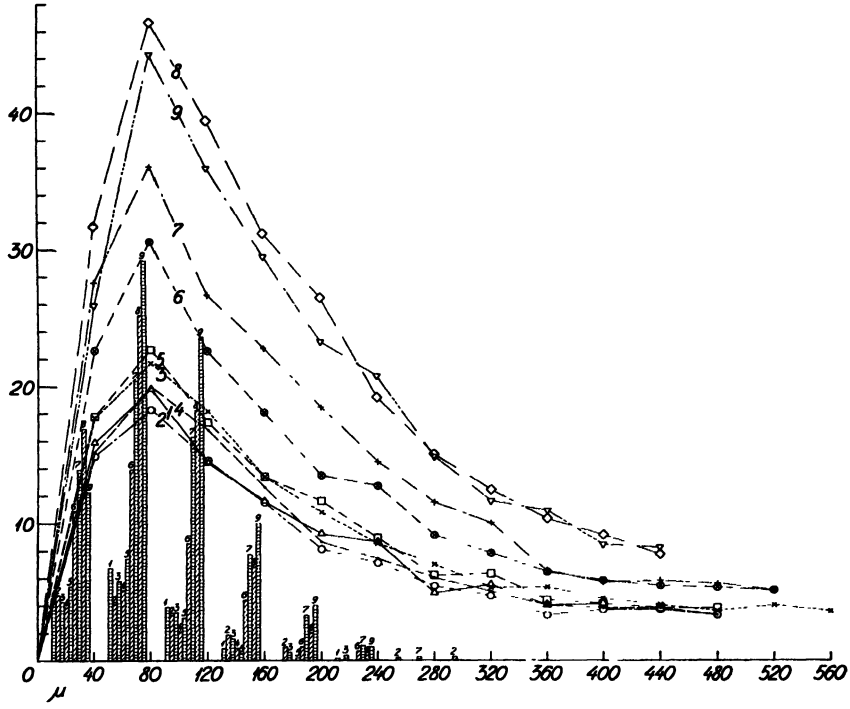


FIG. 1. The curves show the distribution and number of goblet cells (ordinate) in crypts from the small intestine of a cat (S21), starting from the base of the crypt on the left, recording each 40μ (abscissa). Crypts were removed at suitable intervals down through the 120 cm. long intestine from a little more than 2 cm. distal to the pylorus (Curve 1) to about 10 cm. proximal to the caecum (Curve 9). Curve 6 is from the middle of the intestine. Each curve represents the average of 10 or 20 crypts. The columns give the number and distribution of thecae with a minimum diameter of $\leqslant$ 3μ. The numbers of the columns correspond to those of the curves.

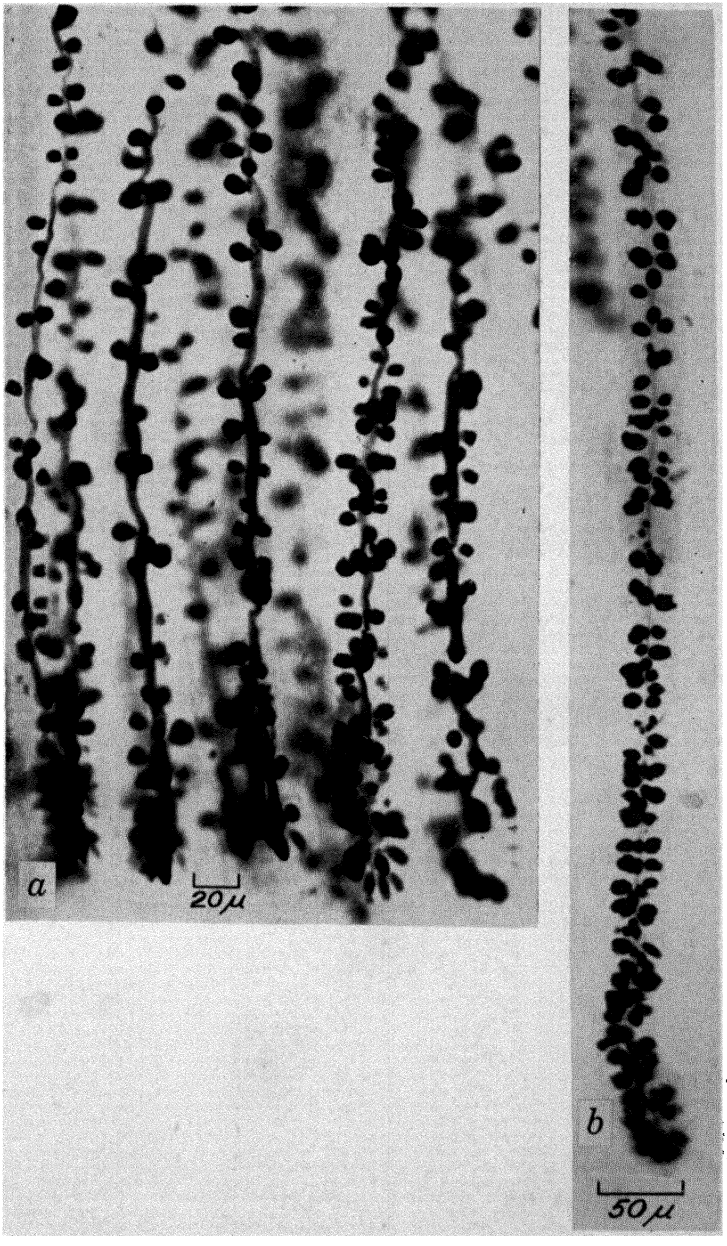


PLATE III. Whole mounts of crypts. Periodic acid-Schiff staining. (a) Intact crypts from the small intestine of a cat showing variations from crypt to crypt. Such large amounts of mucus in the lumen are not common. (b) Intact crypt from the duodenum of a pig. Groups of small thecae.

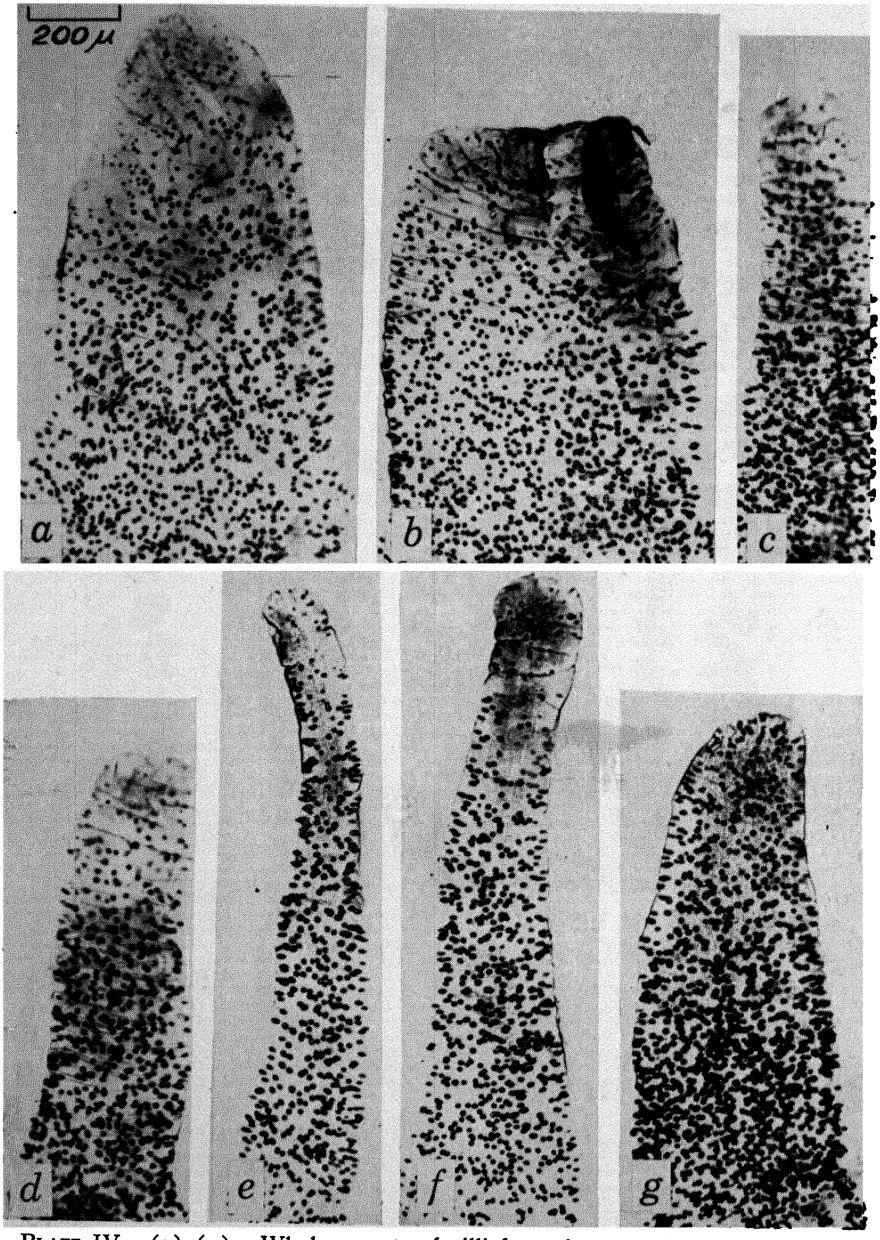


PLATE IV. (a)-(g). Whole mounts of villi from the upper half of the small intestine of a non-fasting cat. Periodic acid-Schiff staining. The villi are set out in rows according to their localization. (a) shows a villus 3 cm. from the pylorus, (g) one in the middle of the small intestine. Same magnification of all villi. In the basal and middle parts of the villi the thecae are large, diminishing near the top. (a) and

of thecae with a minimum diameter $\leqslant 3\mu$ in the crypts of the small intestine of a cat, whereas the curves indicate the number and distribution of all thecae. On the villi, there are almost exclusively large thecae, except in the vicinity of the tops where the size decreases more or less rapidly (Plates IV and V).

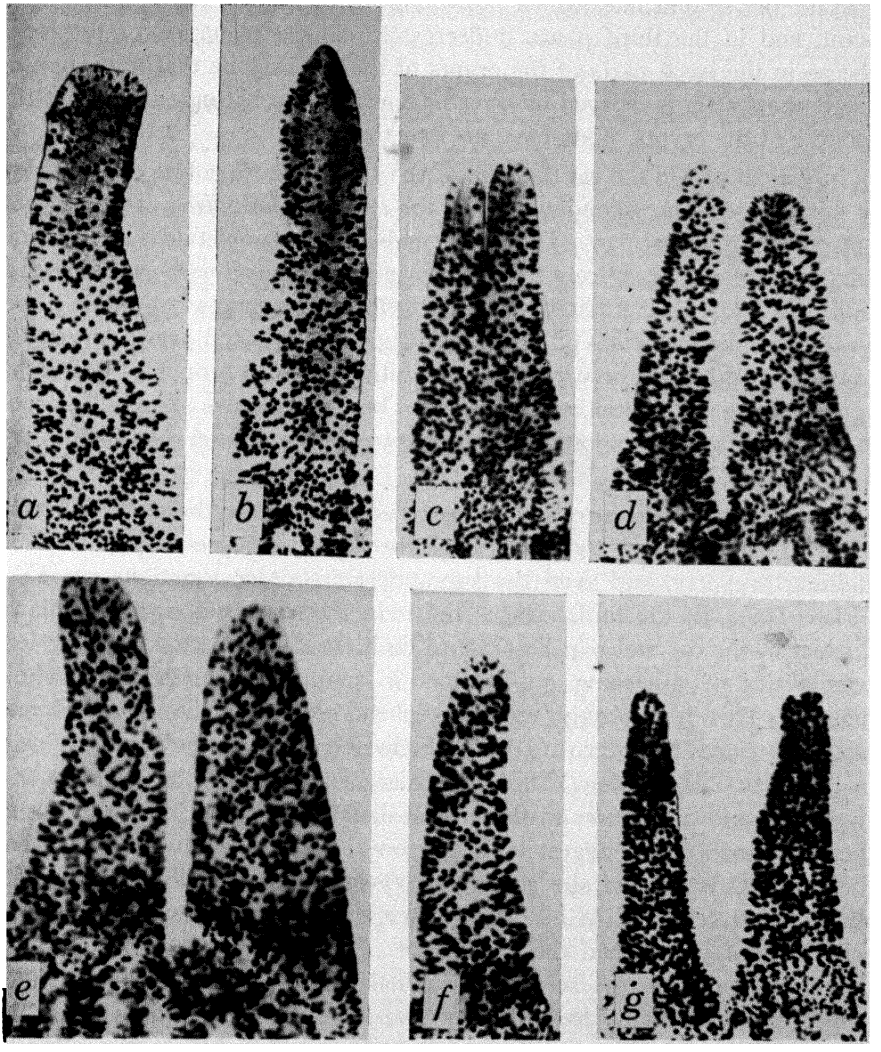


PLATE V. (a)-(g). Continuation of Plate IV. Whole mounts of villi from the distal half of the small intestine. (a) shows a villus near the middle of the small intestine, (g) one a short distance proximal to the caecum. Same magnification as Plate IV. The density of goblet cells is most marked in the distal parts of the intestine.

These findings in the cat and in the rat support the theory that goblet cells are cells *sui generis* and that they pass through only one cycle lasting from their formation in the basal part of the crypt until they perish in some way in the vicinity of, or on the top of the villus. During the migration, the cycle shows three phases. In the first phase the theca increases in size, in the second one, which lasts longest, the thecal size is fairly constant, and in the third phase it decreases. The explanation of the large thecae in the basal parts of the crypts of the cat may be that migration of the deepest cells is delayed or directed towards the bottoms instead of the mouths of the crypts. Cats have no Paneth cells.

In the cat and in the rat the area of the crypts where goblet cells develop is fairly small, corresponding not to the entire mitotic area of the crypts, but only to its basal part. This might indicate that goblet cells and columnar cells are not of entirely the same origin, but that a certain differentiation of the "undifferentiated" dividing cells in the crypts has taken place. In the pig, the formation of goblet cells appears to extend over a large area of the crypts, but the occurrence of the initial stages in groups (Plate IIIb) again indicates a certain independence. The more mature stages also occur in groups, often with a longer distance between the related cells than in the initial stages.

A characteristic feature is the common occurrence in the crypts of pairs of small thecae. Such twin cells have thecae of the same shape, size, and staining property, and even the supranuclear staining reactions are alike (Plate IIc). In the initial stages the twin thecae, which are particularly distinct in the cat and pig, lie close to each other. Sections afford far less possibilities of disclosing goblet cells in pairs and groups than whole mounts. This is probably why this phenomenon has not received any attention since Bizzozero (1892) observed mucus-producing twin cells in the rectum of the dog. The occurrence of twin thecae in the regenerative zones and differences in the mutual distance between small and larger thecae in such pairs suggest both that one is dealing with the initial, not the terminal, stages of the goblet cell cycle and that mucogenesis starts shortly after cell division has taken place, so that the division of adjacent cells has not yet pushed the cells apart. It is not yet clear whether the crypts may also be the scene of terminal stages. The question whether goblet cells can divide has occupied many workers, most of whom have come to the conclusion that they cannot. Discussing this problem, Macklin and Macklin (1932) do not consider it likely that mitosis and production of secretion take place at the same time. A few authors claim that they have observed mitosis of goblet cells in or near the bases of intestinal

crypts (Sacerdotti, 1894; Bizzozero, 1889, 1892; Zipkin, 1903; and others).

In the interpretation of goblet cells as cells *sui generis*, developed from independent elements, it is important to know whether formation can be ruled out in areas outside the regenerative zone in the crypts. In a number of whole mounts from fasting as well as nonfasting cats and rats, no small thecae were observed on the middle half or more of the distance from the bottoms of the crypts to the top of the villi, so that formation of goblet cells within this area may be ruled out. In other instances, there are small thecae, but so few as not to exceed 0.5–1% of all goblet cells within the area mentioned. In a few cases a fairly large number of small thecae has been observed within a limited part or in the major part of the small intestine. At the same time, however, a good many of the goblet cells show mucus extruding from the stoma. Whether this indicates a real activity or is merely a manifestation of postmortem reactions, it is the variability of discharge that seems to be responsible for the varying size of the thecae.

The presence of small thecae is, of course, not tantamount to a formation of goblet cells. Terminal stages or a depression of the cycle also result in small thecae. Goblet cells formed in the basal parts of the crypts may presumably pass through part of a cycle before they are caught by the upward epithelial movement and thus enter into a terminal stage before reaching the upper part of the villus.

Thus, findings in PAS-stained whole mounts of crypts and villi suggest the interpretation that the goblet cells of the cat and rat are formed in the regenerative zone of the crypts and probably not elsewhere. Under physiological conditions, the goblet cells seem to pass through one cycle, but pending studies of other animals and more experimental work we cannot say whether this applies universally.

Of course, the theory that goblet cells are cells *sui generis* does not exclude the occurrence of intermediate varieties with characteristic cytological features as a link in the cytomorphosis. Studies of the number and distribution of such cells in relation to the number and distribution of newly formed and mature goblet cells would seem to be valuable in the elucidation of this problem.

4. *Quantity and Distribution of Goblet Cells*

For years the distribution of goblet cells has been known in its broad features, as it appears at an estimate or after a few determinations of the number of columnar cells between two goblet cells. In numerous species, the quantity of goblet cells has been found to be larger in the distal than in the proximal parts of the intestinal canal; moreover, goblet

cells have been found to be more numerous in the crypts than on the villi and on the colonic surface (cf. Martin, 1910; Clara, 1926; Patzelt, 1936). In the crypts some species have most of the goblet cells in the lower part, others in the middle, and others again in the upper part (Bizzozero, 1889, 1892; Zipkin, 1903; Martin, 1910; Clara, 1926; Kauko, 1928; and others). According to Kauko and other workers the villi show fewer goblet cells near the top than near the base. Kauko did not restrict himself to an estimate, but counted the goblet cells in sections of the small and large intestine. Although his material is limited (a rabbit, a guinea pig, and a mouse) it indicates an orderly distribution of goblet cells from the bottoms of the crypts to the tops of the villi or to the surface of the colon.

On the basis of estimates it is also frequently stated that the number of goblet cells, particularly on the villi, increases greatly during starvation (Paneth, 1888; Beguin, 1904; Williams, 1943; cf. also Macklin and Macklin, 1932), and that it is higher in carnivorous than in herbivorous animals (Martin, 1910; and others).

Detailed data regarding the quantity and distribution of the goblet cells do not appear to have been published. The literature does not tell us anything about normal variations from site to site in the intestine or about the individual variations within the same species, although these are factors of great importance in functional investigations and in comparing the findings from different species.

Two procedures may be chosen in mapping the cells of the small intestine. One is to record them in tissue sections and the other to map them by means of intact crypts and villi. Until recently, only the former procedure was used; it has been employed, e.g. in recording mitoses and argentaffin cells. What may make cell counts in sections inaccurate is mainly that many cells are fragmented. In addition, orientation is a problem, and it is extremely cumbersome to obtain total cell counts for structural units such as crypts and villi. Conclusions based on the relative figures obtained by cell counts in sections must be regarded with particular reserve when the epithelium of the villus and of the crypt is considered as a whole, because the ratio of the total number of epithelial cells on villi and in crypts is unknown. In calculating the rate at which the intestinal epithelium is renewed, Leblond and Stevens (1948) do not take this fact into consideration, interpreting the percentage of mitotic rate in the epithelium from the bottom of the crypt to the top of the villus, where both are cut throughout their length, as the mitotic rate in the entire epithelium. Of course, this may be right, but it presupposes particular ratios of crypt to villus epithelium as regards cell number and size.

The other procedure records the cell types in preparations of intact

villi and crypts after the cells have been stained and the tissue rendered transparent. The main advantages of this method are that the cells are not cut, that the orientation is easy and accurate, and that whole crypts and villi can be analyzed. Its use is limited *inter alia* by difficulties concerning the staining. For goblet cells (Moe, 1952) it is most often well-suited, for argentaffin cells it seems applicable (Moe, unpublished observation), and in certain circumstances columnar cells may be recorded by this method (cf. Plate IIe).

The suitability of intact crypts and villi instead of the epithelial bands of the sections depends on how uniform and regular these structures are. In the cat, the crypts are applicable for purposes of recording, whereas the villi often vary so much in size and shape (Plates IV and V) that in some instances it is more appropriate to record the findings in a uniform, broad strip from the base to the top. In the rat, the villi are far more regular in shape (Plate IId) affording the possibilities of good results in total recording as well as in recording in strips.

In a study of the number and distribution of the goblet cells in the small intestine of the cat (Moe, to be published) to be dealt with below, the total content of goblet cells in the crypts was recorded for each 40μ, starting from the bottom. The goblet cells on the villi were counted in transverse girdles around the entire villus or, when using a strip of the surface, in fields of suitable size from base to top. But the figures obtained (like the results of section studies) carry an uncertainty due to the plastic variation in the size of crypts and villi according to the degree of the intestinal distention, of the contraction of intestinal muscle, and of the amount of blood, lymph, and tissue fluid in the mucosa (Johnson, 1912-13; von Kokas, 1930; Wolff-Heidegger, 1939; Hamperl, 1940; Wells, 1940). This calls for parallel determination of the ratio of goblet cells to other epithelial cells, whenever these factors are expected to disturb the result.

The direct questions that arise regarding the occurrence of goblet cells in the small intestine are: (1) How many goblet cells are there in crypts and on villi? (2) What is their distribution from the bases of the crypts to the tops of the villi? (3) Which are the variations within limited areas of the intestinal mucosa? (4) Which alterations occur from one end of the intestine to the other? (5) Are there any individual differences?

The total number of goblet cells in the crypts varies within limited areas of the mucosa as seen from Plate IIIa. In such areas, the count in crypts with an abundance of goblet cells is about twice that in the crypts with few cells. The variations have little bearing on differences in the length of the crypts, whereas they seem to depend to a considerable extent

on their width. [For information about the morphology and dimensions of the crypts and villi, the reader is referred to Heidenhain (1940)].

Characteristic changes are observed down through the small intestine. The counts are lowest in the proximal third, where the findings are fairly uniform, variations over long distances (with the possible exception of the proximal part of the duodenum) being of similar extent to that mentioned above in the case of limited mucosal areas. As will be seen below, this uniformity also applies to the goblet cells on the villi and to their distribution from the bottoms of the crypts to the tops of the villi. This part of the intestine, therefore, seems well-suited for comparing the findings from cat to cat, and for experimental removal of samples from the same cat from time to time. In the middle part of the intestine, the cryptal content of goblet cells increases in the distal direction, reaching double or triple the initial level in the distal third. An example is given in Table I, where unbranched crypts from various sites of the intestine (abscissa) are distributed according to their content of goblet cells (ordinate). In the distal part of the intestine, the appearances are not always as uniform as indicated by this example.

As might be expected, the total number of goblet cells of the villi within limited mucosal areas varies primarily according to the dimensions of the villi. Usually, the count is between 500 and 1000, but up to 2000 goblet cells may be present on one villus. The total count is, however, of limited interest owing to the marked variations in the size of the villi (Plates IV and V). Instead, in the present study goblet cell counts have been made within surface areas that are, as far as possible, uniform, giving the density of the goblet cells. This reveals that down through the small intestine, the density of goblet cells on the villi largely follows the cryptal content of goblet cells. This may be illustrated by an example (Fig. 2) in which the curves from left to right show the number of goblet cells (ordinate) within successive squares of the surface, $80 \times 80\mu$, from the bases to the tops of the villi (abscissa). Each curve represents an average of several villi from a limited area of the mucous surface, starting with Curve 1 close to the pylorus and finishing with Curve 9 just proximal to the caecum. Curve 6 represents the middle of the small intestine which was 120 cm. long. The tops of the villi are not included. The density of the goblet cells is seen to remain almost unchanged throughout the proximal part of the intestine, then increasing to reach three times the initial count in the distal third.

Owing to the regeneration and migration of the intestinal epithelium, particular interest is attached to the mapping of goblet cells from the bottoms of the crypts to the tops of the villi. Such determination of their dis-

TABLE I

CRYPTS FROM THE SMALL INTESTINE (180 CM LONG) OF A CAT (S26) ACCORDING TO CONTENT OF GOBLET CELLS

Number of goblet cells	Number of crypts								
230							1	2	
220							1		1
210									
200							1	1	1
190							2	1	1
180							4	4	4
170						1	6	6	1
160						2	1	4	
150						5	3	2	2
140	1				1	3			
130	1					2	1		
120	1		1		4	4			
110					2	1			
100	1	1	3	2	7				
90	2	3	2	6	5	1			
80		9	7	5	1				
70	8	4	5	6					
60	6	3	2	1					
50									
Distance from Pylorus in Centimeters	1–4	15–20	35–40	50–55	75–80	95–100	115–120	140–145	160–165

tribution vertically through the intestinal mucosa of the cat shows the largest number in the first 80μ of the crypts. Thereupon, the number decreases, first rapidly and then gradually more slowly until it becomes almost constant in the vicinity of the cryptal mouth. On the villi, the density sometimes decreases slowly towards the top, whereas in others it remains practically uniform until reaching the vicinity of the top, where it usually decreases. This striking regularity in the distribution of goblet cells, applies to the entire small intestine, from one end to the other, as is apparent from Figs. 1 and 2 showing crypts and villi, respectively, from

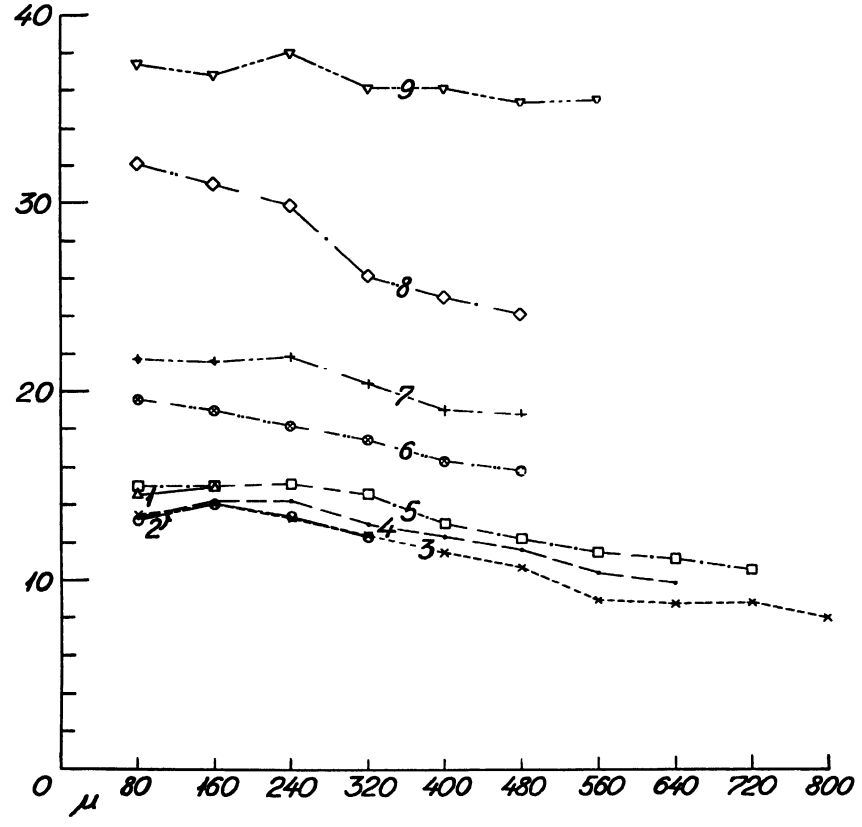


FIG. 2. Distribution and number of goblet cells on the villi from a cat (S21), beginning at the base to the left and recording each 80μ (abscissa). The tops of the villi are not included. The number of goblet cells (ordinate) is given for surface squares, 80 × 80μ. Each curve represents the average of many villi from a limited area of the mucosa, starting with Curve 1, a little more than 2 cm. distal to the pylorus and finishing with Curve 9 about 10 cm. proximal to the caecum. Curve 6 is from the middle of the 120 cm. long small intestine.

the same cat. The curves in Fig. 1 indicate the number of thecae (ordinate) from the bases to the mouths of the crypts, calculated for each 40μ (abscissa). Each curve represents an average of 10 or 20 crypts from the same areas of the mucosa that were used in Fig. 2. As mentioned above, Fig. 2 gives the number of goblet cells on the villi recorded in successive squares of 80 $\times$ 80μ, from the bases up to the vicinity of the tops.

A slight decrease in the goblet cell count up through the crypts may be due to the usually somewhat decreased diameter of the crypts towards the mouths. The question as to what extent this fact and possible alterations of the cell width influence the results calls for determination of the ratio of goblet cell count to the number of other epithelial cells; this has not been attempted in the present work. In the curves representing the villi, minor corrections may be required because the total number of epithelial cells within the surface units is hardly constant. None of these corrections will, however, be able to alter the fact that the goblet cell count in the cat is highest in the deep parts of the crypts, then decreases rapidly until reaching a constant or slowly declining level through the upper half of the crypts and further up the villi.

The curves representing the distribution of goblet cells are assumed to represent the activity of the intestinal epithelium as regards the formation and disappearance of cells. Goblet cells are most ample in the deep portion of the mitotic area and, judging by the thecal size (cf. the columns in Fig. 1), at the site where goblet cells are formed. The marked decrease in the number of thecae takes place in an area which still abounds in mitoses and where the formation of goblet cells is rapidly decreasing.

A possible interpretation may be that the goblet cells formed in the deeper part are pushed apart during migration by other cells that arise by division higher up in the crypts. In that case, there would be in the cat two generative zones that perhaps overlap; a deep one for goblet cells and another one higher up for ordinary columnar cells (Moe, 1953). It would be interesting to determine the distribution of mitoses up through the crypts in order to elucidate this hypothesis. The decline in the distribution curve might also be due to disappearance of goblet cells during the first phase of the migration. No evidence has, however, been advanced of a disappearance so marked as to explain the recorded course of the curves.

The practically uniform distribution of goblet cells from the middle of the crypt up to the middle of the villus seems to indicate that in this area there is approximately a state of equilibrium in the ratio of goblet cells to other cells. Judging by the thecal size, there is no significant increase or decrease in the number of cells in this area. In the proximal part of the

intestine and on long villi, goblet cells often decrease markedly in number at the top gradually as the thecal size decreases (Plate IV). Numerous cells can be recognized as former goblet cells only by the scarce residual material that takes the periodic acid-Schiff stain.

It must be emphasized that the above-mentioned results regarding the distribution of goblet cells are based on the average findings within limited mucosal areas. Although the individual crypts and villi in such an area seldom differ essentially from the average, there are differences of a minor and moderate extent, differences which, like the thecal sizes, might be of interest in the study of the possible rhythms of regeneration and function of the intestinal goblet cells. But no details are known regarding these problems, and the writer will restrict himself to stating that the number of goblet cells in a given area, e.g. the basal part of the villus, represents not only what is going on in the epithelium at the moment of fixation, but expresses also what has taken place previously in the crypts up to the time of the formation of the cells. Increasing or decreasing activity in the formation of goblet cells in relation to the regeneration of the other epithelial cells must, therefore, *ceteris paribus,* be assumed to manifest itself in corresponding alterations in the course of the curve representing the distribution of goblet cells.

Short mention will be made of the individual variations as seen in the proximal part of the small intestine. As far as the crypts are concerned,

TABLE II

CRYPTS FROM THE UPPER THIRD OF THE SMALL INTESTINE OF 10 CATS, ACCORDING TO CONTENT OF GOBLET CELLS*

Cat	Number of crypts																
S21						2	3	8	15	17	16	11	3	2	2	1	
S17					6	6	8	19	9	9	1	2					
S22					7	9	10	13	5	3	2			1			
S19				2	5	13	13	9	4	3			1				
S26				12	23	21	13	7		2	1	1					
S18		5	15	16	1	2											
S24			1	5	7	13	9	10	8	6		1					
S25			2	5	12	15	7	7	1								
S28			6	15	15	3	1										
S27	2	6	11	5	11	4	1										
Number of Goblets	20	30	40	50	60	70	80	90	100	110	120	130	140	150	160	170	

*The first 6 cats were starved for 48 hours; regarding milk, however, only for 24 hours The last four cats were not fasting.

marked differences occur in the total content of goblet cells. This is evident from Table II in which crypts from the upper third of the small intestine of 10 cats—the first 6 fasting and the last 4 not fasting—are set out according to their content of goblet cells. The crypts contain an average of from about 50 to 120 goblet cells in fasting cats and from about 50 to about 90 in nonfasting cats. The variations in goblet cell density on the villi are considerably less marked within each of the two groups, as is evident from Table III, showing the average goblet cell count in a surface

TABLE III

AVERAGE NUMBER OF GOBLET CELLS PER 6400 SQUARE μ OF VILLUS SURFACE IN THE UPPER THIRD OF THE SMALL INTESTINE FROM THE SAME 10 CATS AS IN TABLE II

Cat	80–160 μ from base of villus	160–240 μ from base of villus
S21	14.3	14.0
S17	14.2	13.6
S22	12.2	11.8
S19	13.2	13.1
S26	12 6	12.7
S18	14.5	14.4
S24	11.2	10.7
S25	10.9	10.0
S28	8.0	8.4
S27	8.0	8.1

area, 6400μ^2, 80–160μ and 160–240μ from the base. The counts for S18 are presumably too high as compared with those of the other cats, as an unusually marked contraction of the villi was found in this animal. Such a contraction produces transverse grooves on the surface of the villi (Plate IVc, d) and sections show wrinkling of the epithelium (cf. the tops of the villi Plate Ia, b). Part of the apparent disproportion between the degree of the variations for villi and for crypts may be due to differences in crypt dimensions. Within the same area of villus surface, there were, as apparent from Table III, fewer goblet cells in nonfasting than in fasting cats. This cannot, however, be taken as direct evidence that goblet cells increase in number during starvation, as it is not known whether swelling of the villi occurs during digestion in the same way as it has been observed during secretion (Wells, 1940). The average distribution of goblet cells in crypts and on villi in the upper third of the small intestine is shown in Figs. 3 and 4 illustrating the findings in the 6 fasting cats. Fig. 3 gives the distribution in the crypts, the number of goblet cells being shown on the ordinate and their sites in the crypts on the abscissa. Fig. 4 illustrates the distribution from the bases to the tops

of the villi, the number of goblet cells per 6400 square μ of the surface (ordinate) being calculated for each 80μ (abscissa). Apart from minor differences in the rate at which the goblet cell count decreases up through the crypts, the average distribution in the crypts and on the villi is the same in all 6 cats.

Our knowledge regarding the epithelium of the small intestine is still deficient in fundamental respects, particularly as regards the functional

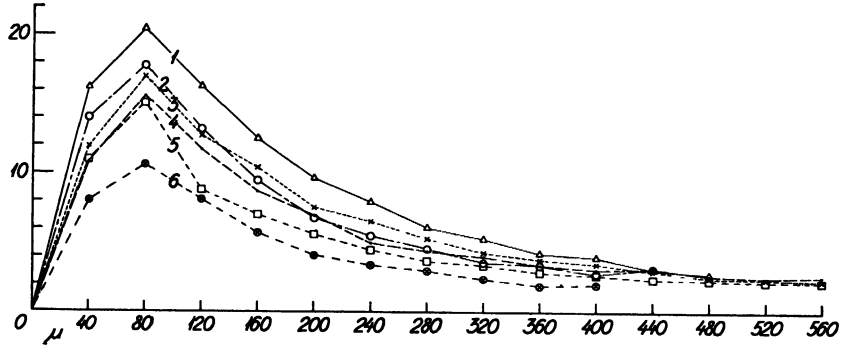


FIG. 3. Diagram showing the average distribution of goblet cells in the crypts of approximately the upper third of the small intestine from 6 fasting cats. The ordinate shows the number of goblet cells recorded for each 40μ of the crypts (abscissa), beginning at the base of the crypt.

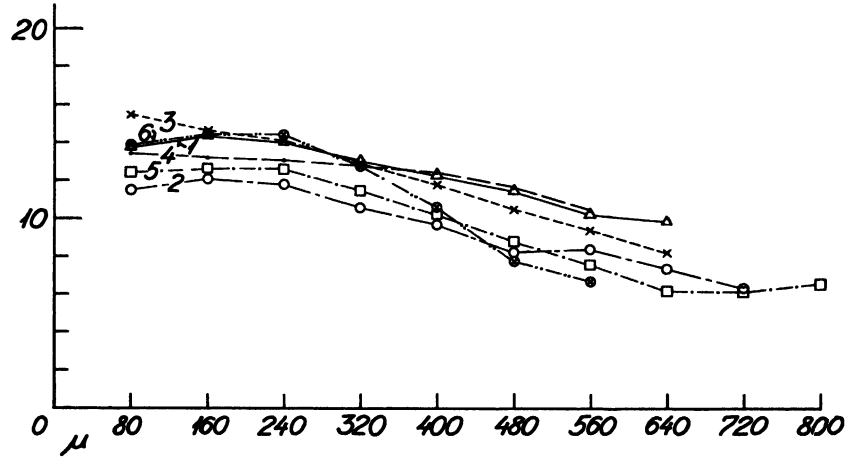


FIG. 4. Average distribution of goblet cells on villi from upper third of the small intestine from the same 6 cats as Fig. 3. The number of goblet cells (ordinate) is calculated in surface areas 6400 square μ, for each 80μ of the villi, starting at the base (abscissa).

reactions. An increased knowledge of the occurrence and frequency of the different cell types under varying conditions, of mitoses (cf. Bullough, 1948), and of nuclear classes (cf. Bergemann, 1952), might perhaps solve some of the obscure aspects. The observations reported in this paper were designed to contribute to such quantitative knowledge which, considering the special regenerative properties and the migration of the intestinal epithelium, could be elaborated with success.

5. *Functional and Pharmacological Aspects of Goblet Cells*

Little has been published regarding the reaction of the goblet cells to the intake of food. Fischer and Ritter (1953) have recently studied differences between starving and fed frogs. In starving animals there was still a number of goblet cells in the process of discharging mucus, but half the intestinal goblet cells were completely filled. As early as 10 minutes after feeding was started, the discharge increased, reaching a maximum activity about 40 minutes later. In the course of 6 hours, the original state had been re-established.

As already mentioned, many workers have found an increase in the number of goblet cells during starvation. It is not known whether this is due to the formation of a relatively larger number of goblet cells than other cells during starvation than during feeding or whether the explanation is merely that more goblet cells discharge their contents when the digestive process is active than during starvation. In fed cats, as already mentioned, goblet cells were less numerous per surface unit of villi than in fasting cats (Table III). The differences are not, however, so marked that this may be taken to mean that goblet cells are more numerous during starvation, as the number of other cell types within the same area is unknown. About the same applies to the goblet cells in the crypts. Perhaps, cats are not suitable experimental animals for studies of this nature. It is strange that goblet cells appear to be more abundant in carnivorous than in herbivorous animals (Martin, 1910; and others). No explanation has been offered, but it may suggest that the function of the goblet cells is something beyond producing substances that in a purely mechanical way facilitate the passage of the feces, even though this is probably an essential function in the most distal parts of the intestine, where the feces are of relatively firm consistency. In the small intestine, where the intestinal contents are fluid, the lubricant properties of the mucus cannot be of much importance, as pointed out by Florey (1933). Applying tiny particles of graphite to the mucous membrane of the small intestine, Florey found that the mucous secretion has as one of its functions the cleansing of the villi from small adherent particles. This is accom-

plished by the movements of the villi and other intestinal motor activities which bring the particles into contact with the sticky mucus secreted by the goblet cells and present as a lacelike meshwork over the mucosal surface. The mucus with adherent particles is then rolled up into small masses by the intestinal movements and propelled onwards by peristaltic action of the bowel. The mucus is, thus, a factor of the utmost importance in the cleansing of the bowel, and (according to Florey) thereby exerts a kind of antibacterial function.

Nothing so far seems to be known regarding a possible role of mucus in the digestive processes.

Certain histological studies indicate that several types of goblet cell mucin are present in the intestinal canal (Lillie, 1949; Boni *et al.*, 1953). For further elucidation of these findings, it would be desirable to improve the possibilities of characterizing the histologically demonstrable mucous substances.

Which are the forces and mechanisms that regulate the activity of goblet cells? This is an important question and, in an extended sense, one that concerns the forces behind cell division, differentiation, regulation of the quantitative ratio between the cell types, synthesis and discharge of secretions, etc.; in a stricter sense it is a question regarding the actions that induce the discharge, and it is in this field that some information is available. The simplest way conceivable is that once the goblet cell has developed, it goes through life autonomously, synthesizing and storing mucus until it overflows and discharges its contents or perishes. Perhaps many goblet cells lead such a mechanical life, at any rate when they are left, so to speak, to themselves. Goblet cells possess considerable autonomous power. This has been evidenced by the fact that even though cell division and epithelial migration is arrested in the small intestine of the rat by X-irradiation, the differentiation and maturation of the goblet cells continue undisturbed, so that in the course of 24 hours fully developed goblet cells accumulate in the crypts (Friedman, 1945). Friedman, furthermore, observed that as migration out along the villi was re-established, the goblet cells appeared in zonal bands at various levels. An effect similar to that of X-irradiation has been obtained with nitrogen mustard (Webber, Craig, and Friedman, 1951). Even following transplantation of embryonic rat intestine to the anterior chamber of the eye, differentiation of goblet cells continues (Kammeraad, 1942), and in one instance Florey (1930) succeeded in developing goblet cells in a tissue culture of an embryonic chick intestine.

Various studies, primarily those of Florey and associates, have shown that a more active secretory function may be induced by various actions,

some of which may be operative under physiological conditions or at least may be kindred to such conditions. As is well-known, higher animals command two indirect mechanisms which elicit discharge of secretion, the humoral or chemical and the nervous reflex mechanism. In experimental work one may try to elicit, imitate, or inhibit these mechanisms. By stimulating the nervi erigentes Wright, Florey, and Jennings (1938) obtained a triad of reactions in the colon of the cat, viz. lively motor activity, dilatation of the mucosal blood vessels, and discharge of secretion. The same effect is obtained by pilocarpine and acetylcholine following administration of eserine. Atropine inhibits these reactions. Stimulation of the sympathetic nerves causes relaxation of the intestinal muscles, contraction of blood vessels, and elicits no secretion. Since mechanical rubbing may provoke lively secretion (Florey, 1930), the experiments were performed on the opened intestine. The experiments that resulted in secretion were accompanied by characteristic histological changes in the basal parts of the crypts, viz. flattening of the epithelium resulting in considerable dilatation of the lumen and marked discharge of mucus from the previously well-filled goblet cells; in the upper part of the crypts, on the other hand, the goblet cells were considerably less affected. The same histological appearance is observed following application of mustard oil in olive oil to the surface of the colon; this injures the surface epithelium and causes the goblet cells in the crypts to discharge their secretion (Florey, 1930; Florey and Webb, 1931). In the small intestine, mustard oil reduces the number of goblet cells in the crypts as a result of discharge, but there is no epithelial flattening or luminal dilatation. Huber (1945) has studied the effect of the alkaloid delphinine on the colon of albino mice. In most cases he observed complete discharge of the mucus from the goblet cells in the deep and middle portions of the crypts, whereas those in the necks of the crypts and on the surface seemed unaffected. The emptied goblet cells quickly resume the formation of mucus and become completely restituted in 12 hours. In Florey's experiments using mustard oil on the cat, the recovery stage took 24–48 hours. Florey and associates came to the conclusion that the goblet cells at the bases of the crypts undergo discharge and exhibit their cycle of changes before those nearer the surface that remain well-filled despite powerful stimulation.

Nothing seems to have been published regarding the nervous regulation of the goblet cells of the small intestine. A hormone called enterocrinin, with a secretagogue effect on the small intestine, has been described by Nasset and co-workers (Nasset, 1938; cf. also Babkin, 1950; and Grossman, 1950), but no experiments with this hormone appear to have been combined with an investigation of the goblet cells.

The effect of pilocarpine on the duodenum of the guinea pig has been studied by Schumann (1936). He found pilocarpine to induce emptying of most goblet cells, but since it also stimulates the synthesis of mucus, the goblet cells had refilled in two hours. According to Schumann, histamine also effects an emptying of the goblet cells, but without stimulating the synthesis. Florey (1930), on the other hand, found no effect of histamine on the goblet cells in the cat colon.

To sum up, the intestinal goblet cells can be induced to give off secretion by mechanical action upon the intestinal surface and by injury to the latter due to application of chemical agents. In addition, they may indirectly be induced to give off their mucus, but workers who have inspected the intestinal mucosa at the same time have observed simultaneous violent contractions of the muscles and dilatation of the mucosal vessels. The significance of these phenomena attendant to the process of discharge is not yet clear. The continual shifting of the mucous membrane due to contractions of the muscularis externa and the muscularis mucosae can probably not be disregarded as an eliciting factor, even though the mechanical rubbing of the mucosal surfaces against each other is prevented when the bowel is cut open. And increased amounts of fluid in the stroma, due to vasodilatation, might be supposed to entail stretching of crypts and swelling of villi (cf. Wells, 1940) with resulting epithelial flattening which perhaps facilitates the emptying of the goblet cells.

To these problems are added those raised by the migration of the intestinal epithelium. Cells situated at a certain level in the crypts or on the villi, e.g. 24 hours after the administration of a secretagogue, cannot be taken to show the degree of restitution for cells of this localization at the moment of the action, but for more deeply placed cells or, if the localization is near or in the zone of regeneration, for cells that were perhaps not yet formed! Whereas this is of considerable interest in the small intestine where epithelial regeneration is rapid, it may be less significant in the colon, judging by Huber's (1945) findings of the mitotic rate in this part of the intestine. It may be added, however, that not only cell division in the crypts, but also epithelial desquamation from the surface are presumably accompanied by migration.

This is of course not saying that the various secretagogues exert no direct effect on the goblet cells, but only that probably it is difficult to obtain a clear idea of the functional reactions of the goblet cells and the underlying mechanisms without elaborating our knowledge of the reactions of the intestine and intestinal epithelium as a whole.

Acknowledgments

This work was aided by grants from King Christian the Tenth Foundation and The Danish State Research Foundation.

IV. References

Babkin, B. P. (1950) "Secretory Mechanism of the Digestive Glands," 2nd ed. Hoeber, New York.
Beguin, F. (1904) *Arch. Anat. microscop.* **6**, 385.
Bensley, R R. (1908) *Anat. Record* **2**, 92.
Bergemann, T. (1952) *Z. mikroskop. anat. Forsch.* **58**, 196.
Bizzozero, G. (1888) *Anat. Anz.* **3**, 781.
Bizzozero, G. (1889) *Arch. mikroskop. Anat. u Entwicklungsmech.* **33**, 216.
Bizzozero, G. (1892) *Arch. mikroskop. Anat. u. Entwicklungsmech.* **40**, 325.
Bizzozero, G. (1893) *Arch. mikroskop Anat. u. Entwicklungsmech.* **42**, 82.
Boni, M., Caprino, G., and Virno, M. (1953) *Bull. Assoc. Anat.* **75**, 590.
Bullough, W. S. (1948) *Proc. Roy. Soc.* **B135**, 212.
Cajal, S. Ramon y (1915) quoted from Nassonov (1923).
Chlopkow, A. (1928) *Z. Zellforsch. u. mikroskop. Anat.* **7**, 502.
Clara, M. (1926) *Z. mikroskop. anat. Forsch* **6**, 256.
Cloetta, M. (1893) *Arch. mikroskop. Anat. u Entwicklungsmech.* **41**, 88.
Dalton, A. J., Kahler, H., and Lloyd, B. J. (1951) *Anat. Record* **111**, 67.
Dornesco, G.-Th., and Valverde, R.-E. (1930) *Compt rend. soc. biol.* **103**, 684.
Duthie, E. S. (1933) *Proc. Roy. Soc.* **B113**, 459
Eklöf, H. (1914) *Anat. Hefte* **51**, 1
Fischer, M. H., and Ritter, U. (1953) *Pflügers Arch. ges. Physiol.* **257**, 161.
Florey, H. (1930) *Brit. J. Exptl. Pathol.* **11**, 348.
Florey, H. (1931) *Brit. J. Exptl Pathol* **12**, 301.
Florey, H. (1932) *Brit. J. Exptl. Pathol.* **13**, 349.
Florey, H. W. (1933) *J Pathol. Bacteriol.* **37**, 283.
Florey, H., and Webb, R. A. (1931) *Brit. J. Exptl Pathol* **12**, 286.
Friedman, N. B. (1945) *J. Exptl. Med.* **81**, 553.
Galeotti, G. (1895) *Intern. Monatsschr. Anat. Physiol.* **12**, 116.
Gersh, I. (1949) *Arch. Pathol.* **47**, 99.
Glick, D. (1949) "Techniques of Histo- and Cytochemistry." Interscience, New York.
Gomori, G. (1952) "Microscopic Histochemistry." Univ. of Chicago Press, Chicago.
Grossman, M. I. (1950) *Physiol. Revs.* **30**, 33.
Hamperl, H. (1940) *Virchow's Arch. pathol. Anat. u. Physiol* **305**, 432.
Heidenhain, M. (1940) *Z. mikroskop. anat. Forsch.* **47**, 522.
Hotchkiss, R. D. (1948) *Arch. Biochem.* **16**, 131.
Huber, P. (1945) *Vierteljahrsschr. naturforsch. Ges. Zürich* **90**, Beihefte 4.
Johnson, F. P. (1912-13) *Am. J. Anat.* **14**, 235.
Kammeraad, A. (1942) *J. Exptl. Zool.* **91**, 45.
Karasek, H. (1933) *Z. Zellforsch. u. Mikroskop. Anat.* **20**, 206.
Kauko, I. (1928) *Ann. Acad. Sci. Fennicae, Ser. A* **27**, No. 7.
Keiser-Nielsen, H. (1953). "Mucin," Thesis. Dansk videnskabs forlag, Copenhagen.
Kokas, E. von (1930) *Pflügers Arch. ges. Physiol.* **225**, 416.
Kopsch, F. (1926) *Z. mikroskop. anat. Forsch.* **5**, 221.

Leblond, C. P., and Stevens, C. E. (1948) *Anat. Record* **100**, 357.
Lillie, R. D. (1949) *Bull. intern. Assoc. Med. Museums* **29**, 1.
Macklin, C. C., and Macklin, M. T. (1932) *in* "Special Cytology" (Cowdry, ed.) 2nd ed., Vol. 1, p. 253. Hoeber, New York.
McManus, J. F. A. (1948) *Stain Technol.* **23**, 99.
Martin, F.-P. (1910) Thesis. Dresden.
Meyer, K. (1945) *Advances in Protein Chem.* **2**, 249.
Moe, H. (1952) *Stain Technol.* **27**, 41.
Moe, H. (1953) *Nature* **172**, 309.
Nasset, E. (1938) *Am. J. Physiol.* **121**, 481.
Nassonov, D. N. (1923) *Arch. mikroskop. Anat. u. Entwicklungsmech* **97**, 136
Paneth, J. (1888) *Arch. mikroskop. Anat. u. Entwicklungsmech.* **31**, 113.
Patzelt, V. (1936) *in* "Handbuch der mikroskopischen Anatomie des Menschen" (von Möllendorff, ed.), Vol. 5, Part 3, p. 111. Springer, Berlin.
Pearse, A. G E (1953) "Histochemistry." J. and A. Churchill, London.
Popoff, N. W. (1939) *Arch. Pathol.* **27**, 841.
Romeis, B. (1948) "Mikroskopische Technik." Leibniz Verlag. Munchen.
Sacerdotti, C. (1894) *Intern. Monatsschr. Anat. Physiol* **11**, 501.
Schaffer, J. (1927) *in* "Handbuch der mikroskopischen Anatomie des Menschen" (von Möllendorff, ed.), Vol. 2, Part 1, p. 1. Springer, Berlin.
Schofield, G. (1951) *Acta Anat.* **11**, 414.
Schofield, G. (1952) *Acta Anat.* **16**, 1.
Schofield, G. (1953) *Acta Anat.* **18**, 256.
Schulze, F. E. (1867) *Arch. mikroskop. Anat. u. Entwicklungsmech.* **3**, 145.
Schumann, G. (1936) *Z. Zellforsch. u. mikroskop. Anat.* **24**, 540.
Seillers, R. F. von (1891) *Arch. mikroskop. Anat. u. Entwicklungsmech.* **38**, 177.
Stacey, M. (1946) *Advances in Carbohydrate Chem.* **2**, 162.
Steedman, H. F. (1950) *Quart. J. Microscop. Sci* **91**, 477.
Tschassownikow, S. (1914) *Arch. mikroskop. Anat. u. Entwicklungsmech.* **84**, 150.
Webber, B., Craig, B. R., and Friedman, N. B. (1951) *Cancer* **4**, 1250.
Wells, H. S. (1940) *Am. J. Physiol.* **130**, 410.
Werner, I. (1953) *Acta Soc. Med. Upsaliensis* **58**, 1.
Williams, M. A. (1943) *Anat. Record* **85**, 195.
Wolff-Heidegger, G. (1939) *Z. wiss. Mikroskop.* **56**, 417.
Wright, R. D., Florey, H. W., and Jennings, M. A. (1938) *Quart J Exptl. Physiol.* **28**, 207.
Yokochi, C. (1951) *Arch. histol. Japan* **3**, 37.
Zipkin, R. (1903) *Anat. Hefte* **23**, 113.

Localization of Cholinesterases at Neuromuscular Junctions

R. COUTEAUX

Laboratoire de Biologie animale (P.C.B.), Faculté des Sciences, Université de Paris, Paris, France

		Page
I.	Introduction	335
II.	General Features of Neuromuscular Junctions	336
	1. The Mammalian Motor End-Plate	337
	2. Neuromuscular Junctions of Other Vertebrates	350
III.	Junctional Cholinesterases and Their Histochemical Localization	355
	1. Biochemical Data	357
	2. Histochemical Data	361
	3. Conclusions	373
IV.	References	373

I. Introduction

The morphology and physiology of the neuromuscular junction have long been studied almost independently of each other. This has resulted in two rather different trends in the general interpretation of this junction.

Experimental data on the transmission of excitation from motor nerve to striated muscle have led most physiologists to consider that, for the neuromuscular junction, a satisfactory hypothesis would be the particularly simple one of a "synaptic membrane" separating the cytoplasm of the two excitable elements.

To most histologists on the other hand, the neuromuscular junction appeared from the outset as an extremely complex structure, corresponding not only to the interlocking of structures already existing at other points of the nerve fiber and the muscle fiber, but also to the presence of special structures located exclusively at this level. It is understandable that faced with the tangle of varied and partly original structures which characterize the synaptic areas of striated muscle, many of these authors, even if they adhered to the neuron theory, may have regarded the hypothesis of a synaptic membrane as an oversimplification. Their reserve seems all the more legitimate since, in spite of repeated efforts, histologists like Cajal who had no doubts about the individuality of the nerve cells, were unable to establish positively the existence of a boundary separating the two main components of the neuromuscular junction.

In reality, these failures were not conclusive and were probably largely due to an almost exclusive use of neurofibrillar staining methods, unsuitable for distinguishing cell boundaries. Recent histological research has

in fact shown that a sharp discontinuity can be seen between the branches of the nerve endings and the sarcoplasm at the level of their junction, which to a certain extent justifies the hypothesis formulated long ago by the majority of physiologists.

An agreement on a capital issue would thus begin to appear between morphological and experimental data, so that the schism which has been dividing biologists for so long should come to an end, at least as far as the neuromuscular junction is concerned.

The recent, but already fruitful, introduction of histochemical methods, and, in particular, methods of enzymatic histochemistry in the analysis of nerve-muscle connections is also likely to establish new links between morphological and experimental research.

All the data at present available are derived from the use of the light microscope, but the results already obtained by electron-microscopic examination of certain axo-axonic synapses (Robertson, 1953) show the potential advantages of the electron microscope in the study of the neuromuscular junction. The benefit in this field will be all the greater since the principal structures on which interpretation is based are at the limit of visibility when observed with the light microscope.

It is obviously on the development of histochemical methods and their adaptation to the working conditions of the electron microscope with maximum magnification that the future progress of analysis in this field will mainly depend. The combined use of really specific staining methods and observation instruments with a much higher resolving power than the light microscope will make it possible to come fairly close to the molecular level, the only level at which morphologists, biochemists, and physiologists will be able to solve in collaboration some of the fundamental problems raised by the study of synapses.

This paper which discusses the results hitherto obtained in the localization of cholinesterase activities at neuromuscular junctions will show what is to be expected from further research at submicroscopic level.

II. General Features of the Neuromuscular Junctions

Wagner (1847) was the first to reveal two fundamental features of the neuromuscular junction of vertebrates, when he concluded from his observations on the hyoidean muscles of the frog that the motor nerve fiber, after branching, loses its myelin sheath and pierces through the sarcolemma surrounding the muscle fiber. This suggested the idea of an intimate union of the nerve substance and the muscle substance.

Two equally important characters, although less constant throughout the various types of neuromuscular junctions found in vertebrates, were

discovered subsequently: the first, by Kühne (1862) who showed, also on frog muscles, that, after piercing through the sarcolemma, the motor nerve fiber branches again and thus produces an arborization under the sarcolemma; the second, by Rouget (1862) who reported the presence, in muscles of reptiles, birds, and mammals, of a flattened heap of granular, nucleated substance at the level of the neuromuscular junction, which he interpreted as the spreading of the axis cylinder substance at the surface of the myofibrils and to which he gave the name of end-plate ("plaque terminale"). This term is today often applied, conveniently but improperly, to all neuromuscular junctions, whether or not they present this plate of granular, nucleated protoplasm.

1. The Mammalian Motor End-Plate

a. A Brief Description and General Interpretation. In mammals the presence of a heap of granular nucleated substance at the junction of the nerve fiber and the muscle fiber is almost general. It is the first character of the motor end-plate described in this class of vertebrates. It was observed by Rouget even before the terminal ramifications of the neurite were perceived, since the latter were first described in mammals (in cats) by Krause in 1863, and at that time their very existence could still be disputed by Kühne (1863).

The accumulation of nuclei and granular cytoplasm of the junctional area forms a rounded heap at the surface of the myofibrillar bundle, but is only very slightly in relief when the muscle fiber is not contracted. It is this plate-like heap of protoplasm lying against the myofibrillar bundle and at the level of the ramification of the nerve ending that Kühne called the sole (Fig. 1, Plate Ia).

Some of the various nuclei observed in the vicinity of the nerve ending are closely connected with the terminal nerve branches. These are the nuclei described by Ranvier (1878) under the name of "arborization nuclei". Then there are the "fundamental nuclei" of Ranvier (Kühne's "Sole nuclei," 1864), which are larger and much less stainable than the former and which contain much larger nucleoli. In addition to these two categories of nuclei there are the endothelial nuclei of the blood capillaries, sometimes very closely attached to the end-plate, and lastly, nuclei which are not constantly present and which are linked to the connective coating of the ending ("vaginal nuclei" of Ranvier).

Of these four kinds of nuclei which may thus be observed at junction level, the only ones which are frequently easy to identify are the fundamental nuclei, as these are generally distinguished by their size and especially their structure. But this is not the case for the arborization

nuclei, the endothelial nuclei of the blood capillaries, and the vaginal nuclei, at least in adult muscles. This doubtlessly explains the long, inconclusive debate on the end-plate nuclei between Kühne and Ranvier and the numerous controversies on this subject which have arisen constantly since the end of the last century.

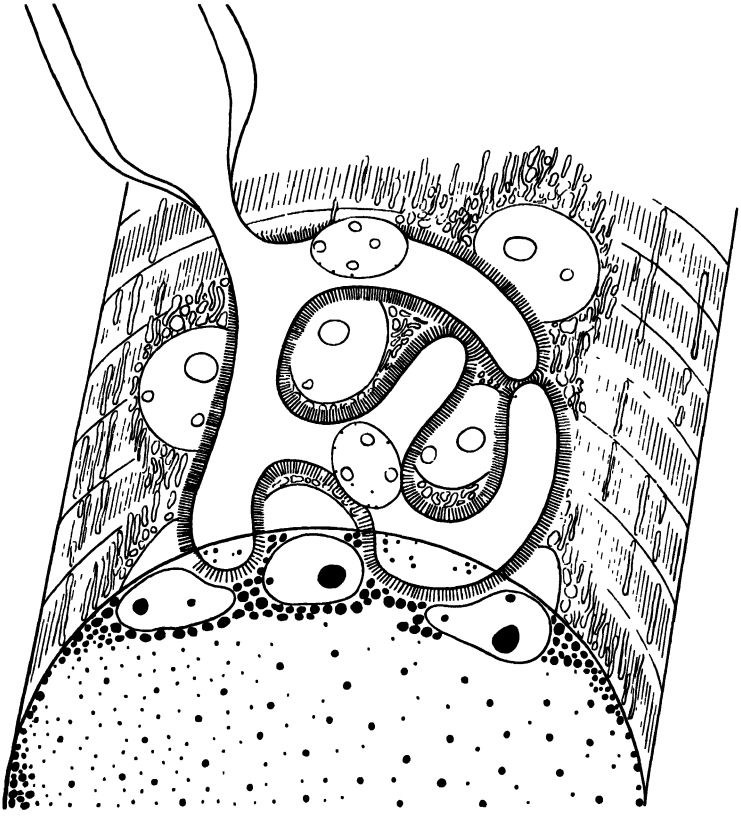


FIG. 1. Schematic drawing of motor end-plate (surface view and cross-section). Only arborization nuclei and fundamental nuclei are represented.

As regards the nature of these nuclei and the corresponding interpretation of the whole motor end-plate, the inconclusive and frequently contradictory results of the extensive morphological research, carried out on the muscles of adult animals, led to an attack on the problem by embryological methods.

If the various phases of the formation of the motor end-plate are followed by methods which give not only intensive staining of the neurites

but also, as is the case with the Bielschowsky-Gros method, very heavy staining of the various nuclei, it is then easy to establish the origin of the fundamental nuclei and the arborization nuclei; the former come from embryonic muscle nuclei, whereas the latter belong to cells of the

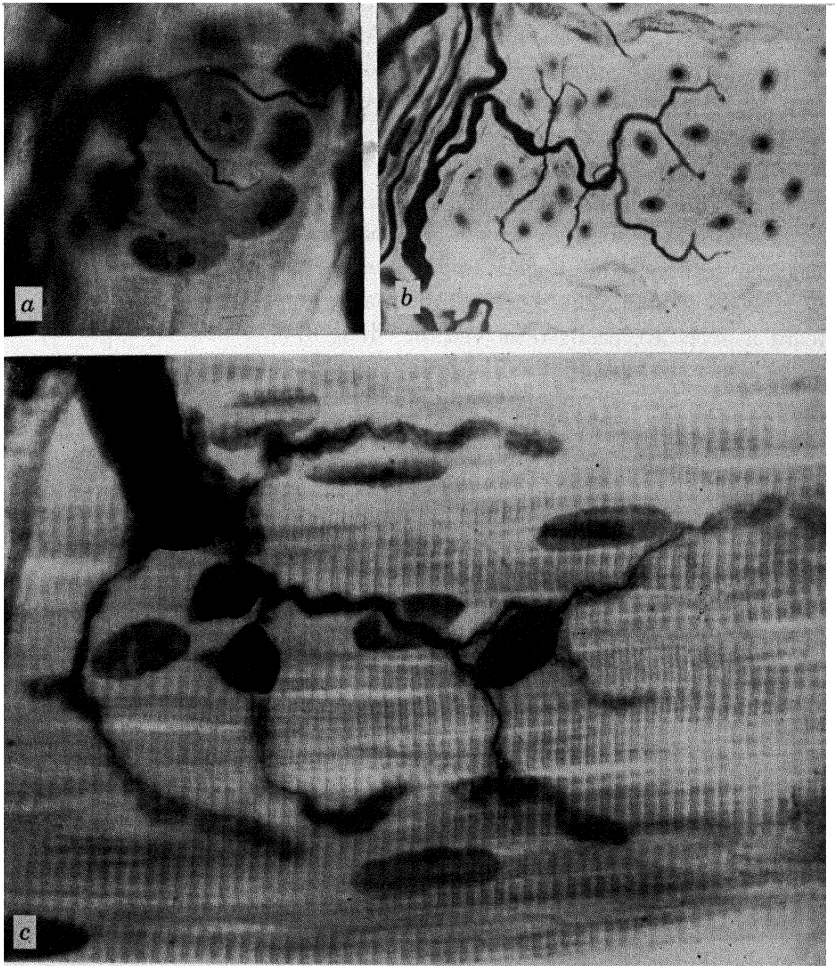


PLATE I. Silver preparations of neuromuscular junctions showing neurofibrils of nerve endings and nuclei of motor junctional area. (a): Guinea pig motor end-plate. Magnification: 880 ×. (b): Catfish neuromuscular junction with arborized nerve ending (original photomicrograph from Barets. Magnification: 540 ×. (c): Frog neuromuscular junction; only a part of the end-bush is seen here. Magnification: 800 ×.

The fish and frog neuromuscular junctions do not possess protoplasmic plate.

same origin as Schwann's cells, which accompany the branches of the nerve ending just as Schwann's cells accompany the neurite before its ending.

At certain stages of muscular development, in particular in guinea pigs, where the aspects are especially clear when the branches of the nerve ending are still not very greatly developed, the embryonic end-plate appears to be formed of two separate parts, one consisting of the nerve ending to which one, two, or three nuclei are closely attached, which will later turn into the arborization nuclei, and the other consisting of a heap of large nuclei accompanied by granular substance, which will produce the fundamental nuclei.

The distinction between these two parts of the end-plate at this stage of its development is made particularly clear by the fact that the first part, the "neuro-Schwannian expansion", depresses the second and often deforms the fundamental nuclei as if it were exerting pressure on them. Nevertheless it should be pointed out that whereas these particularly significant aspects are easily observed on whole muscle fibers, they are much more difficult to find on the sections. This is a possible source of error which may perhaps explain certain disagreements.

On the basis of the data obtained from the study of development, it is possible to distinguish three different protoplasms in the motor end-plate (Couteaux, 1938a, b; 1941). One is nervous in origin and corresponds to the terminal arborization of the motor neurite; the second (of the same origin as Schwann's cells which sheath the nerve fibers) accompanies the nerve ending—this is the teloglia, to which the arborization nuclei are attached; and the third, to which the fundamental nuclei belong, originates from and continues with the sarcoplasm of the subjacent muscle fiber. The research carried out by Boeke (1942) produced very similar conclusions for this point.

The study of development made with classical neurohistological methods does therefore make it possible to identify the various protoplasms involved in the construction of the motor end-plate, but it does not provide any valid data on the connections between them.

By using new methods it has been possible to trace in the adult a definite limit between the nervous and telogliar part, and the muscular part of the motor end-plate.

The first methods were postvital staining with stains normally used to distinguish the chondriome vitally, e.g. Janus green B, methyl violet and dahlia violet, the stained muscles being observed fresh or after fixing with ammonium molybdate (Couteaux, 1947). With these methods it was possible to delimit the sarcoplasm at the level of the motor end-plate and to describe in the superficial layer of this sarcoplasm (under the name of

the subneural apparatus) a complex lamellar structure situated at the deep face of the nerve branches wherever they come into contact with the sarcoplasm.

Thanks to the development of methods of enzymatic histochemistry and in particular to the discovery of methods for localizing cholinesterases, these postvital methods have been replaced by far more specific methods which are much more reproducible and which, applied to formaldehyde-fixed muscles, gave images quite as conclusive as the preceding methods (Couteaux and Taxi, 1951, 1952; Coërs, 1953a; Holt, 1954) (see Plates II and III).

Lastly, the classical cytological methods, such as staining with ferric hematoxylin or Altmann's aniline fuchsin, associated with other stains, provide images of the subneural apparatus on muscles fixed with osmic mixtures, which are less sharp than those obtained with histochemical methods, but which are nevertheless very clear.

In preparations of this kind, the subneural apparatus, by its presence on the surface of the junctional sarcoplasm, divides the end-plate into two parts. The branches of the nerve ending accompanied by the arborization nuclei are completely separated from the fundamental nuclei and the granular sarcoplasm which surrounds them.

Like the nuclei, the granules are also divided between the two parts of the end-plate. Two types of granules may easily be perceived. Those inside the nerve branches correspond to numerous small neurosomes, more numerous it seems than at the level of the axon; the others surround the fundamental nuclei, are always very numerous, and vary greatly in size. Their form, which also varies greatly, depends to a certain extent, as is found with the other sarcoplasmic granules, on the degree of contraction of the muscle fiber after its fixation. These granules were stained by Regaud's method by Boeke and Noël (1925a, b) and Noël (1925, 1950), having been observed first by Rouget (1862) and then by Held (1897) at the level of the muscles of various animal species, using a very inelective staining method. From their staining affinities and sometimes also from their form, Boeke and Noël concluded that these granules are probably mitochondria.

Granules entirely equivalent to those surrounding the fundamental nuclei of the end-plate may be observed around the muscle nuclei found at the surface of the "sarcoplasm-rich fibers" (Fig. 2).

b. Synaptic Gutters. The muscular portion of the motor end-plate which, together with the fundamental nuclei and its granules, forms a slight projection at the surface of the uncontracted muscle fiber, is depressed by gutters in which the branches of the nerve aborization wind.

PLATE II. Subneural apparatus. (a-f): Mouse intercostal muscle. Acetylthiocholine method after fixation by formaldehyde (photomicrographs from Couteaux and Taxi, 1952). Magnification: (a-c) 300 ×), front view without counterstaining. Magnification· (d-f) 1500 ×) cross-sections with staining of nuclei by carmine. (g, h): Lizard muscle (pelvic plate). Subneural apparatus positively stained with Janus B green (fixation of the staining with ammonium molybdate). Magnification: (g) (1000 ×), front view. Magnification: (h) 2250 ×), front view of small portion of the end-plate. The subneural apparatus is prolonged onto the surface of the end-plate by a system of threads. The distribution of these superficial threads (hardly visible in this photograph) is subject to very precise laws. Certain of them unify lamellae of the same or of two neighboring gutters. (i-l): Frog muscle (gastrocnemius). End-bushes (photomicrographs from Couteaux and Taxi, 1952). Magnification: (i) (250 ×), silver preparation; (j) (250 ×); (k) 2000 ×); (l) 2000 ×), acetylthiocholine method, front view of subneural apparatus without counterstain.

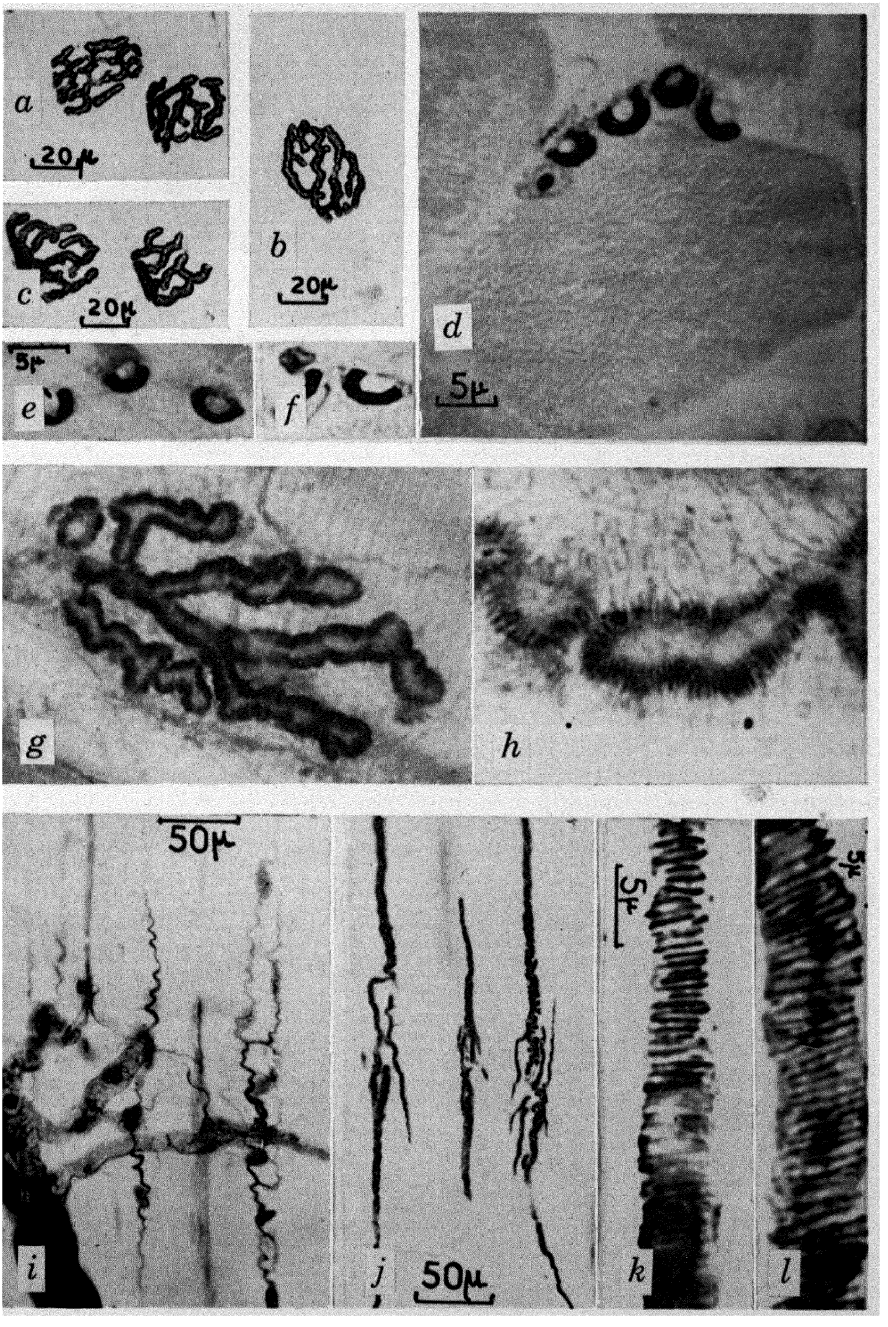
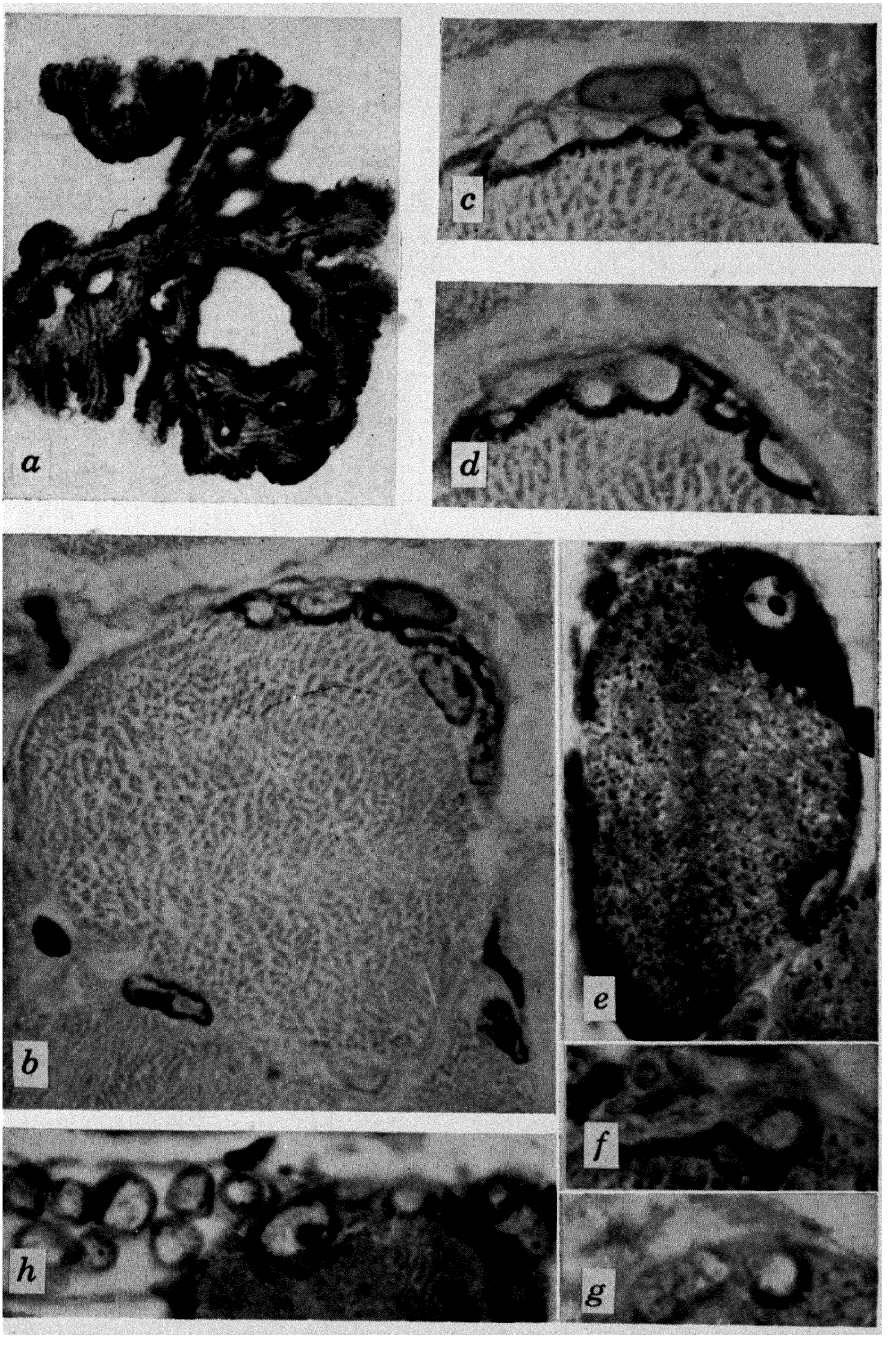

a
20μ
b
20μ
c
20μ
d
5μ
e
5μ
f
g
h
50μ
i
j
50μ
5μ
k
5μ
l

PLATE III. Hedgehog motor end-plate (intercostal muscle). (a-d): Acetylthiocholine method after fixation by formaldehyde. Magnification: (a) 1590 ×), front view of the subneural apparatus without counterstaining. Magnification: (b) 1590 ×); (c): 2120 ×); (d): 2120 ×), cross-sections through a motor end-plate showing synaptic gutters with subneural lamellae; nuclei are stained by hematoxylin. (e-h): Fixation by the osmio-chromic J fluid of Laguesse, aniline acid fuchsin-methyl green; cross-sections through motor end-plate. Magnification: (e) (1325 ×), cross-section outside the synaptic gutters showing the granular plate and one fundamental nucleus. Magnification: (f) (2120 ×); (g): (2120 ×), two consecutive sections showing synaptic gutters and subneural apparatus. Magnification: (h) (1590 ×), end-plate and cross-section of several motor fibers (left).


a
c
d
b
e
f
h
g

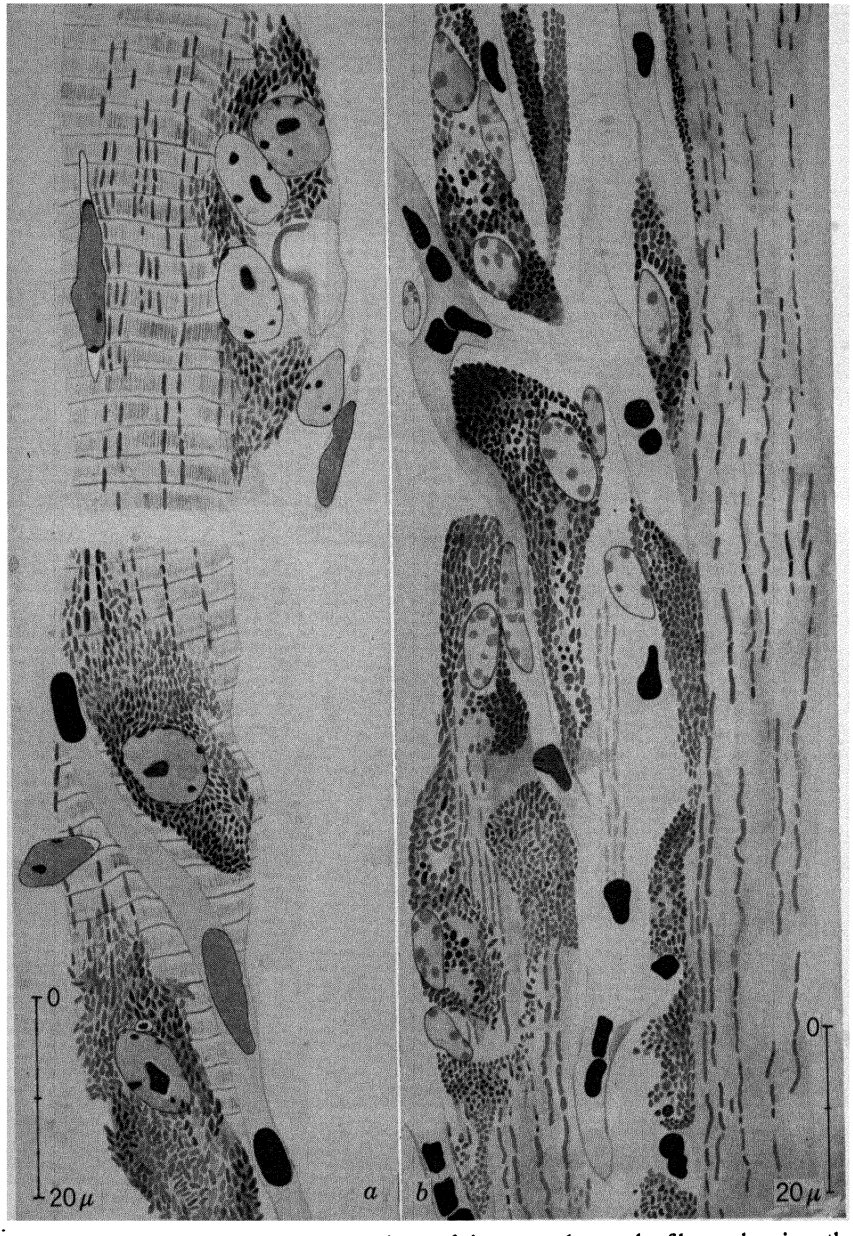


FIG. 2. Drawings of tangential sections of intercostal muscle fibers showing the peripheral sarcoplasmic mitochondria. (a) hedgehog muscle fiber, fixation by the osmio-chromic J fluid of Laguesse, aniline acid fuchsin-methyl green stain. In the upper portion of the muscle fiber, the motor end-plate is seen. (b) mouse muscle fibers, fixation by Regaud's fluid, aniline acid fuchsin-methyl green stain. These

This superficial position, which is not consistent with traditional opinion, was observed by Gutmann and Young (1944) in lateral views of end-plates with silver preparations, and by Couteaux (1944, 1945a) when describing the subneural apparatus stained with Janus green B in cross-sections of mouse muscle fibers.

It is at the bottom of these "synaptic gutters" of varying depth, sunk in the sarcoplasm of the motor end-plate, that the nervous substance and the muscular substance confront each other.

The problem of capital importance which arises here is to know what happens at the level of the surface of this concave junction, first, to the membranes coating respectively the nerve fiber and the muscle fiber outside the junction level and, second, to the teloglia.

A first point, which may be considered as established, is that no coarse membrane of the type that envelops the end-plate is interposed between the terminal nerve branches and the sarcoplasm. Henle's sheath, which prolongs the system of lamellar nerve sheaths to the level of the isolated nerve fiber, continues with its flattened nuclei at the surface of the end-plate, but passes above the nerve branches lying within the synaptic gutters.

The argyrophil networks of Plenk-Laidlaw and Maresch, which envelop the nerve fiber and the muscle fiber, respectively, appear at the surface of these fibers rather late, at a stage of muscle development when the junction of the nerve fiber and the muscle fiber is already accomplished. These networks could not be detected in adults at the bottom of the synaptic gutters either by the Bielschowsky-Maresch method or by the Laidlaw method.

Like the membrane prolonging Henle's sheath at the level of the end-plate, these networks thus seem to exist only at the surface of the end-plate and do not penetrate within the neuromuscular articulation. It is in this sense that the situation of the nerve ending may today still be considered as hypolemmal.

It remains to be seen what happens to the teloglia accompanying the terminal nerve branches at the surface of junction of the nerve ending and the sarcoplasm.

Tello (1944) admits that the end arborization is completely enclosed in a sheath which he regards as a probable continuation of the Schwann cells sheath. Through comparison of aspects of the nerve ending obtained by postvital staining with methylene blue or Janus green B, and by silver methods, Couteaux (1945a, b) described two distinct parts in the end-bush of the frog. One, axial, extending the axon, and the other which surrounds the first and which he regarded as belonging to the teloglia.

This peripheral part forms a continuous sheath around each nerve branch. Couteaux compared it to the "stroma" described by Kühne (1887) by means of gold methods, but which Kühne had regarded as a component of the nervous end arborization.

This interpretation of Kühne's stroma as a sheath made of non-nervous protoplasm was not new. Tiegs (1932) showed on reptilian muscles that the stroma (which he called "perilemma" to stress its independence from the axon) survives degeneration of the nerve after section and remains in direct continuity with the cord of Büngner, i.e. Schwann cells of the degenerated nerve. But the examination of sections made through the motor end-plate after fixation by means of good osmic fixatives (Bensley, osmiochromic fluid of Laguesse) and staining by mitochondrial methods, shows that there is no room in the synaptic gutters for a periaxoplasmic layer as thick as Kühne's stroma. If the teloglia completely surrounds the axon ramification, this stroma (which Kühne found to be more granular in its peripheral part) probably represents not only telogliar sheath but, when located underneath the nerve branch, represents the subneural apparatus as well. There is even very likely some sort of a retraction space comparable to the "sheath" described by Mauthner between the axoplasm and the myelin sheath.

The teloglia is very likely capable, as are the Schwann cells, of forming extremely thin membranes. The impossibility of observing it in the light microscope even after good cytological fixation cannot, therefore, be sufficient to eliminate the hypothesis of its interposition between the axoplasm and the sarcoplasm. Only observations with the electron microscope after osmium fixation will permit tackling this problem in a profitable manner.

c. The Subneural Apparatus. Below the nerve branches, occupying the synaptic gutters, there is a collection of long narrow lamellae attached by one edge and along their whole length to the sarcoplasmic membrane; these lamellae together constitute the subneural apparatus (Couteaux, 1944, 1947; Couteaux and Taxi, 1952). These subneural lamellae are inserted along fairly equidistant parallel lines, which confers on the subneural apparatus the character of a periodic structure. The lamellae insertion lines are oriented in different ways in relation to the gutter axis—transversally, obliquely, or longitudinally. When the lamellae are oriented transversally to the gutter axis, they assume a disposition reminiscent of radiator gills. But this disposition is rather rarely seen in mammals and generally only in short stretches of the gutters. It seems that the best idea for the general disposition of the lamellae at the deep face of the nerve branches is obtained by comparing it with the papillary crests of fingertip skin. The orientation at the surface of the lamellae seems subject to certain fixed

rules. Geometrically speaking, the subneural lamellae seem to be regulated surfaces, generated by perpendiculars to the cylindrical surface of the synaptic gutters.

In following the curve of the gutters, each of the subneural lamellae crops out here and there at the surface of the end-plate; it is prolonged at this level by threads which are sometimes very long.

If we stain only the superficial part of these lamellae, we obtain, from the face view of an end-plate, a palisade or ciliate fringe appearance, similar to the one described by Kühne (1883) at the surface of the end-plate and which he interpreted as a superficial structure situated "in or on" the membrane covering the end-plate. Although Kühne observed this aspect only on the end-plates of reptiles and was unable to find the equivalent in mammals, there seems no doubt that it corresponds to the outcropping of the subneural lamellae at the surface of the sole. The observations made on the motor end-plates of lizards by means of postvital staining are entirely convincing on this point.

On a section cut perpendicular to one of the branches of the terminal nerve arborization of an end-plate of a mouse, the subneural apparatus appears at the deep face of the nerve branch as a very thin layer, curved into an arc. This characteristic aspect can only be observed on sections cut exactly perpendicular to the plane of the nerve arborization through an uncontracted muscle fiber. More or less oblique sections, or those cut through a motor end-plate projecting considerably owing to the contraction of the myofibrils show the subneural apparatus in very different aspects and do not always show its edges cropping out at the surface of the end-plate.

The thickness of the subneural apparatus, i.e. the thickness of the layer formed by its combined lamellae, is certainly less than one micron, perhaps even less than half a micron. It cannot be correctly assessed on sections with a thickness of several microns or even of one micron. Ultrathin sections are necessary.

What is the nature of this subneural apparatus situated at the boundary of the nerve ending and the sarcoplasm?

The most important fact which may help to elucidate this problem at the present time is provided by the study of the neuromuscular junction after section of the motor nerve. On mice, one week after section, when the nerve endings have completely disappeared and the synaptic gutters are more or less deformed, postvital staining with Janus green B showed subneural lamellae of practically normal aspect. Two weeks after section, the subneural apparatus is still easily recognizable and the lamellae of a number of end-plates even appear almost normal in aspect. The only

change in this case seems to be in stainability. Coërs (1953c) confirmed and supplemented these observations by means of Koelle's modified method. He was also able to recognize subneural apparatus in the gastrocnemius of the rat three months after section of the sciatic nerve.

If at the same time we remember that the subneural lamellae dip into the sarcoplasm and seem to be attached to the sarcoplasmic membrane, the first hypothesis which springs to mind is that the subneural apparatus is of a muscular nature (Fig. 3a, b).

The recent electron microscopic study of the electric plates of *Torpedo* suggests, however, that it might not be exclusively muscular. The common embryological origin of the electric organ and striated muscle and, moreover, the striking morphological, biochemical, and physiological homologies established between the nerve-muscle and nerve-electric organ junctions permit us to consider these two organs as closely related. It then becomes natural to compare (Couteaux, 1944) the subneural apparatus with the collection of small rods described long ago by Remak and many authors in the electroplasm layer situated immediately below the nerve arborizations covering the electric plate. Examining sections of the electric plates of *Torpedo*, with the electron microscope, Bernhard and Couteaux (unpublished data) found, first, that the electric rods do not end freely in the electroplasm but are united with each other at the deep end forming, as it were, croquet hoops; and, second, that the substance of the fine network in which the nerve fibers terminate on the surface of the electric plate penetrates within the electric rods and seems to form their axis.

Although these are only preliminary observations, they oblige us to consider it possible that the protoplasm contained in the synaptic gutters penetrates within the subneural lamellae. In this hypothesis we should still have to determine whether the axoplasm penetrates these lamellae alone (Fig. 3c) or accompanied by the teloglia (Fig. 3d) or whether the teloglia penetrates alone (Fig. 3e).

As the teloglia persists for some time in the end-plate after section of the motor nerve and degeneration of the neurons, it is not easy to rule out the hypothesis of teloglia participation in the constitution of the subneural lamellae.

It seems that these problems of the nature of the subneural apparatus can only be solved by electron microscopic examination.

2. *Neuromuscular Junctions in Other Vertebrates*

The extensive reviews of Hinsey (1934) and Tiegs (1953) provide very full information on the various types of neuromuscular junctions found in the striated muscles of vertebrates. Here we shall only consider very

briefly some types of neuromuscular junctions which, like the mammalian motor end-plate, have already been the subject of histochemical research or which recent observations indicate as a particularly favorable field for further histochemical or histophysiological research.

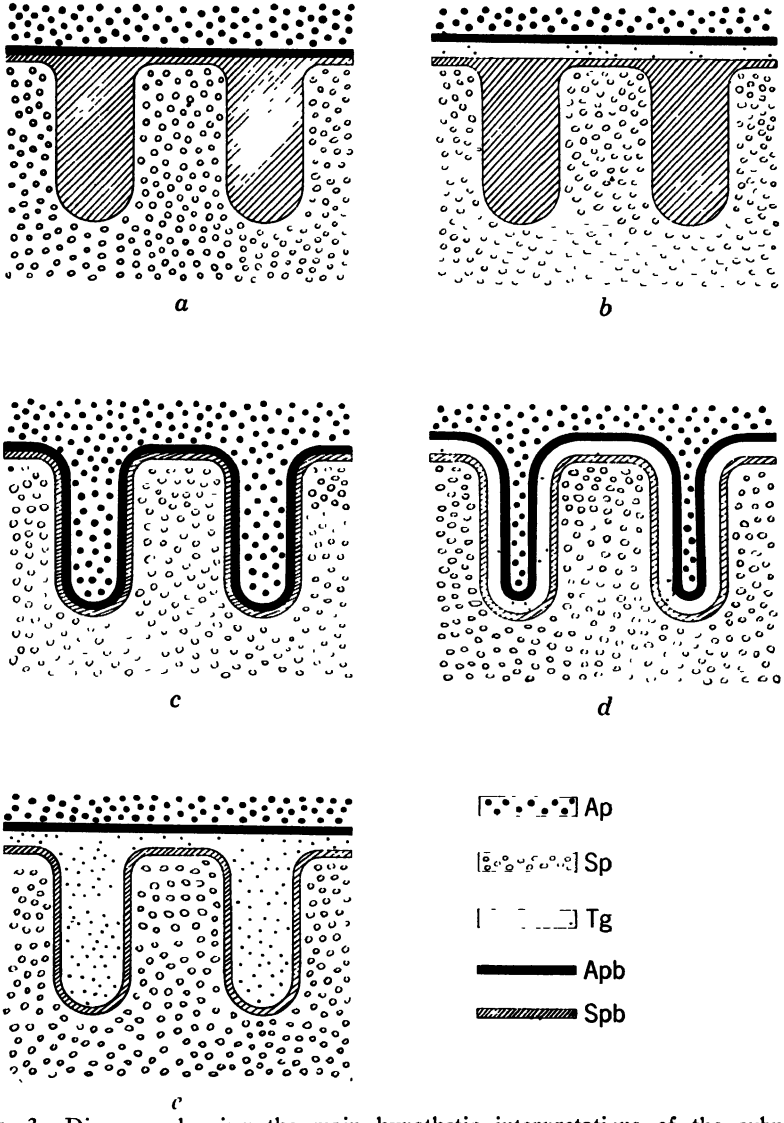


FIG. 3. Diagram showing the main hypothetic interpretations of the subneural apparatus. *Ap.*: axoplasm; *Sp.*: Sarcoplasm; *Tg.*: teloglia; *Apb.*: axoplasmic boundary; *Spb.*: sarcoplasmic boundary.

Of the neuromuscular junctions corresponding to large motor fibers, those of the lizard (frequently used in histology because of their size) (Plate IIg, h) and the gecko are a type closely comparable with the mammalian end-plate and have been used jointly with the latter to elucidate the structure of the subneural apparatus.

In frogs, the large motor fibers terminate in very large arborizations (Kühne's "Endbusch") with rectilinear branches parallel to the main axis of the muscle fiber and often several hundred microns long (Fig. 4, Plates

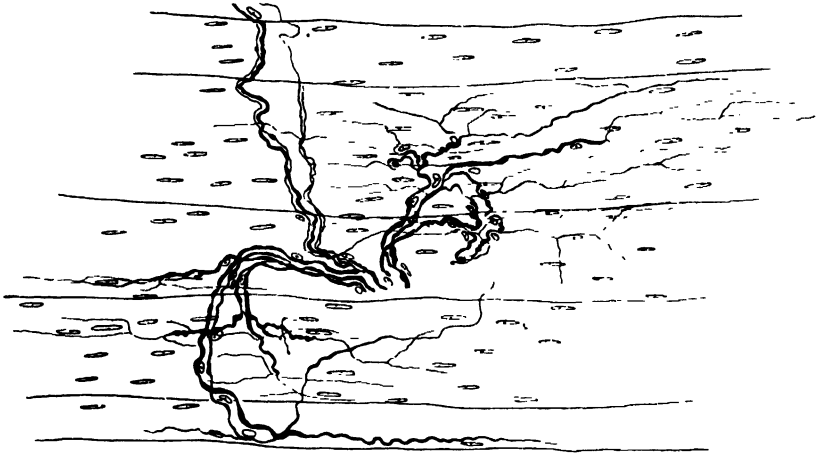


FIG. 4. Drawing of frog end-bushes (silver preparations).

Ic and IIi). At the level of these end-bushes there is no protoplasmic plate comparable to the mammalian and lizard end-plates; instead there are numerous small heaps of granular protoplasm, often uni-nucleated, distributed along the terminal branches. One of the advantages of these end-bushes in the study of the subneural apparatus is that their synaptic gutters generally present only a very slight curvature and are very shallow. Accordingly, from the front view of an end-bush it is very easy to observe the subneural lamellae over practically their whole extent, by focusing on the bottom of the gutter. Another peculiarity of these end-bushes is that orientation of the subneural lamellae is almost regularly transversal to the gutters.

In fish, where little work has been done so far on the motor innervation of the striated muscles, certain neuromuscular junctions corresponding to large motor fibers would seem to merit attention because of the quite unusual site which they occupy on the muscle fiber. It is some time since Giacomini (1898a, b) and others after him reported that nerve endings, which they considered as sensory and whose function might compensate

for the absence of neuromuscular spindles, were situated at the extremities of the muscle fibers of selachians, teleosts, and even of muscles of vertebrates belonging to other classes. A convergent collection of morphological findings recently challenged this interpretation and led to the view that in some cases, if not all, Giacomini's "basket endings" at the extremities of the muscle fibers are not sensory but motor terminations. At the level

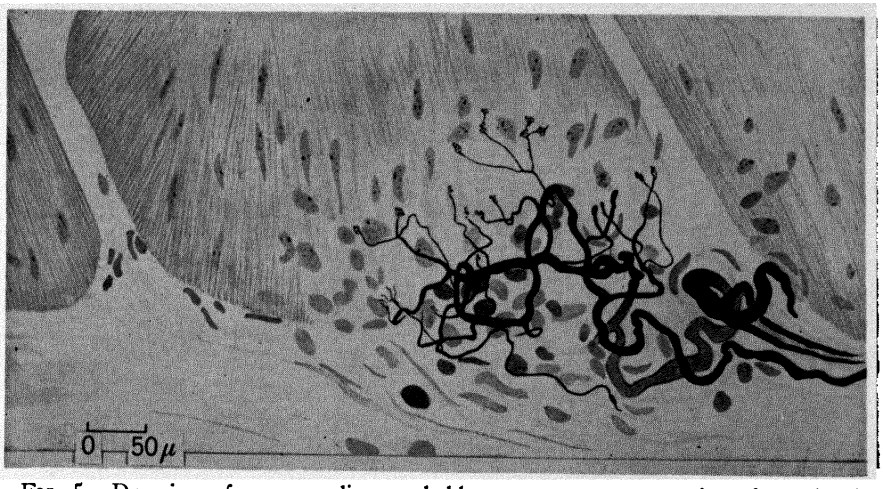


FIG. 5. Drawing of nerve ending probably motor at an extremity of a selachian muscle fiber (skate).

of the tail myomers of selachians (*Raja* and *Torpedo*), numerous endings, which were situated at the extremities of the muscle fibers (Fig. 5) and described by Giacomini as sensory, would in this case be motor (Couteaux, 1950). In certain teleosts, in particular catfish, eels, and congers, Barets (1952) also considered that the nerve endings situated at the extremities of the muscle fibers of the deep lateral muscle (Fig. 6) were probably motor. This hypothesis is supported by recent electrophysiological observations on the deep lateral muscle of catfish (Cuypers and Fessard, 1954). The double innervation observed in this case deserves to be compared with the observations of Katz and Kuffler (1941) on the end-bushes of frog sartorius.

Thanks to the further data recently provided by Kuffler and his collaborators (1947, 1953a, b) on the "small nerve motor system" of frogs, it has become possible to undertake more successfully the functional interpretation of muscular innervations by small myelinated fibers found in frog. Suggestive comparison has already been made by Günther (1949) between experimental and histological findings, which seems to indicate that the

endings of the small nerve motor system correspond to some of the hitherto very enigmatic nerve endings (Fig. 7) described in frogs by various authors, in particular Murray (1924). It does not seem that endings of this kind can co-exist on the same muscle fiber with the endings of the

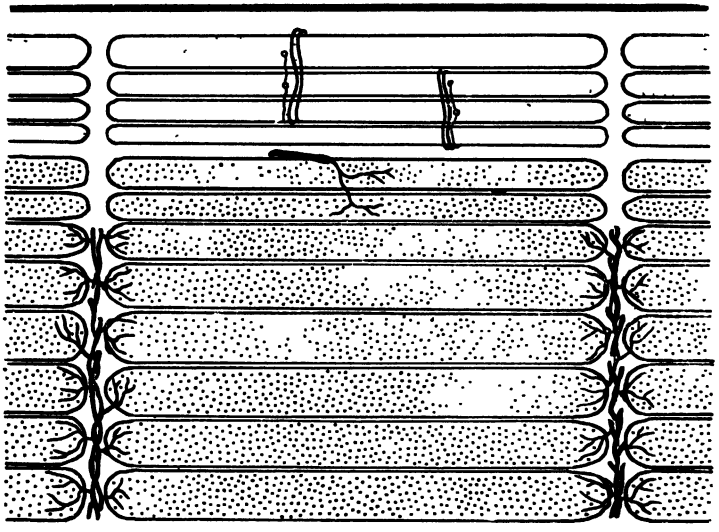


FIG. 6. Diagram showing the kinds of innervation by myelinated fibers in the catfish lateral muscle. Superficial part of the lateral muscle is represented by fine stippling, deep part by coarse stippling (from Barets, 1952).

large motor fibers. Moreover, they are disposed at several levels on the same muscle fiber (Couteaux, 1952) sometimes at short intervals (Fig. 8). These two peculiarities in the distribution of these endings seem to agree with the latest experimental results of Kuffler and Vaughan Williams (1953a, b) establishing that in frogs there are two functionally distinct nerve muscle systems (the small nerve, slow muscle fiber system and the large nerve, twitch system).

The observations of Barets (1952) make it highly probable that the somatic motor innervation of the superficial lateral muscle of catfish is ensured solely by small myelinated fibers whose endings, of very small dimensions (Fig. 9), differ morphologically from the other endings of the lateral muscle (Plate Ib and Fig. 6). Further research will have to show how far this innervation is functionally assimilable to the small nerve motor system of frog.

The histochemical study of neuromuscular junctions corresponding to small myelinated fibers is still only in its initial stages, but it seems likely

to provide important knowledge on this subject. It may also be expected to evolve new tests to elucidate the obscure question of the grape-like terminations and the termination of unmyelinated fibers, which have not yet been conclusively classified as motor or sensory.

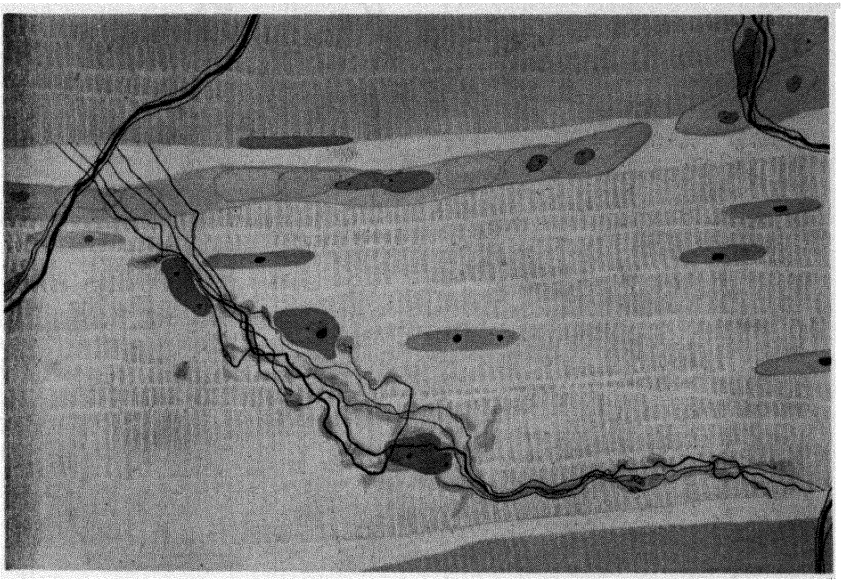


FIG. 7. Drawing of nerve endings probably motor corresponding to small myelinated fibers (pectoral muscle of frog). Magnification: 400 ×.

III. JUNCTIONAL CHOLINESTERASES AND THEIR HISTOCHEMICAL LOCALIZATION

The research undertaken on the cholinesterase activities of the striated muscle appears as a direct consequence of the work of Dale and Feldberg (1934), and Brown, Dale, and Feldberg (1936), which led to the hypothesis that acetylcholine intervenes as a chemical agent in the transmission of excitation from the motor nerve to the striated muscle.

If it is assumed that in the neuromuscular transmission of excitation acetylcholine may play a comparable role to that which it plays in the transmission of vagal excitation at the level of the heart (Loewi, 1921–1924), the supposition must be put forward at the same time that acetylcholine released by excitation of the motor nerve is destroyed during the refractory period, i.e. in a few milliseconds. Among the mechanisms capable of such a rapid destruction of acetylcholine, the hydrolyzing action of cholinesterases was naturally one of the first to be considered, since

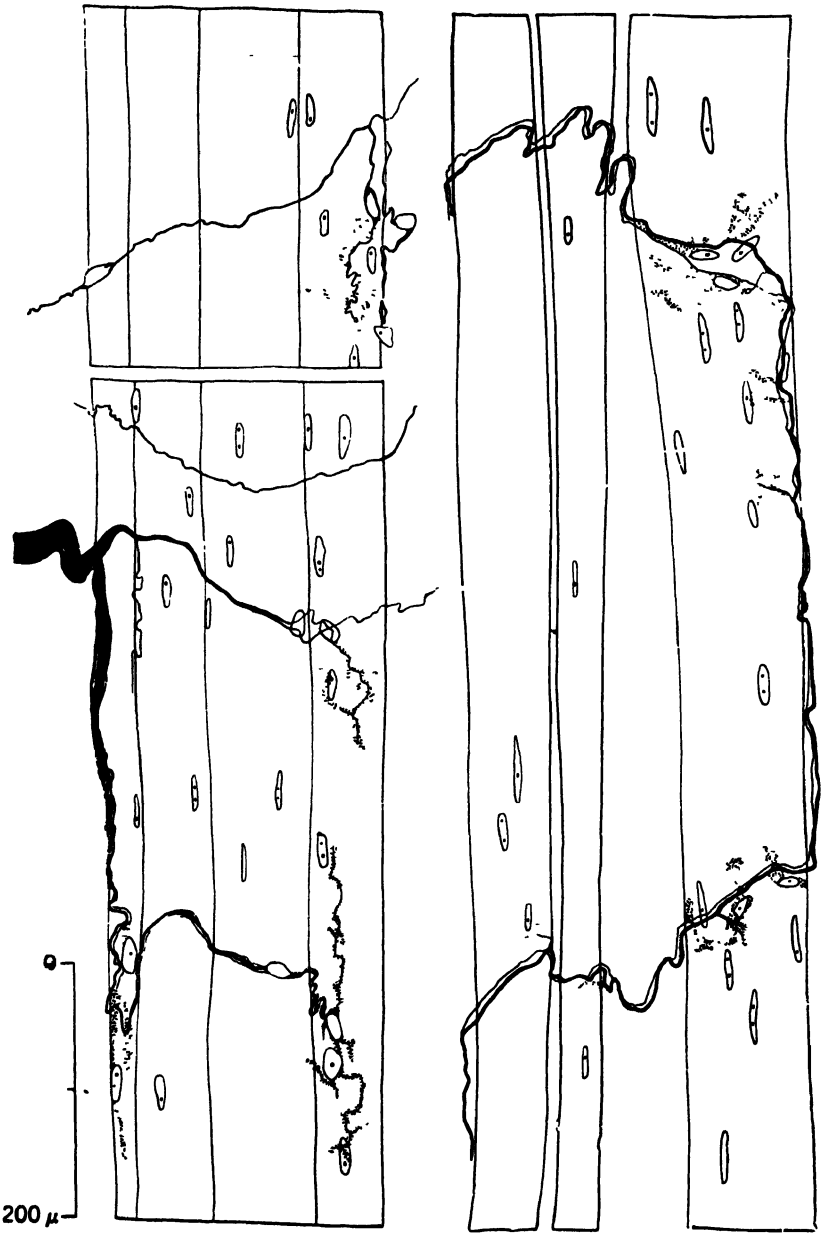


FIG. 8. Drawings of nerve endings, probably motor, corresponding to small myelinated fibers (pectoral muscle of frog). The two portions of the same muscle fibers bundle drawn on the left are separated by 700μ.

the presence of these enzymes had been demonstrated in almost all animal tissues (Plattner and Hintner, 1930). There was, nevertheless, a difficulty. The average concentration of the enzyme in the striated muscle is much too low to explain such an extraordinarily rapid action as is required in

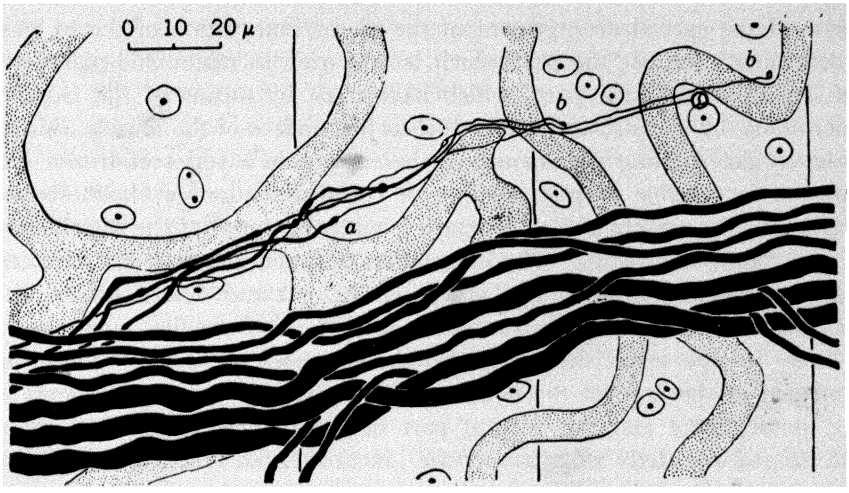


FIG. 9. Drawing of nerve endings, probably motor, corresponding to small myelinated fibers in superficial part of the catfish lateral muscle (from Barets, 1952). See also Fig. 6.

the hypothesis of a chemical transmission of excitation. A study of the distribution of the enzyme in the striated muscle has shown that in reality the cholinesterase is very unequally distributed and is so highly concentrated at the level of the neuromuscular junction that it has now become conceivable that this enzyme does hydrolyze the acetylcholine released at each nervous impulse during the brief refractory period.

1. *Biochemical Data*

a. Cholinesterase Activity of the "Neural" and "Aneural" Parts of the Muscle. In an early stage of research on muscular cholinesterases, it was established manometrically that enzymatic hydrolysis is faster in the area of the striated muscle containing the motor nerve endings ("neural" part) than in the areas with no motor nerve endings ("aneural" part) or at the level of the motor nerve itself.

This fact was demonstrated on the sartorius of frog (Marnay and Nachmansohn, 1937a) and toad (Feng and Ting, 1938) by comparing the aneural pelvic part and the neural part, whose motor endings belong to the end-bush category.

For mammalian muscles, the first significant results enabling the conclusions reached for the end-bushes of batrachians to be extended to motor end-plates proper were obtained only later on the m. gastrocnemius internus (Couteaux and Nachmansohn, 1940, 1942). In this last research it was possible to determine the distribution of the muscle cholinesterase because of the special arrangement of the motor innervation of the m. gastrocnemius internus; the part which is very rich in motor end-plates can be separated from the parts which have none by means of the freezing microtome, by cutting sections parallel to the surface of the muscle. Moreover, by determining the amount of the enzyme in a series of frozen sections, it is possible to plot a curve of the distribution of cholinesterase activity in the muscle and to compare it with the curve of the distribution of the motor end-plates. The correlation between the two distributions may be accurately determined by alternate enzyme determination and staining of nerve endings on successive sections cut from the same muscle.

Once it was established that the cholinesterase is more highly concentrated at the level of the neuromuscular junctions than at the level of the motor nerve and the aneural part of the muscle, it still had to be determined in exactly what part of the junctional area this large amount of enzyme was located.

An important contribution to the solution of this problem came from the study of the variations in the cholinesterase content of the striated muscle after section of the motor nerve and the consequent disappearance of the presynaptic part of the neuromuscular junction.

b. Changes in Cholinesterase Activity after Section of the Motor Nerve. The first research on the cholinesterase of the striated muscle after section of the motor nerve was carried out by Martini and Torda (1937) with the aim of explaining the hypersensitivity of the denervated muscle to acetylcholine. They observed on the gastrocnemius of rat, two weeks after section of the motor nerve, that the enzymatic concentration fell to under half its normal value. These results, which provided a convenient explanation of the hypersensitivity of the denervated muscles to acetylcholine, might also lead one to think that the junctional cholinesterase is highly concentrated in the nerve endings themselves.

Marnay and Nachmansohn (1937b), repeating this experiment on gastrocnemius of guinea pig, found contrary to all expectations that, after section of the motor nerve and degeneration of the neurites, there was a substantial increase in the cholinesterase concentration in the weeks following denervation. Without discussing any possible difference between the rat and the guinea pig, it should be pointed out that in these experiments on the guinea pig the manometric determinations were made after grind-

ing the muscle, whereas Martini and Torda (1937) worked on fragments of unground muscle.

Couteaux and Nachmansohn (1938, 1940, 1942) showed that the increase in the cholinesterase concentration observed in the guinea pig can easily be explained by the persistence of high cholinesterase activity at the level of the soles of the denervated motor end-plates. Owing to the atrophy of the muscle fibers caused by denervation, the number of muscle fibers, and consequently the number of soles of denervated motor end-plates, does in fact increase per unit of muscle weight in the weeks following the section of the motor nerve, and the persistence of a large quantity of cholinesterase at the level of these soles has the unexpected result of raising the average concentration. But this is only an apparent increase in muscle cholinesterase. By comparing the variations in enzymatic concentration with the variations in weight reflecting muscle atrophy, Couteaux and Nachmansohn showed that in reality, in the case of gastrocnemius of guinea pig, the total content of muscle cholinesterase decreases after denervation and may in 3 or 4 weeks fall to 50% of its initial value or less.

The principal conclusion to be drawn from this work is that the junctional area which has had its nerve endings removed several weeks before probably still retains a considerable part of the cholinesterase which it contained before section of the motor nerve, and so, even after denervation, it still has a cholinesterase concentration higher that at any other point of the striated muscle. This leads to the supposition that a large part of the cholinesterase attached to neuromuscular junctions is located outside the nerve endings. As regards the endings themselves, the fall in the total quantity of muscle cholinesterase observed in the denervated muscle occurs too late for it to be directly related to the disappearance of any cholinesterase which might be contained in the endings.

It seems interesting to compare these experiments on the guinea pig with those of Feng and Ting (1938) on the toad sartorius. In batrachians the muscle fibers atrophy extremely slowly after denervation and, one month after section of the motor nerve, these authors observed, not an increase in cholinesterase concentration as with the guinea pig, but a decrease of 30%; this confirms indirectly the interpretation given by Couteaux and Nachmansohn of the increase in the enzyme concentration in the case of muscles where denervation provoked rapid atrophy of the muscle fibers.

A number of denervation experiments similar to the foregoing were carried out by other authors on mammalian muscles, but the results obtained by no means tally. Leibson (1939) on the soleus of rabbit finds

an increase in concentration up to the sixth week. Meng (1940) working with the gastrocnemius of rat observes a slight decrease in concentration, which he considers not significant; Stoerck and Morpeth (1944) on the same animal and the same muscle find the concentration falling by 30% in 4 weeks. Sawyer, Davenport, and Alexander (1950) indicate in a preliminary report that on the muscles of rats, guinea pigs, and rabbits they recorded first a definite decline and then an increase in enzymatic activity after section of the motor nerve.

To return to the problem, already tackled by Martini and Torda, of the hypersensitivity of the denervated muscle to acetylcholine, Brooks and Myers (1952) recently working on the m. serratus anterior of guinea pig, resumed the study of the variations in the cholinesterase content following section of the motor nerve. The special interest of this work lies in the fact that the authors subjected the figures obtained in their experiments to statistical analysis in order to determine how far the changes observed are significant. They found that the enzyme concentration, assessed by using acetyl-β-methylcholine as substrate, increases in direct relation to the degree of atrophy. These results of Brooks and Myers imply the persistence at the level of the denervated soles of a high concentration of cholinesterase; in this they agree with the findings of Couteaux and Nachmansohn. But an important difference appears regarding the total cholinesterase content of the denervated muscle. Brooks and Myers find that this content is not affected by denervation. In their experiments, when the muscle weight is reduced by half owing to atrophy, the enzyme concentration amounts to double that of the control. Contrary to the results obtained on the gastrocnemius of guinea pigs, the denervation of m. serratus anterior of the same animal does not therefore bring about a decrease in the total cholinesterase content.

It should be pointed out that in a final note added in proof, Brooks briefly reports the results of additional research carried out on the gastrocnemius of guinea pigs and rats. This further research has enabled him to reproduce the figures obtained by Couteaux and Nachmansohn, showing a fall in the total cholinesterase content to about half its initial value three weeks after denervation; but the repetition of the experiments made on m. serratus anterior could only confirm the results previous obtained, showing no decline in total muscle activity for the same degree of atrophy. So sharp a difference in the behavior of two striated muscles of the same animal can apparently only be interpreted after further research.

The results reported so far relate only to variations in the average cholinesterase concentration and the total quantity of muscle cholinesterase after denervation. They provide only an indirect assessment of the cholin-

esterase concentration at the level of the denervated soles of the motor end-plates. By applying to the denervated muscle the frozen sections technique already used to compare the cholinesterase activities of the "neural" and "aneural" parts of the normal muscle, Couteaux and Nachmansohn (1940, 1942) showed that the "neural" area of the gastrocnemius of guinea pigs does indeed contain, two weeks after denervation, a higher cholinesterase concentration than the "aneural" areas. This result verifies the hypothesis stated previously, that much of the enzyme of the "neural" part is located outside, and not inside, nerve endings.

Whereas the denervation experiments enable us to establish clearly that in the non-nervous part of the motor innervation area there is a higher cholinesterase concentration than at any other point of the muscle, they do not provide figures which can be used for the cholinesterase content of the nerve endings themselves. What is certain, however, is that the cholinesterase content of the nerve ending is low in comparison with the rest of the junctional area, so relatively low that the total content of cholinesterase in the striated muscle, at least in the guinea pig, shows no change which can be regarded as significant during the first week following the section of the motor nerve.

2. *Histochemical Data*

By measuring cholinesterase activities on homogenates of normal or denervated muscles, it has been possible to establish that there is a high cholinesterase concentration in the motor innervation area and that this high concentration is located outside the nerve endings. By its very nature, however, this method cannot take us any further as regards the location of cholinesterase activity in various non-nervous structures of the junctional area. At this stage of analysis histochemical methods become absolutely essential.

By comparing various biochemical-physiological data with certain morphological data, Couteaux (1947) was led to formulate the hypothesis that the junctional cholinesterase is mainly located at the level of the subneural apparatus. But this was still only a conjectural opinion, for which positive support could be found by recourse to methods of enzymatic histochemistry.

a. Methods for Localizing Cholinesterases. Several histochemical methods have already been applied to the study of the cholinesterases of the striated muscle.

Gomori's method (1948) was to hydrolyze, by the cholinesterases of the tissues, choline esters of long-chain fatty acids. If a cobalt salt is added to the incubation medium, these acids precipitate in the form of insoluble

cobalt salts which can be transformed into a black precipitate of cobalt sulphide. In this method the sections are cut after fixation with cooled acetone and embedding in paraffin.

As Gomori himself has shown, the substrates used here are not hydrolyzed by certain acetylcholinesterases, e.g. that of the electric organ of the electric eel, whereas they may be very readily by acetylcholinesterases of other origins. The same applies to unspecific cholinesterases which will or will not hydrolyze these substrates, as the case may be. A classification of cholinesterases based on the ability or inability to hydrolyze these long-chain fatty acid esters would thus have no common basis with the currently accepted biochemical classification (Gomori, 1952a, b).

In 1949 Koelle and Friedenwald elaborated a new technique using acetylthiocholine as substrate. The chief advantage of using this substrate is that it is hydrolyzed very rapidly by the acetylcholinesterases, more rapidly even than acetylcholine at certain pH values. The thiocholine released by enzymatic hydrolysis in an incubating mixture containing copper glycinate precipitates as a mercaptide, of which the white precipitate can easily be converted into copper sulphide.

The method is practiced on fresh frozen sections. The acetylthiocholine is attacked not only by the acetylcholinesterases, but also by the unspecific cholinesterases. These two types of enzyme cannot therefore be separated by the method in this first form. In order to remedy this deficiency, Koelle (1950) made certain additions to the original method. In order to localize the acetylcholinesterases, he had recourse to the properties of diisopropylfluorophosphate (DFP), an irreversible inhibitor, which inactivates unspecific cholinesterases in most animal species at lower concentrations than those required for the inhibition of acetylcholinesterases (Adrian, Feldberg, and Kilby, 1947; Hawkins and Mendel, 1947; Adams and Thompson, 1948).

According to Koelle's experiments, the unspecific cholinesterases of tissues of cats are practically completely inhibited when treated with DFP at a concentration of 10^{-6} *M*, at 38° (at a pH of 6.4 for 30 minutes), whereas in the same animal the activity of acetylcholinesterases is reduced by only 40% with the same conditions.

By treating with DFP prior to incubation it is thus possible to prevent unspecific cholinesterase activities and to leave more than half the acetylcholinesterase activity which it is desired to localize.

Later Koelle (1951) reduced the DFP concentration designed to inactivate the unspecific cholinesterases from 10^{-6} to 10^{-7} *M*, which produces complete inhibition of unspecific cholinesterases and reduces the activity of acetylcholinesterases by only 5%. More recently (1954) he has even

stated that 2.5×10^{-8} *M* inactivates unspecific cholinesterases "relatively selectively."

Moreover, in order to localize unspecific cholinesterases, he used another thiocholine ester, butyrylthiocholine, which in cat is practically only hydrolyzable by such cholinesterases, judging at least from the comparison of the results obtained at pH 6.4 on homogenates of some organs.

Despite this improvement in specificity, this method using thiocholine esters remained very imperfect with regard to the precision of its histological localizations. It involved a number of artifacts, in particular diffusion artifacts, which Koelle (1951) and Couteaux and Taxi (1951, 1952) endeavored to eliminate. As the method is practiced on fresh frozen sections of muscle, far-reaching deteriorations of the muscle fibers may also result, both post gelum and during incubation. The problems raised by these various artifacts will be considered later.

Nachlas and Seligman's method (1949a), in which the substrate is β-naphthyl acetate, was at first used only for the localization of aliesterases. Naphthol liberated under the action of the enzyme is converted into an intensely colored, insoluble azo dye by the action of a diazonium salt.

Since further research has shown that specific and unspecific cholinesterases hydrolyze β-naphthyl acetate as well as aliesterases (Ravin, Tsou, and Seligman, 1951; Barnett and Seligman, 1951), positive results might be expected if this method were applied to the striated muscle. But the use of β-naphthyl acetate as substrate produces diffusion artifacts which prevent any precise localization of the enzymes. The substitution of α-naphthyl acetate for β-naphthyl acetate eliminates the diffusion artifacts and thereby considerably increases the sharpness of the images obtained (Gomori, 1950, 1952a, b). It was also with α-naphthyl acetate that Denz (1953) obtained the best results on motor end-plates; he also showed histochemically that the effect of the various cholinesterase inhibitors is the same with α-naphthyl acetate as with β-naphthyl acetate and established by means of the colorimetric method of Nachlas and Seligman (1949b) that these two substrates are hydrolyzed to the same extent by acetylcholinesterases.

Lastly, new methods have been suggested for use on all hydrolytic enzymes, with which it is hoped to make great progress in the precision of cholinesterase localization. Their principle is the hydrolysis of tissues of indoxyl derivatives by the enzymes. Such hydrolysis releases indoxyl, which when oxidized gives insoluble indigo dyes.

Barnett and Seligman (1951) elaborated the first method of this kind, choosing indoxyl acetate as substrate. But with this substrate the indigo deposit formed by atmospheric oxidation of enzymatically liberated indoxyl

is too coarse a precipitate for the requirements of cytological localization (Gomori, 1952a).

Holt and Withers (1952) showed that with other indoxyl derivatives it is possible to obtain satisfactory localizations. In addition to the sharpness of the images they obtained, there is apparently even great sensitivity, since staining of motor end-plates to an intensity equivalent to that obtained by the acetylthiocholine method after 10 minutes incubation may be achieved in 10 seconds, using 5-bromoindoxyl acetate as a substrate and potassium ferricyanide as an oxidizing agent.

The systematic research undertaken by Holt (1954) to improve the precision of localizations even further has already produced very interesting results. Using as test objects the motor end-plates of mouse intercostal muscle, he has obtained localizations of remarkable sharpness with 5,5'-diindoxyl diacetate as substrate.

As may be seen from this brief survey of existing methods for the histochemical study of cholinesterase activities, there are already a fairly large variety of substrates which can be used. There is an even larger number of cholinesterase inhibitors which may be used to supplement the above methods by making it possible, not only to control the enzymatic nature of the reaction, but also, when these substances are selective inhibitors, to separate histochemically the different esterase activities located in the same organ, either cholinesterases from aliesterases or two cholinesterases of different types. Eserine is still useful for the first separation, as cholinesterases are almost entirely inhibited by a concentration of 10^{-5} *M*. For the separation of specific and unspecific cholinesterases a series of highly selective inhibitors which can be used histochemically is now available. At very low concentrations the reversible inhibitors Nu 1250 (Hawkins and Mendel, 1949) and 284.c.51 (Austin and Berry, 1953) prevent the staining caused by acetylcholinesterase activities and at the same concentrations leave intact the activities due to unspecific cholinesterases. On the other hand, several inhibitors, apart from DFP and other alkyl phosphates, may electively inhibit unspecific cholinesterases at low concentrations, e.g. Nu 683 (Hawkins and Gunter, 1946) which is reversible, and E.600 (Paraoxon) (Aldridge, 1950; Davison, 1953) and iso-OMPA (Aldridge, 1953) which are both irreversible.

b. Artifacts. In its first form and even in the second improved form evolved later by Koelle (1950), the acetylthiocholine method involves a number of artifacts, in particular diffusion artifacts, which considerably limit the value of its localizations on the histological level.

These diffusion artifacts lead to various false localizations, nuclear in particular, wherever the method yields positive results. Wherever bio-

chemical methods have revealed the existence of a high cholinesterase concentration in a particular area of an organ, the cell nuclei in that area, irrespective of the tissue to which they belong, are more or less heavily stained. At the level of the motor end-plates, the different kinds of nuclei may all be stained, and in general all the more heavily the nearer they are to the nerve ending; even ordinary muscle nuclei are quite often stained when they are not very far away from the motor end-plate. Other false localizations are also possible, especially after prolonged incubation. In particular it is quite common to observe staining in that portion of the myofibrils lying immediately under the motor end-plate. It was when using this acetylthiocholine method in its original 1949 form that Noël and Bressaud (1952) saw the sole of the motor end-plate in cross sections "almost uniformly stained" from Henle's sheath to the surface of the myofibrillar bundle, and concluded that there was "impregnation" by cholinesterase of all sole components.

These diffusion artifacts, whether due to the diffusion of the enzyme or of the reaction products prior to their precipitation, are well known in other fields of enzymatic histochemistry (Danielli, 1946; Lison, 1948; Jacoby and Martin, 1949; Gomori, 1950). Koelle (1951) himself demonstrated their existence in his method and in order to avoid them made further modifications, the principle being to keep the sections at the different stages of the technique in solutions containing very high concentrations of sodium sulphate. Thus modified, the acetylthiocholine technique no longer stains the nuclei in the neighborhood of enzyme-rich structures, and in particular does not stain the nuclei of the motor end-plate.

Koelle's purpose in modifying his technique was to prevent diffusion of the enzyme. For this purpose he used very highly concentrated sodium sulphate solutions (24% for acetylcholinesterases and 28% for unspecific cholinesterases), because with these concentrations the cholinesterases contained in solutions prepared from the brain and plasma of cat may be completely precipitated at pH 6.0.

Is the elimination of nuclear staining, which Koelle succeeded in obtaining by the latest modifications of his method, sufficient to establish that such staining is due to a diffusion of the enzyme with secondary adsorption by the nuclear structures?

Several facts lead rather to the view that enzyme diffusion in the case of cholinesterases is not probable.

In the first place, it seems a little surprising that the cholinesterases, which have a very high molecular weight (according to Rothenberg and Nachmansohn (1947) about 3 million for the acetylcholinesterase of the electric organ of electric eel), should so easily leave their initial location,

move within the section crossing intracellular and extracellular structures, and finally present a distribution so different from the original one. It is of course possible that part of the enzyme might escape from the preparation at the level of the section surface; it is less understandable how these large molecules, especially in the case of very short incubations, e.g. 15 minutes for the motor end-plates, can diffuse so easily in the thickness of the preparation.

Another more positive fact which hardly fits in with the hypothesis of an enzyme diffusion is that prolonged washing of the muscle sections in distilled water or in physiological saline before incubation does not lead to nuclear staining, at least if incubation takes place at a suitable pH and with an appropriate buffer (Couteaux and Taxi, 1951, 1952; Couteaux, 1951).

These two factors, pH and nature of buffer, seem to play a role of primary importance in the mechanism of diffusion artifacts.

In the acetylthiocholine method, pH not only affects the speed of hydrolysis but also other factors. The optimum pH for the various types of cholinesterase is about 8.0. (Bernheim and Bernheim, 1936; Easson and Stedman, 1936; Glick, 1938; Allen and Hawes, 1940), but, as was shown by Koelle (1950), if the incubation medium is buffered at this pH value, no precipitation of cuprothiocholine is observed and it is necessary to come down considerably below the optimum value for precipitation to appear. Koelle admits that this phenomenon is due to the effect of pH on the relative affinities of glycine and thiocholine for the cupric ion. Couteaux and Taxi (1951, 1952) have shown that pH is also decisive in the production of diffusion artifacts and that, according to the pH value selected, nuclear staining may or may not be observed. They also drew attention to the great importance of the choice of a buffer in the appearance of diffusion artifacts. Sörensen's diphosphate mixture, originally used by Koelle, appears to be one of the causes of the artifacts observed, and these would not arise if other buffers were used, such as the mixture, acetic acid-sodium acetate or sodium maleate. It is at present difficult to explain the difference in the action of the various buffers, but it does not seem that these can be due in all cases to unequal buffer capacity.

It is to be noted that, when modifying his method in order to avoid enzyme diffusion, Koelle did not confine himself to keeping the sections in highly concentrated solutions of sodium sulphate, but that at the same time he lowered the pH of the incubation medium from 6.4 to 6 and replaced the diphosphate buffer by sodium maleate.

We may, therefore, ask whether the elimination of nuclear staining is not due largely to these changes of pH and buffer. In any case, the improve-

ment obtained does not seem to be attributable to the precipitation of the enzyme by sodium sulphate.

Recent observations by Holt (1954) (during the elaboration of a cytochemical localization technique for esterases using indoxyl derivatives) are also opposed to the hypothesis of cholinesterase diffusion, at least at the level of the striated muscle. This author reports that "washing in saline for several hours fails to reduce the measured cholinesterase activity of the tissue and storage of formalin fixed muscle in aqueous solutions for as long as one month has no effect on the precision with which the enzyme can be localized by the staining reaction."

If enzyme diffusion may be considered in the case of muscle cholinesterases as entirely improbable and at any rate practically negligible, we still have to consider the hypothesis of a diffusion of one of the hydrolysis products, thiocholine or its cupric mercaptide. Koelle thought he could disregard this hypothesis because his method, in accordance with the suggestion of Friedenwald and Becker (1948), included previous saturation of the incubation media with the reaction product, precisely in order to avoid any diffusion of the hydrolysis products. But it seems improbable that this previous saturation of the incubation medium is sufficient to prevent the diffusion of thiocholine released by enzymatic hydrolysis. As regards the striated muscle, the saturation of the incubation medium by cuprothiocholine does not seem to have any effect on the results of the method (Couteaux and Taxi, 1952; Coërs, 1953b; Denz, 1953).

Accordingly there does not seem to be any reason why we should not interpret any diffusion artifacts which may be observed with the acetylthiocholine method as the result of a diffusion of hydrolysis products starting from the location of the enzyme.

Diffusion artifacts are not the only ones to be feared in the application of methods of enzymatic histochemistry to the localization of muscle cholinesterases. In addition there are, of course, the ordinary artifacts found with classic histological methods. In the case of the acetylthiocholine method applied to preparations of fresh muscle tissue, the structures may deteriorate very seriously if the sections are cut by the freezing process. In itself the freezing of the fresh muscle is already capable of producing extensive artifacts. Frozen sections cut longitudinally may indeed present post gelum a very far-reaching deterioration of the muscle-fibers, and sometimes even complete disorganization as described in the last century and studied more recently by Nageotte (1937). Other artifacts may also result if the fresh muscle tissue sections are kept for a fairly long time in an incubation medium containing substances capable by their chemical properties, or by their concentration, of attacking fine, fragile structures.

To check the validity of the observations made on preparations of fresh tissue, it was therefore necessary to find a fixation process which would leave sufficient residual activity in the fixed pieces for it to be detected histochemically. Of the various fixatives tested, formaldehyde proved most suitable for this purpose (Couteaux and Taxi, 1951, 1952; Holt and Withers, 1952).

The effect of formaldehyde on cholinesterase activities has already been the subject of some research, e.g. by Ravin, Tsou, and Seligman (1951) on unspecific cholinesterases of various tissues by colorimetric methods and by Taxi (1952) by means of manometric determinations on various preparations of cholinesterases, either acetylcholinesterases or unspecific cholinesterases. The total results obtained by the latter show that in the various cholinesterase preparations studied, the enzyme offers very unequal resistance to the inactivating effect of formaldehyde. When endeavoring to explain this unequal resistance to the action of formaldehyde, it is difficult to know whether to attribute it to a difference in the nature of the enzyme or to a "protective" effect which might be exerted by the various tissue components accompanying the enzyme in the homogenates and which would differ according to the organ studied.

Whatever the causes, the difference in resistance observed from one preparation to another must be taken into consideration in interpreting the histochemical results between homologous cholinesterase activities like those at the level of the neuromuscular junctions of mouse and frog. On mouse muscles the residual activity is still sufficient, after proper fixation with formaldehyde, for the histochemical localization of the cholinesterase to be achieved in satisfactory condition; the inactivation of cholinesterases by the same fixative was on the other hand extremely rapid on frog muscles.

c. Results. The principal histochemical data available on the localization of cholinesterases at the level of the neuromuscular junction were obtained by methods using thiocholine esters as substrates.

Koelle and Friedenwald (1949) were the first to show histochemically, and particularly convincingly, the existence of a high cholinesterase activity at the level of the motor end-plates. In the preparations obtained by these authors, with acetylthiocholine as substrate, staining was strictly limited to the motor end-plates, but it was difficult to identify with stained structures with any certainty, although they seemed closely connected with the branches of the nerve ending and their appearance was to some extent reminiscent of the subneural apparatus stained with Janus green.

After a first modification of this method Koelle (1950) obtained further images suggesting that the enzyme of the neuromuscular junction was

chiefly concentrated at the level of "a continuous bandlike structure," but was also found in the membranes of the neighboring nuclei. This cholinesterase localization at the level of the nuclei of the junctional area is linked with the diffusion artifacts mentioned above. Another modification made in the acetylthiocholine method by Portugalov and Jakovlev (1951) and applied by them to the striated muscle also has the same disadvantages.

With the modifications made to his method in 1951, Koelle observed no nuclear localizations. Changing both the pH of the medium and the buffer, Couteaux and Taxi (1951, 1952) also eliminated these diffusion artifacts and, moreover, by fixing the muscles with formaldehyde before incubation and then comparing them with those found in fresh frozen sections, they were able to attempt a more accurate histological identification of the stained structures at the level of the motor end-plates.

In mouse and frog muscles, the structures stained by the acetylthiocholine method at the level of the neuromuscular junction seemed to them absolutely identical with those of the subneural apparatus (Plates II and III).

The results obtained from muscular preparations of the mouse, whether fixed with formaldehyde or not, have always been identical for pH values of the incubation medium well below those causing diffusion artifacts with nuclear staining. But it should be noted that, with fixed mouse muscles and with these alone, a quite different type of staining may be obtained at a slightly higher pH value, nearer to that at which nuclear staining is observed. In this case it is the arborization of the nerve ending which is electively stained and the subneural apparatus is only affected later and to a slight extent. This staining of the nerve ending, observed only after fixation, affects only the portion of the arborization situated within the synaptic gutters and, therefore, in direct contact with the subneural apparatus. Couteaux and Taxi (1952) have expressed the hypothesis that this aspect is the result of a diffusion of the reaction products starting from the subneural apparatus with secondary precipitation at the level of the nerve arborization.

With very similar technical conditions and after fixation with formaldehyde, Coërs (1953a, b) electively stained the subneural apparatus of the motor end-plates of humans, which had not previously been done by any other method; in this case he observed no staining of the nerve arborization.

So far we have considered only the results obtained with the acetylthiocholine method. By using butyrylthiocholine as substrate, Koelle (1950) observed only practically negligible staining of the motor end-plates which

led him to conclude that there is "almost exclusively" acetylcholinesterase at the level of the neuromuscular junction.

With an incubation period eight times longer than that used by Koelle, i.e. four hours, Denz (1953) obtained with butyrylthiocholine, on the diaphragm of rat, staining entirely comparable to that obtained with the acetylthiocholine method. The effect of selective inhibitors suggests that an unspecific cholinesterase is involved.

Two other methods, the long-chain fatty acid method of Gomori (1948) and the azo dye method of Nachlas and Seligman (1949a), which had not given any results for the cholinesterases of the neuromuscular junction, also enabled Denz to observe, with the diaphragm of rat, comparable aspects to those obtained with the thiocholine ester methods.

It should be pointed out that Denz's results were obtained in slightly different conditions from those governing the original methods. Gomori's (1948) method was here applied to fresh frozen sections and Nachlas and Seligman's method (1949a), modified by the use of α-naphthyl acetate as substrate, was also used successfully on fresh frozen sections, whereas in paraffin sections of acetone-fixed tissues no significant staining appeared at the level of the motor end-plates.

The results of these various methods, and in addition those obtained with Gomori's method for lipase (1945) which was applied to the striated muscle by Denz, have been subjected by this author to a detailed analysis with the aid of selective inhibitors of cholinesterases. Moreover, he has verified with acetylcholinesterase preparations that these enzymes are to some extent capable of hydrolyzing the substrates used in the long-chain fatty acid method and in the azo dye method, which were in his experiments myristoylcholine and α-naphthyl acetate, respectively. His total research has led him to conclude that the staining of motor end-plates obtained with myristoylcholine and α-naphthyl acetate as substrates is due, as in the acetylthiocholine method, to an acetylcholinesterase. From the results obtained with butyrylthiocholine, myristoylcholine, and α-naphthyl acetate, he deduces that at the neuromuscular junction there are "two enzymes sharply confined to the synaptic gutter," an acetylcholinesterase and an unspecific cholinesterase, the second of these enzymes being present in a very much smaller quantity than the first. In addition to these junctional cholinesterases there is an aliesterase distributed throughout the muscle "without focal concentration" at the neuromuscular junction.

It is interesting to note that a similar problem arises about the cholinesterase activity which is histochemically demonstrated by the acetylthiocholine method at the muscle-tendon junction on mouse and frog muscles (Couteaux, 1953) and on fish muscle; at this level the same results are

also obtained by using butyrylthiocholine as substrate after longer incubation.

Although observations of Holt and Withers (1952) and Holt (1954) on motor end-plates, by means of indoxyl derivatives, were not carried out up to now with the intention of identifying the exact nature of esterases which they include, they have, however, the great interest of confirming, by means of methods using very different substrates from those of other methods, the location of the cholinesterases at the level of subneural lamellae. By trying to limit as much as possible the diffusion artifacts through a suitable choice of the substrate and of the oxidizing agent, these authors did obtain an absolutely elective staining of the subneural lamellae as shown by photomicrographs of motor end-plates of the rat intercostal muscles after fixation by formaldehyde.

The concordance of results achieved with methods using various substrates—esters of thiocholine, long-chain fatty acid esters, α-naphthyl acetate, and indoxyl derivatives—reduces to a minimum the uncertainty by which diffusion artifacts often taint the results of histochemistry and more specially of enzymatic histochemistry. This concordance of course would be delusive if enzymatic diffusion was observed here, but on the contrary the muscle cholinesterases seem to be strongly linked to structures.

Therefore it can be regarded as very probable that the greater part of the junctional cholinesterase activity is located at the level of the subneural lamellae.

As these lamellae survive for some time after the section of the motor nerve, it was important to specify histochemically what happens to the cholinesterase activity of the motor end-plate after degeneration of the nerve endings. In their preliminary report, Sawyer, Davenport, and Alexander (1950) indicate only for the rat, the guinea pig, and the rabbit, persistence of cholinesterase concentration at level of motor end-plates after section of motor nerve. Coërs (1953c), resuming this study in a more thorough manner on the rat gastrocnemius, did follow by means of the modified Koelle method the changes in cholinesterase activity of the subneural apparatus occurring after degeneration of the nerve endings. His observations show that long after the degeneration of motor axons, it is still possible to demonstrate histochemically a cholinesterase activity at the level of the subneural apparatus; even after three months, this activity can still be demonstrated. But the weakening of the intensity of staining obtained after degeneration of the neurites, however, indicates an important diminution of this activity. These histochemical results fit satisfactorily with the biochemical results achieved by Couteaux and Nachman-

sohn (1940, 1942) when studying changes of concentration and total content of muscle cholinesterases after section of the motor nerve and also fit with their interpretation. The biochemical and histochemical studies of the denervated muscle thus establish clearly that cholinesterases of the subneural lamellae are not immediately linked to the axoplasm of nerve endings and cannot therefore be regarded as presynaptic.

Although the various methods used till now only demonstrate cholinesterases at the level of the subneural apparatus, should one consider that there are no other sites of cholinesterase activity in the motor junctional area?

Based upon biochemical data, it can already be admitted that outside the subneural lamellae there exists a certain amount of cholinesterase, as the "aneural" part includes a noticeable part of the total quantity of enzyme contained by the muscle. The failure to find the localization of this moiety of the esterase by the acetylthiocholine method should not surprise us since this method is not sufficiently sensitive to demonstrate the acetylcholinesterase of red blood cells, as was shown by the results of Harris, Cohen, and Bergner (1953), studying the correlation between quantitative methods for determination of cholinesterase activity and Koelle's histochemical technique.

It also seems probable that there is a not negligible cholinesterase concentration at the level of the nerve ending, since the cholinesterase activity of the sectioned nerve diminishes on the degeneration of its neurites. The rapid decrease in the cholinesterase of the sympathetic ganglion following the section of the preganglionic fibers (von Brucke, 1937; Couteaux and Nachmansohn, 1940, 1942; Sawyer and Hollinshead, 1945) and the no less rapid decrease in medullar cholinesterase after section of the dorsal roots (Nachmansohn and Hoff, 1944) also lead to the view that the terminal arborizations of the neurites generally have a relatively high cholinesterase content.

Lastly, Zacks and Welsh (1951) showed that unspecific and specific cholinesterases are present in rat liver mitochondria. We have therefore to admit as possible a similar location at the level of the numerous mitochondria of end-plates.

The results indicated above concern only neuromuscular junctions corresponding to large nerve fibers. The histochemical study of neuromuscular junctions corresponding to small nerve myelinated fibers in the superficial part of frog gastrocnemius shows that here also there is a subneural location of cholinesterase, limited to very small junctional areas through which small fibers, extremely thin-ended, get in touch with a muscle fiber (Couteaux, unpublished data). To know if these histochemical observations

apply to the "small nerve motor system" described by Kuffler *et al.* (1947, 1953a, b) it is better to wait for further progress in the morphological study of innervations by small myelinated nerve fibers.

3. *Conclusions*

The whole of the biochemical and histochemical results achieved up to now suggest that the cholinesterases of the motor junctional area, including acetylcholinesterase and probably a small quantity of an unspecific cholinesterase, are chiefly located at the lamellae of the subneural apparatus, situated at the frontier of axoplasm and sarcoplasm. These extremely thin lamellae are distributed in a protoplasm layer with a thickness of less than a micron.

If the nature of the subneural lamellae was not exclusively muscular but a composite, a more precise localization of the junctional cholinesterase activity would require histochemical methods with the use of ultrathin sections and electron microscopic examinations. But one fact established by the biochemical and histochemical studies of the denervated muscle dominates all interpretations; it is the fact that a great part of the junctional cholinesterase activity does not disappear as quickly as the nerve endings.

IV. References

Adams, D. H., and Thompson, R. H. S. (1948) *Biochem. J.* **42**, 170.
Adrian, E. D., Feldberg, W., and Kilby, B. A. (1947) *Brit. J. Pharmacol.* **2**, 56.
Aldridge, W. N. (1950) *Biochem. J.* **46**, 451.
Aldridge, W. N. (1953) *Biochem. J.* **53**, 62.
Allen, G. A., and Hawes, R. C. (1940) *J. Biol. Chem.* **133**, 357.
Austin, L., and Berry, W. K. (1953) *Biochem. J.* **53**, ix.
Barets, A. (1952) *Arch. Anat. microscop. Morphol. exptl.* **41**, 305.
Barnett, R. J., and Seligman, A. M. (1951) *Science* **114**, 579.
Bernheim, F., and Bernheim, M. L. C. (1936) *J. Pharmacol. Exptl. Therap.* **57**, 427.
Boeke, J. (1942) *Koninkl. Ned. Akad. Wetensch. Proc.* **45**, 444.
Boeke, J., and Noel, R. (1925a) *Compt. rend. Soc. Biol.* **92**, 263.
Boeke, J., and Noel, R. (1925b) *Compt. rend. Assoc. Anat.* **20**, 133.
Brooks, V. B., and Myers, D. K. (1952) *J. Physiol. (London)* **116**, 158.
Brown, G. L., Dale, H. H., and Feldberg, W. (1936) *J. Physiol. (London)* **87**, 394.
Brucke, F. T. von (1937) *J. Physiol. (London)* **89**, 429.
Coers, C. (1953a) *Arch. biol. (Liège)* **64**, 133.
Coers, C. (1953b) *J. belge Pathol. Med. exptl.* **22**, 306.
Coers, C. (1953c) *Acad. roy. Belg. Classe sci. Mem.* **39**, 447.
Couteaux, R. (1938a) *Compt. rend. soc. biol.* **127**, 218.
Couteaux, R. (1938b) *Compt. rend. soc. biol.* **127**, 571.
Couteaux, R. (1941) *Bull. Biol.* **75**, 101.
Couteaux, R. (1944) *Compt. rend. soc. biol.* **138**, 976.

Couteaux, R. (1945a) *Compt. rend.* **220**, 567.
Couteaux, R. (1945b) *Compt. rend. soc. biol.* **139**, 641.
Couteaux, R. (1947) *Rev. Can. Biol.* **6**, 563.
Couteaux, R. (1950) *Compt. rend. Assoc. Anat.* **61**, 80.
Couteaux, R. (1951) *Arch. intern. Physiol.* **59**, 52.
Couteaux, R. (1952) *Compt. rend. Assoc. Anat.* **73**, 264.
Couteaux, R. (1953) *Compt. rend. soc. biol.* **147**, 1974.
Couteaux, R., and Nachmansohn, D. (1938) *Nature* **142**, 1481.
Couteaux, R., and Nachmansohn, D. (1940) *Proc. Soc. Exptl. Biol. Med.* **43**, 177.
Couteaux, R., and Nachmansohn, D. (1942) *Bull. Biol.* **76**, 14.
Couteaux, R., and Taxi, J. (1951) *Compt. rend. Assoc. Anat.* **70**, 1030.
Couteaux, R., and Taxi, J. (1952) *Arch. Anat. microscop. Morphol. exptl.* **41**, 352.
Cuypers, A., and Fessard, A. (1954) *J. physiol. (Paris)* **46**, 322.
Dale, H. H., and Feldberg, W. (1934) *J. Physiol. (London)* **81**, 320.
Danielli, J. F. (1946) *J. Exptl. Biol.* **22**, 110.
Davison, A. N. (1953) *Biochem. J.* **54**, 583.
Denz, F. A. (1953) *Brit. J. Exptl. Pathol.* **34**, 329.
Easson, L. H., and Stedman, E. (1936) *Proc. Roy. Soc.* **B121**, 142.
Feng, T. P., and Ting, Y. C. (1938) *Chinese J. Physiol.* **13**, 141.
Friedenwald, J. S., and Becker, B. (1948) *J. Cellular Comp. Physiol.* **31**, 303.
Giacomini, E. (1898a) *Atti. regia accad fisiocritici Siena sez. med. fis.* **10**, 371.
Giacomini, E. (1898b) *Atti. regia accad. fisiocritici Siena sez. med fis.* **10**, 560.
Glick, D. (1938) *J. Gen. Physiol.* **21**, 289
Gomori, G. (1945) *Proc. Soc. Exptl. Biol. Med.* **58**, 362.
Gomori, G. (1948) *Proc. Soc. Exptl. Biol. Med.* **68**, 354.
Gomori, G. (1950) *J. Lab. Clin. Med.* **35**, 802.
Gomori, G. (1952a) "Microscopic Histochemistry" Univ. Press, Chicago.
Gomori, G. (1952b) *Intern. Rev. Cytol.* **1**, 323.
Günther, P. (1949) *Anat. Anz.* **97**, 175.
Gutmann, E., and Young, J. Z. (1944) *J. Anat.* **78**, 15
Harris, C., Cohen, B. S., and Bergner, A. D. (1953) *J. Histochem. Cytochem.* **1**, 405.
Hawkins, R. D., and Gunter, J. M. (1946) *Biochem. J.* **40**, 192
Hawkins, R. D., and Mendel, B. (1947) *Brit. J. Pharmacol.* **2**, 173.
Hawkins, R. D., and Mendel, B. (1949) *Biochem. J.* **44**, 260.
Held, H. (1897) *Arch. Anat. u. Physiol. Anat. Abt.* 204.
Hinsey, J. C. (1934) *Physiol. Revs.* **14**, 514.
Holt, S. J. (1954) *Proc. Roy. Soc.* **B142**, 160.
Holt, S. J., and Withers, R. F. J. (1952) *Nature* **170**, 1012.
Jacoby, F., and Martin, B. F. (1949) *Nature* **163**, 875.
Katz, B., and Kuffler, S. W. (1941) *J. Neurophysiol.* **4**, 209.
Koelle, G. B. (1950) *J. Pharmacol. Exptl. Therap.* **100**, 158.
Koelle, G. B. (1951) *J. Pharmacol. Exptl. Therap.* **103**, 153.
Koelle, G. B. (1954) *J. Comp. Neurol.* **100**, 211.
Koelle, G. B., and Friedenwald, J. S. (1949) *Proc. Soc. Exptl. Biol. Med.* **70**, 617.
Krause, W. (1863) *Z. f. rationelle Med.* **18**, 136.
Kuffler, S. W., and Gerard, R. W. (1947) *J. Neurophysiol.* **10**, 383.
Kuffler, S. W., LaPorte, Y., and Ransmeier, R. E. (1947) *J. Neurophysiol.* **10**, 395.
Kuffler, S. W., and Vaughan Williams, E. M. (1953a) *J. Physiol. (London)* **121**, 289.

Kuffler, S. W., and Vaughan Williams, E. M. (1953b) *J. Physiol. (London)* **121**, 318.
Kuhne, W. (1862) "Ueber die peripherischen Endorgane der motorischen Nerven." Engelmann, Leipzig.
Kuhne, W. (1863) *Virchow's Arch. pathol. anat. u. Physiol.* **27**, 508.
Kuhne, W. (1864) *Virchow's Arch. pathol. anat. u. Physiol.* **29**, 433.
Kuhne, W. (1883) *Z. Biol.* **19**, 501.
Kuhne, W. (1887) *Z. Biol.* **23**, 1.
Leibson, R. (1939) *Bull. biol. med. exptl. U.R.S.S.* **7**, 514.
Lison, L. (1948) *Bull. histol. appl. et tech. microscop.* **25**, 23.
Loewi, O. (1921) *Pflüger's Arch. ges. Physiol.* **189**, 239.
Loewi, O. (1924) *Pflüger's Arch. ges. Physiol.* **204**, 361.
Marnay, A., and Nachmansohn, D. (1937a) *Compt. rend. soc. biol.* **125**, 41.
Marnay, A., and Nachmansohn, D. (1937b) *Compt. rend. soc. biol* **126**, 785.
Martini, F., and Torda, K. (1937) *Klin. Wochenschr.* **16**, 824.
Meng, C. W. (1940) *Chinese J. Physiol.* **15**, 143.
Murray, P. D. F. (1924) *Proc. Linnean Soc. N. S. Wales* **49**, 371.
Nachlas, M. M., and Seligman, A. M. (1949a) *J. Natl Cancer Inst.* **9**, 415.
Nachlas, M. M., and Seligman, A. M. (1949b) *J. Biol. Chem.* **181**, 343.
Nachmansohn, D., and Hoff, E. C. (1944) *J. Neurophysiol.* **7**, 27.
Nageotte, J. (1937) *Z. Zellforsch. u. mikroskop. Anat.* **26**, 603.
Noel, R. (1925) *Bull. histol. appl. et tech. microscop.* **2**, 124.
Noel, R. (1950) *Biol. méd. (Paris)* **39**, 273.
Noel, R., and Bressaud, R. (1952) *Compt. rend. Assoc. Anat.* **71**, 73
Plattner, F., and Hintner, H. (1930) *Pfluger's Arch. ges Physol.* **225**, 19
Portugalov, V. V., and Jakovlev, V. A. (1951) *Doklady Akad. Nauk. S.S.S.R.* **78**, 1021.
Ranvier, L. (1878) "Leçons sur l'Histologie du Système nerveux" Savy, Paris.
Ravin, H. A., Tsou, K. G., and Seligman, A. M. (1951) *J. Biol. Chem.* **191**, 843.
Robertson, J. D. (1953) *Proc. Soc. Exptl. Biol. Med.* **82**, 219.
Rothenberg, M. A., and Nachmansohn, D. (1947) *J. Biol. Chem.* **168**, 223.
Rouget, C. H. (1862) *Compt. rend.* **55**, 548.
Sawyer, C. H., Davenport, C., and Alexander, L. M. (1950) *Anat. Record* **106**, 287.
Sawyer, C. H., and Hollinshead, W. H. (1945) *J. Neurophysiol.* **8**, 137.
Stoerk, H. C., and Morpeth, E. (1944) *Proc. Soc. Exptl. Biol. Med.* **57**, 154
Taxi, J. (1952) *J. Physiol. (Paris)* **44**, 595.
Tello, J. F. (1944) *Trabajos inst. Cajal invest. biol. Madrid* **36**, 1.
Tiegs, O. W. (1932) *J. Anat.* **66**, 300.
Tiegs, O. W. (1953) *Physiol. Revs.* **33**, 90.
Wagner, R. (1847) "Neue Untersuchungen über den Bau und die Endingungen der Nerven." Leipzig (cited by Ranvier, 1878).
Zacks, S. I., and Welsh, J. H. (1951) *Am. J. Physiol.* **165**, 620.

Evidence for a Redox Pump in the Active Transport of Cations

E. J. CONWAY

Department of Biochemistry, University College, Dublin, Ireland

		Page
I.	Introduction	377
II.	The Active Transport of Cations in Yeast	378
	1. Evidence for the Theory Based on the Effect of Redox Systems External to the Cell	378
	2. Effect of Redox Dyes	379
	3. Effects on the External Redox Potential	379
	4. Effects on the Active Transport of K^+ Ions	380
	5. Effects on the Excretion of H^+ Ions	381
	6. Effect on the Excretion of Na^+ Ions during Anaerobic Fermentation	381
	7. Effect of Inorganic Redox Systems	382
	8. The Nature of the Effect Produced by the External Redox Systems	382
	9. Relation of Phosphate Energy to Active Transport of Na^+ and K^+ Ions	383
III.	The Active Transport of Na^+ Ions through Frog Skin	385
	1. Structure of Frog Skin	385
	2. Relation of the Active Na^+ Transport or the Current in a Short-Circuited Skin to the Oxygen Consumption	386
	3. Comparison of Sodium Transport through Frog Skin and from Sodium-Rich Yeast Cells	386
IV.	Secretion of H^+ Ions by the Gastric Mucosa	387
	1. The Analogy with Yeast	387
	2. Active Transport of Succinate Ions from the Yeast Cell and Chloride Ions from the Oxyntic Cell	390
	3. The Ratio Q_{HCl}/Q_{O2}	391
	4. The $\Delta H^+/\Delta O_2$ Ratio for the Mouse Stomach	392
V.	Further Observations on Active Transport in Plants	394
	1. Active Transport of Calcium during Photosynthesis	394
	2. Active Transport of Na^+ Ions in *Ulva lactuca*	395
VI.	References	395

I. Introduction

In the previous communication (Conway, 1953) the theory of the redox pump (Conway, 1951, 1953) for the active transport of ions was described in some detail. It was shown how the energy for the performance of osmotic work could be transferred from the electron energy levels described by electrical potential measurement into osmotic energy described by chemical potentials. The theory in general has the initial merit of

limiting the number of hypotheses or possible systems in active transport in so far as the carrier system and the immediate energy donating system for the performance of osmotic work are one and the same system.

All theories of active transport of ions as a continuous or long sustained process may be expected to interpret the following requirements:

(1) The ions actively transported are not carried across the membrane barrier as free ions, but are bound in some complex.

(2) The carrier system in the membrane operates in a cycle.

(3) At the instant of release of the free ion to a higher chemical potential, there must be a corresponding release or transference of the required free energy.

The redox pump theory fulfills these three conditions and is specially suitable for interpreting the third.

Briefly, according to this theory (as concerns cations) and considering for example, the active transport of Na^+ from the cell, there is formed a complex of Na^+ with the reduced form of a redox system on or towards the internal aspect of the membrane. The complex moves or orients in the membrane so that in the next stage, where the electrons are transferred to another redox system, the carrier is oxidized at the outer aspect, the free inorganic ion being then liberated into the external fluid, and the potential it develops is sufficient to draw out an associated small anion, or alternatively such an anion may itself be actively carried. On the other hand the Na^+ ion so excreted may exchange for a K^+ or other inorganic ions.

In the excretion of H^+ ions, these are carried as metabolic hydrogen atoms in the reduced form of a redox system and, when the electrons are transferred, H^+ ions are set free, the subsequent events being similar to that described for the excretion of Na^+ ions.

As previously pointed out, such a theory is a development from redox theories first proposed by Lund (1928) and by Stiehler and Flexner (1938), and subsequently developed by Lundegardh (1940, 1947, 1948, for active transport of anions in root hairs.

II. The Active Transport of Cations in Yeast

1. Evidence for the Theory Based on the Effect of Redox Systems External to the Cell

It had been previously shown that when yeast ferments 5% glucose (1 part of yeast to about one part of solution) without any K^+ ions or buffering outside, succinic acid is excreted in a relatively high concentration, of the order of 50 meq./l. The amount of succinic acid so excreted can be greatly reduced by previous oxygenation of the yeast over about

30 hours. If the fermentation is carried out in the presence of 0.1 or 0.2 *M* KCl, K^+ ions exchange for the H^+ ions previously linked with the succinic ions as succinic acid, and free H^+ ions corresponding to a pH in the region of 1.4–1.8 appear in the external fluid, a corresponding amount of K^+ ions being taken up by the yeast cells. This process is practically completely inhibited by azide (0.005 *M*), and is also considerably inhibited by 2,4-dinitrophenol which at the same time scarcely affects the fermentation. These findings are described in a series of papers (Conway and O'Malley, 1946; Conway and Downey, 1950; Conway and Brady, 1950; Conway, Brady, and Carton, 1950).

In this case K^+ ions are actively taken up in exchange for H^+ ions. At the same time, under the conditions studied (Conway, Brady, and Carton, 1950), a degree of alkalinity is produced inside the cells equal to acidity outside them. Besides this active transport of K^+ ions into the cell, the active excretion of Na^+ ions from the yeast cell can be readily demonstrated (Conway, Ryan, and Carton, 1954). In this demonstration, a sodium-rich yeast is prepared first by fermenting for two hours in the presence of *M*/5 sodium citrate (Conway and Moore, 1954), and then washed and resuspended either in water when Na ions are actively excreted with anions or in *M*/10 KCl when they are much more rapidly excreted in exchange for K^+ ions. In this process the active excretion of Na^+ is inhibited by anoxia or cyanide, but Na^+ can be actively extruded anaerobically if the sodium-rich yeast ferments a 5% glucose solution. It is upon such active transporting systems operating anaerobically that the effects of external redox systems were investigated.

2. *Effects of Redox Dyes* (Conway and Kernan, 1955)

Washed bakers' yeast was suspended in 20 times its weight of a solution containing 5% glucose and 0.04 *M* potassium succinate at a pH of approximately 4.5, one or another redox dye being included in 0.0001 *M* strength, the effect of the dye being compared with a control in which it was absent. Throughout the experiment pure nitrogen gas was bubbled through the mixture.

3. *Effects on the External Redox Potential*

The curves of potentials for resting yeast (Curve A) in the mixture without glucose and for fermenting yeast (Curve B) appear in Fig. 1. Figure 2 shows the effects of Nile blue, with a characteristic potential of +19 mv. at a pH of 4.5, and of phenol indo 2,6-dichlorophenol, with a characteristic potential of +388 mv. at a pH of 4.5. The initial

steep decline of potential is attributable to the residual oxygen being used up. After this period, fermentation in itself produces a marked increase in potential over the resting yeast and reaches a figure of about +180 mv.

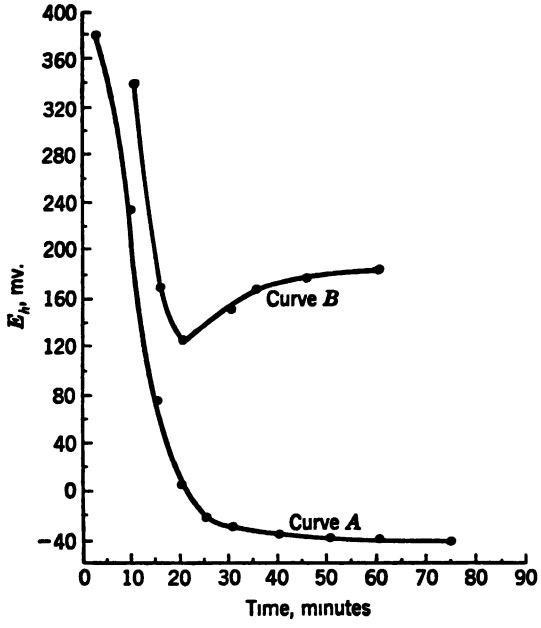


FIG. 1. The redox potentials of yeast suspensions (1:20 of mixture described in text) at pH 4.5 plotted against the time in minutes from the introduction of a stream of nitrogen gas. Curve A: without glucose. Curve B: suspending fluid contains 5% glucose. (Conway, 1952)

after 45 minutes. The presence of Nile blue, which may be termed in this context a reducing dye, causes a marked fall in potential which tends to stabilize, after about 45 minutes, to a level of 47 mv. On the other hand, with the oxidizing dye the level of about +320 mv. is reached. Marked changes in potential can therefore be produced by such dyes in concentrations of only 0.0001 *M*, depending on their characteristic potential.

4. *Effects on the Active Transport of K^+ Ions*

This effect is measured by finding the K^+ absorption after 45 minutes of anaerobic fermentation in the presence of the redox dye and for the same period in the control mixture, and then expressing the percentage change in the K^+ absorption attributable to the dye.

As appears from Fig. 3, when the redox potential in the presence of

the dye is reduced below +180 mv., the uptake of potassium is inhibited and tends toward zero absorption at a potential of about +100 mv. On the other hand, when the potential is raised above the control level, there

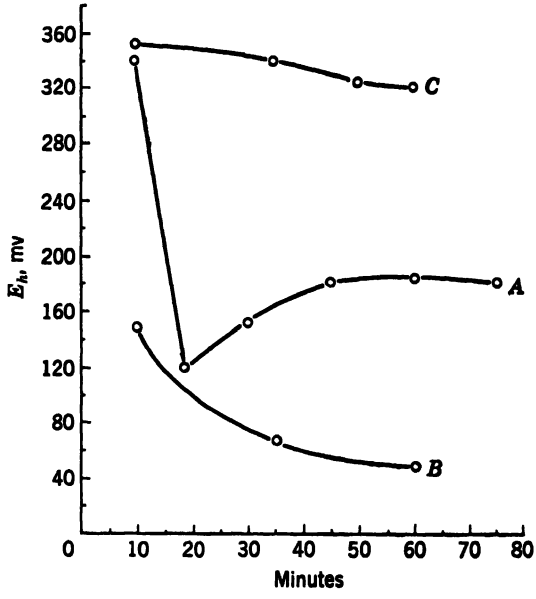


FIG. 2. Conditions as in Fig. 1 with suspending fluids containing 5% glucose. Curve C: with phenol indo 2,6-dichlorophenol. Curve B: with Nile blue (0.0001 *M*). Curve A: with no redox dye. (Conway, 1952)

occurs a marked increase in the uptake, depending on the level to which the external redox potential is raised.

5. *Effect on the Excretion of H^+ Ions*

The effect on the excretion of H^+ ions appears in Fig. 4. As K^+ ions are taken up, under the stated conditions, in exchange for H^+ ions, it is to be expected that the effect on the H^+ ion excretion will be the same as on the K^+ ion excretion, which is borne out by the observations.

6. *Effect on the Excretion of Na^+ Ions during Anaerobic Fermentation*

It is curious that the change of potential produced by the redox dyes has an effect on the excretion of Na^+ ions which is the opposite of its effect on the absorption of K^+ ions. It is to be noted that the potential

of the control mixture without any redox dye is much elevated above that found with a similar control for a normal yeast instead of a sodium-rich yeast, being +260 mv. as compared with +180 mv.

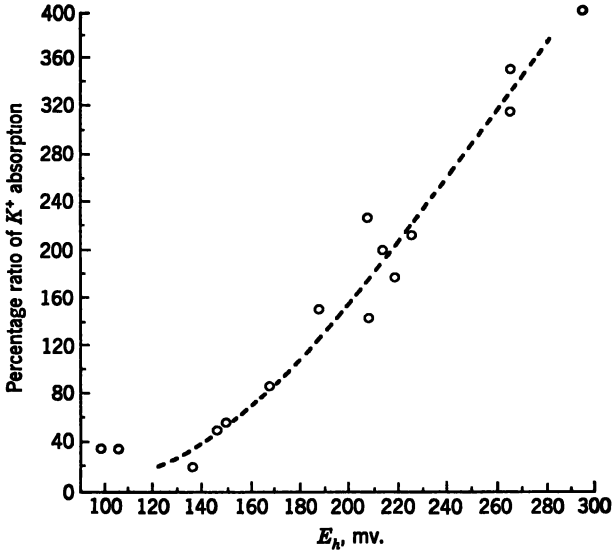


FIG. 3. Percentage ratio of K^+ absorption after 90 minutes fermentation K^+ absorption without redox dyes being 100, plotted against redox potentials of the yeast suspensions using various redox dyes as described in text. (Conway, 1952)

7. *Effects of Inorganic Redox Systems*

Ceric sulphate and chromic chloride were used as oxidizing systems and titanous chloride and stannous chloride as reducing systems. These substances were used in the same manner as the redox dyes. The results showed a trend similar to the effect of the redox dyes. The high-potential systems produced an increase in potential over the control and carried in turn an increased potassium absorption, the low-potential systems acting in the reverse way.

However, the following difference appears: it requires a larger potential difference with the inorganic system to produce effects similar to the organic redox dyes.

8. *The Nature of the Effect Produced by the External Redox Systems*

It is apparent that the effects are rather closely correlated with the potential changes they bring about. Yet the result is not interpretable in the simple terms of reversible systems ready to give or receive electrons.

It would appear necessary to suppose some kind of correlation of the dye with a redox system in the membrane. For example, with the reducing dyes, a combination may be assumed to occur between the oxidized form of the dye and the reduced form of what may be termed as normally the K^+

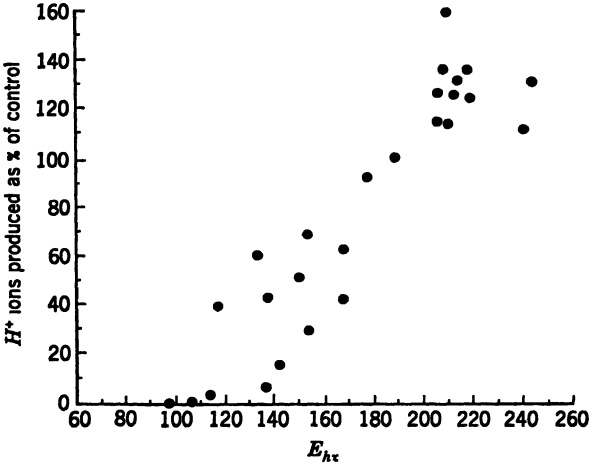


FIG. 4. Percentage excretion of H^+ ions after 90 minutes fermentation (excretion without redox dyes being 100) plotted against the redox potentials of yeast suspensions using various redox dyes as described in text.

carrier, and that this blocks the further transference of electrons from the carrier. This system will then be unable to complete its cycle and not only is the K^+ absorption inhibited but likewise the H^+ excretion.

This type of explanation, however, would appear more difficult when the K^+ absorption or H^+ ion excretion is increased by oxidizing dyes.

Whereas the details of the action are not clear, it is in any case evident that such marked effects, produced by very small amounts of redox dyes and related to the change of potential they produce, are good evidence for the assumption that the active carriers are themselves redox systems.

9. *Relation of Phosphate Energy to Active Transport of Na^+ and K^+ Ions*

As is well known the formation of energy-rich phosphates would appear to be the main way in which the free energy of electron transference across redox systems (proceeding from the original organic metabolite) is fixed in a form available for various processes of a chemical or physical kind requiring free energy (see Lipmann's reviews, 1941, 1946). As already shown the redox pump system can utilize the electron energy directly, without indirect passage through energy-rich phosphate or other

bonds. Whereas it is practically certain with regard to active uptake of K^+ ions that the phosphate bond as in polyphosphates is not *directly* associated with the actual carriage of the ions, none the less this process is inhibited by 2,4-dinitrophenol which is considered to act more or less specifically in the inhibition of phosphate bond formation.

The reason why it is concluded that the immediate carrier is not a phosphate bond is that when yeast is suspended in 5% glucose (1 part of yeast in 20 parts of solution) containing 0.05 *M* magnesium acetate at a pH of 6.0 with no other salt present, then Mg^{++} is steadily transported into the yeast cells in exchange for H^+ ions, but the presence of K^+ even in one two-hundredth part of the concentration of the Mg^{++} ion can almost completely inhibit the Mg^{++} uptake. Similarly if Mg^{++} ions (0.1 *M*) only are present outside the resting sodium-rich yeast they are taken up in exchange for Na^+, but a very small K^+ ion concentration can inhibit the Mg^{++} uptake. Investigating other ions in a similar way, relative affinities of various ions for the K^+ carrier are given in Table I (Conway and Ryan, 1954).

TABLE I

RATES OF ENTRY OF Ca^{++}, Mg^{++}, Li^+, Cs^+, Rb^+ INTO SODIUM-RICH YEAST AS COMPARED WITH ENTRY OF K^+ IONS

Ion examined	External concentration of ion (meq./l.)	External K^+ concentration at 50% uptake (meq./l.)	Ratio of data in columns 3 and 2	Uptake in absence of K (meq./kg.)
Ca^{++}	200	0.32	1:625	8.9 (3 hours)
Mg^{++}	200	0.47	1:425	15.8 (3 hours)
Li^+	100	0.93	1:107	21.5 (1½ hours)
Cs^+	100	20.0	1:5	38.0 (1½ hours)
Rb^+	100	37.0	1:2.7	43.0 (1½ hours)

Affinities for a phosphate or polyphosphate bond carrier would follow quite a different order.

Also it may be noted that, whereas 2,4-dinitrophenol or azide inhibits the active transport of K^+ ions either exchanging for H^+ ions during fermentation or exchanging for Na^+ ions in a resting sodium-rich yeast, the active excretion of Na^+ ions (not exchanging for K^+ ions) from such yeast is not inhibited by 2,4-dinitrophenol or azide but is inhibited by anoxia or by cyanide (Conway, Ryan, and Carton, 1954).

III. The Active Transport of Na^+ Ions through Frog Skin

The study of the active transport of salt through frog skin has had a long history, which has been reviewed by Ussing in various communications (e.g. Ussing, 1949a, b, 1951; Ussing and Zerahn, 1951). A good summary has also been given by Linderholm (1952). The introduction of isotopic tracers opened an intensive period of study (1939, 1940) in which it was shown that Na^+ ions pass through the frog's skin at a greater rate inwards than outwards when the same saline mixture was used on both sides.

Ussing extended this study demonstrating (1948, 1949b) that Na^+ ions could be transported across the skin against a steep electrochemical potential gradient and showed, in various papers with collaborators, the effects of various factors on the active transport. By short circuiting the inner and outer surfaces of the skin in an outer circuit and reducing the potential difference across the skin towards zero, Ussing and Zerahn (1951) reported that the current then passing in the outer circuit could be attributed entirely in such isolated skins to the active transport of Na^+ ions, no net passage of Cl^- ions occurring. In short, when NaCl was actively transported across the skin, Na^+ ions were actively carried and Cl^- ions followed passively due to the potential development. This was fully confirmed by Linderholm, 1952. Under certain conditions, evidence for active Cl^- ion transport could be obtained. This appeared to occur when the skin was stimulated by adrenaline (Koefoed-Johnsen, Ussing, and Zerahn, 1951.)

1. Structure of Frog Skin

Frog skin is composed of an inner connective tissue layer and an outer epithelial layer. The connective tissue layer allows for free diffusion, and active transport can only occur in the epithelial layer. This consists of two or three layers of cells. The nearest layer to the connective tissue is constituted of cylindrical epithelial cells (stratum germinativum), the cells external to this being more or less keratinized, and fully keratinized at the outermost edge.

If, as Ussing points out, we regard these epithelial cells as having the general property of active Na^+ extrusion, the loss of this property in keratinization will impart an inward bias or direction to the active transport of Na^+ ions.

As noted by Linderholm (1953), Ottoson *et al.* (1953) found in electron micrographs a continuous basement membrane 200–300 Å thick which covers the basal epidermal cells and separates the chorion connec-

tive tissue from the epithelium. They were able to show also (using microelectrode and optical techniques) that the essential electrical potential drop is located at the dermo-epidermal junction. Furthermore, it would appear that the epithelial cells are separated by intercellular spaces.

Linderholm (1952) pointed out that the redox pump theory "has the advantage that it readily explains the current produced by the skin, especially the direct relation between the net transport of sodium ions and the equivalent amount of current produced in the short-circuited skin." Also, "it explains the dependence of the electrical potential on the oxygen supply as well as the inhibition of the potential by several respiratory inhibitors."

2. *Relation of the Active Na^+ Transport or of the Current in a Short-Circuited Skin to the Oxygen Consumption*

For every gram molecule of oxygen consumed in the acceptance of electrons passing through the redox pump, $4F$ of electricity are required. Thus for the short-circuited skin the ratio:

$$\frac{\text{current (expressed as meqs. net sodium extrusion)}}{\text{millimoles } O_2 \text{ consumed by the skin}}$$

should have an upper limit of 4.0. The ratio should be experimentally less than 4.0 since, as would seem probable, the oxygen consumption of the skin very largely arises from the epithelial cells (Erdman and Schmerl, 1926) and is there largely associated with the transport mechanism and may be expected to be not much less than 4.0. The respiration was measured in skins which were effectively short-circuited by the body fluid and the Q_{O_2} values are therefore best compared with the sodium flux in short-circuited skins. From the mean value of 1.33 $m\mu l.\ O_2 cm.^{-2}\ sec.^{-1}$ and a mean sodium transport in short-circuited skins corresponding to a current of $197/\mu\mu F$ $cm.^{-2}\ sec.^{-1}$ a ratio of 3.28 is obtained. Linderholm discusses the data much further and in general the conclusion to be drawn is that the ratio obtained is in harmony with what might be expected from the redox pump theory.

3. *Comparison of Sodium Transport through Frog Skin and from Sodium-Rich Yeast Cells*

The active transport of Na^+ ions appears to be a general endowment of cells if one may judge from its occurrence in the yeast cell as well as in muscle and nerve and other animal tissue cells. This endowment is certainly a curious one from the chemical standpoint, though its physiological origins would seem plain enough if one considers the origin and

development of the cell in an early marine environment (see Conway, 1943, 1947). It is very probable that the essential nature of the carrier system is the same wherever it appears though the energy supply and final electron acceptor may differ in kind. If we were to compare the system as it operates in yeast and in the frog skin, there are the following similarities:

(1) The carrier in each case is highly specific for Na^+ ions as compared with K^+ ions. Thus, in a sodium-rich yeast, although K^+ ions are present as well as Na^+ ions, and often in a greater concentration, no detectable amount of K^+ ions are excreted when Na^+ is being excreted in quantity. Similarly, it has been found (Ussing and others) that the transport of Na^+ ions is very specific for Na^+ as against K^+.

(2) Although specific as against K^+, the carrier system in yeast and frog skin treat Li^+ and Na^+ ions very similarly (though this does not apply apparently to the mammalian erythrocyte, Maizels, 1954). Thus a sodium-lithium-rich yeast excretes Na^+ and Li^+ in about the same proportion as they appear in the yeast, while no K^+ is excreted (Conway and Ryan, 1954).

(3) Both carrier systems, the one operating in resting sodium-rich yeast and the other in the frog skin are inhibited by anoxia and by cyanide.

Besides these similarities, the following differences may be mentioned: (a) The active transport of sodium in frog skin is inhibited by 2,4-dinitrophenol (Ussing, 1954) whereas this does not inhibit the sodium carrier in yeast; it does, however, inhibit the potassium carrier in yeast (Conway, Ryan, and Carton, 1954). (b) The active transport of sodium in the frog skin is inhibited also by drugs which inhibit choline esterase, such as tetraethylpyrophosphate and eserine (Ussing, 1954). These have no effect on the active sodium transport in yeast.

Reviewing these facts it would seem reasonable to conclude that the systems are essentially the same for yeast and frog skin, and that the differences in action of certain inhibitors arise from indirect rather than direct actions on the carrier.

IV. Secretion of H^+ Ions by the Gastric Mucosa

1. The Analogy with Yeast

Washed bakers' yeast suspended in about 0.6 of its volume in a solution of 5% glucose containing 0.1 *M* KCl produces a pH in the suspending fluid of about 1.8 (Conway and O'Malley, 1946). Within about 30 minutes the suspending fluid contains free HCl in amount corresponding to a pH of 1.8; it contains also free succinic acid (see Rothstein and

Enns, 1946), the total titratable acidity being about half due to the free succinic acid though the proportion is variable. The succinic acid excreted in such experiments appears to come largely from succinate ions already present in the yeast cells (Conway and Brady, 1950), although if the

TABLE II

ACID PRODUCTION BY THE FERMENTING YEAST CELL AND THE ACTIVE OXYNTIC PARIETAL CELL

Type of cell	pH reached by secretion or external fluid	Secretion rate as meq./kg./hour (wet weights)
Parietal cells, human mucosa	0.9	2,500
Yeast cells (bakers' yeast) 1:250 of 5 per cent glucose (38°C.) (oxygenated)	3.2	625
Parietal cells, rat mucosa	0.9	300
Frog gastric mucosa (25°C.)	1.0–2.0	30 (72 maximum).
Yeast cells, 1:0.6 of 5 per cent glucose containing 5/*M* KCl (prior oxygenation) (18°C.)	1.65 (minimum of 1.4)	50 (maximum of 100)

From Conway (1952).

In the above table, the order of figure for the acidic secretion rate has been stated rather than the mean as a formal statistical expression. The data for the human secretion rate are taken from Ihre (1939), with mucosal weight and fractional volume of the parietal cells from Davies (1948). Data for the rat are from Davenport (1946) and for the frog mucosa from Davies (1948), the average secretion rate being roughly calculated from his figures. To obtain the rate from the oxyntic cells of the frog mucosa the figures above should be multiplied by a factor which is probably of the order of 2 to 4. Yeast cell data are from the author's laboratory (Conway and O'Malley, 1946; Conway and Brady, 1947, 1950; and Conway and Carton, 1950). With the concentrated suspension of yeast (1:0.6) the mean rate for the first 30 minutes has been taken.

volume of the suspending fluid be much increased, as for example to 20 times the volume of yeast, succinic acid is newly formed and secreted over a long period (which agrees with the experimental findings of Kleinzeller, 1941).

When no KCl is present in the external fluid, the secretion of succinic acid is greater than when KCl is present and the external pH is about 3.0 because of the free succinic acid.

When the washed yeast is oxygenated for many hours the yeast cells are much depleted of succinic ions, and when the yeast is then suspended in 0.6 its volume of 5% glucose, the succinic acid secretion is very much reduced. If, however, 0.1 *M* KCl is present, the free K^+ ion concentration

produced is much greater than before and the pH reaches about 1.6. Also, if the suspending fluid is progressively reduced in value, a pH of 1.5 is reached outside the yeast cells, produced by their metabolic activity.

Tables II and III illustrate the analogous H^+ ion secreting power of the

TABLE III

COMPARISON BETWEEN YEAST SUSPENDING FLUID AFTER 30–40 MINUTES FERMENTATION AND THE HISTAMINE JUICE OF THE GASTRIC MUCOSA

	Yeast fluid	Parietal secretion of dog (Gray, 1943)	Parietal secretion of man (Fisher and Hunt, 1950)
pH	1 4 (minimum observed)	0.9	0.9
Cl^-	142 mM./l.	166 mM./l.	170 mM./l.
H^+	40 mM./l.	159 mM./l.	160 mM./l.
K^+	102 mM./l.	7 mM./l.	10 mM./l.
Na^+	0 mM./l.	0 mM /l.	0 mM./l.
Organic acid anions	2 mM./l. minimum observed	None	None

The yeast was previously oxygenated for 30 hours and then suspended (1 part centrifuged yeast to 0 6 suspending fluid) in a solution containing 5% glucose and M/5 KCl (Conway, 1952)

yeast cell and that of the oxyntic cell of the gastric mucosa in higher animals. It may be pointed out that when the yeast cell excretes H^+ ions in exchange for K^+ ions, or in association with succinate ions, such H^+ ion excretion occurs through the same mechanism, as is shown by various facts reviewed elsewhere (Conway, 1952). Thus, when the cells are excreting only succinic acid in the absence of external K^+ ions, this excretion is inhibited by various substances or factors to the same degree as the excretion of H^+ ions in exchange for K^+ ions.

The fact that the highest concentrations of free H^+ ions appear during fermentation as a result of an exchange of K^+ and H^+ ions may (as already noted) obscure a more significant analogy between the excretion of succinate ions and of Cl^- ions.

This analogy is shown by the effect of azide, which can inhibit almost completely the excretion of succinic acid or the exchange of H^+ ions with K^+ ions. It is also illustrated by the fact that, whether the acidity outside the cell is entirely due to succinic acid or to free H^+ ions, there is an equivalent alkalinization within the cell using concentrated yeast suspensions.

If, then, in yeast without added KCl only succinate ions are available in quantity to accompany the H^+ ions liberated by the ionization of the re-

duced metal catalyst, whereas in the parietal cell Cl^- ions play the same role, the significant parallel lies between the secretion of succinic acid by yeast and HCl by the parietal cell.

The above considerations relate to analogies of levels and rates of H^+ ion secretion.

Further close parallels will be found in the following list of facts explaining the "redox theory" of gastric acid secretion (Conway, 1953). It explains: (a) how H^+ ions can be excreted in high concentrations without their passing directly from the cellular fluid across a very high concentration difference. (b) why no indication of a metabolite of glucose, acting as a direct H^+ donor, can be found for the production of parietal or yeast acidity. (c) how H^+ ions can be formed metabolically at a low pH. (d) why, for each equivalent of H^+ ions excreted, a H^+ ion equivalent is removed from within the cell. (e) the action of azide in inhibiting the acid secretion from the parietal or oxyntic cell while increasing the oxygen consumption, and inhibiting the yeast acidity while increasing the alcohol production. (f) the action of other inhibitors, such as monoiodoacetic acid which, cutting off the entire supply of hydrogen atoms from dehydrogenases, eliminates the formation of both acid and alcohol in yeast. (It also explains why cyanide ($N/200$) completely inhibits H^+ ion secretion in the parietal or oxyntic cell, by blocking the path of the electrons to oxygen, but has no appreciable effect on H^+ ion excretion from anaerobically fermenting yeast). (g) why HCl, once formed, cannot readily return. (h) how the secretion of HCl from the parietal cell can be quantitatively greater than the oxygen consumed.

2. *Active Transport of Succinate Ions from the Yeast Cell and Chloride Ions from the Oxyntic Cell*

In the case of the yeast cell, resting or during fermentation, neither succinic acid nor its ions appear to penetrate into the cells when introduced into the suspending fluid, but succinate ions which are excreted in quantity during fermentation, associate with the special H^+ ion excretion and appear as succinic acid. When the volume of the suspending fluid is relatively low, the succinate ions excreted are already very largely present in the cells. It would seem probable that the succinate ions are actively transported rather than brought out passively by the potentials developed by the H^+ ions excreted. Evidence has also been brought by Hogben (1951) in Ussing's laboratory that the chloride ions of the gastric secretion are actively transported, and it is obvious that such active transport is quite in harmony with the redox theory of gastric acid secretion.

3. *The Ratio Q_{HCl}/Qo_2*

There remains the consideration of a further important implication of such a theory, namely that where oxygen is the final acceptor the ratio:

$$\frac{\text{H}^+ \text{ ions secreted}}{\text{oxygen molecules consumed}} \text{ or } Q_{HCl}/Qo_2$$

may be expected to have an upper limit of 4.0 in accordance with the equation:

$$O_2 + 4e + 4H^+ \longrightarrow 2\ H_2O$$

In the case of gastric acid secretion, taking the extra oxygen after histamine action on the isolated stomach of the frog as associated with the extra acid secreted, Davies and his collaborators support higher ratios than 4.0 (see Davies and Ogston, 1950) and an energetic efficiency for isolated stomachs of about 100%.

Davenport (1952) carried out a large number of observations on frog gastric mucosal sacs, prepared as described by Davies (1948) in which the acid production and O_2 consumption were measured. The results of 147 such observations, with and without histamine (this being used in various concentrations, at a pO_2 of 610 mm. Hg.), are shown in Fig. 5. It may

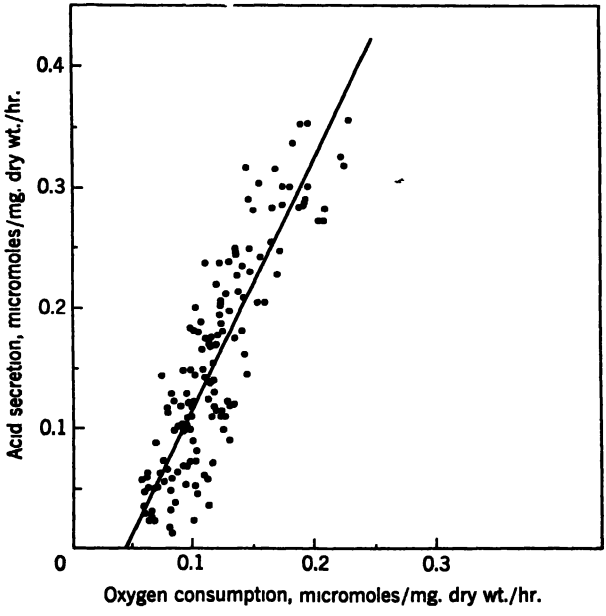


FIG. 5. From Davenport (1952).
(Courtesy of Federation Proceedings)

be noted that he always found that unstimulated mucosae secrete acid, sometimes at a high rate.

The line through the observations is a regression line (the correlation coefficient = +0.86), the equation being expressed as $Y = -0.100 + 2.15\ x$, where 'Y' is the acid secretion (μM./mg. dry weight/hour) and 'x' is the oxygen consumption (μM./mg. dry weight/hour).

The slope 2.15 ($\Delta H^+/\Delta O_2$) may be considered as the ratio of moles H^+ produced to moles O_2 consumed or Q_{HCl}/Q_{O_2}. The intercept on the x-axis may be taken as the nonacid-producing oxygen consumption.

If the experiments are carried out as a series of two stages in which the acid secretion and oxygen consumption are measured in the first stage and, after washing out the sac and refilling with salt solution, the observations are repeated, a difference in slope of the line relating acid production to oxygen consumption may be observed. This applies also where, in the first stage, histamine is not used, but is used in the second stage. Such change of slope is of the greatest importance with regard to the interpretation of efficiency of acid production.

Davenport (1952) illustrates this in a diagram, where there are two slopes, corresponding to $Q_{HCl}/Q_{O_2} = 2$ or 4 (Fig. 6). One possibility in this diagram is shown by line A. In this the rate of acid secretion increases as a result of stimulation "*but the rate of oxygen consumption goes down, as it often does in fact.*" The apparent ratio in this case is the observed one of —1.8. Lines B and C indicate possibilities in which the apparent ratios are +22 and +0.8. He remarks further "we have not been able to design experiments which will establish the true efficiency independently of the slope of the regression line obtained for a large number of data. All that can be said is that we have never found the molar ratio of acid secreted to oxygen consumed to be significantly greater than 4. If this is actually the maximum ratio, the thermodynamic efficiency of the secretory process is only about 30 per cent. A ratio of 4 molecules of acid secreted to 1 molecule of oxygen used up suggests that the underlying process is an oxidation-reduction reaction, involving the transfer of one electron for each proton secreted."

4. *The $\Delta H^+/\Delta O_2$ Ratio for the Mouse Stomach*

The observations of Davenport and Chavré (1953) were extended to the isolated mouse stomach, the experimental treatment of which has been greatly improved (Davenport and Chavré, 1953). The values for the ratios were investigated in a series of paired observations, one before and one after administration of carbaminoylcholine (0.1 mg.%) on the same stomach.

None of the ratios obtained for the mouse stomach had negative values such as were found for frog gastric mucosae (range of —46 to +50). For the latter no significant statement of the ratio could be made apart from the regression line for a large number of observations. However, with the

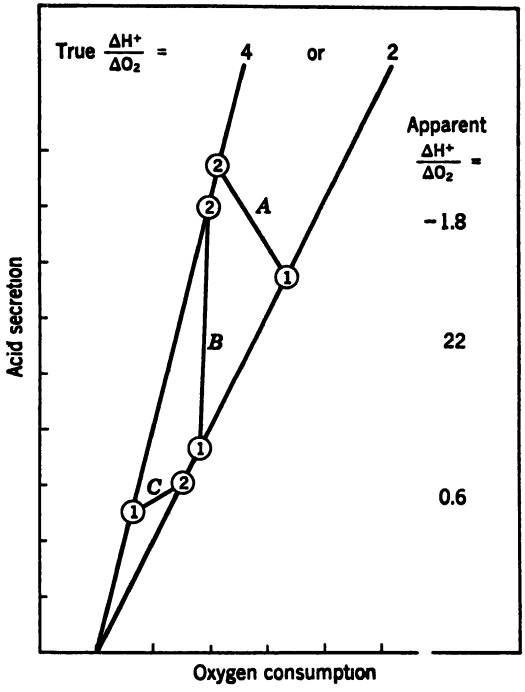


FIG. 6. From Davenport (1952). (Courtesy of Federation Proceedings)

mouse mucosae, the values obtained for each pair of observations on the one stomach before and after the action of carbaminoylcholine lay within a narrow range, and could be regarded as giving significant ratios. The ratios were in fact of the same order as the slope of the regression line for a number of data.

The conclusions of Davenport and Chavré (1954) may be quoted. Referring to the acid secretion by the gastric mucosa of the frog and the mouse they write "although the proof is not absolutely rigorous, it appears that the efficiency measured by the ratio $\Delta qH^+/\Delta qO_2$ in both species is 4 or less." They add, "If the efficiency is really 4 or less, the thermodynamic efficiency is low and it is unnecessary to postulate mechanisms giving nearly 100% thermodynamic efficiency as Davies and Ogston (1950) have done."

They also note that reducing substances, cysteine and glutathione, appear to increase acid secretion, and that this supports the redox theory. Their very recent communication (Davenport and Chavré, 1954) is thus of much interest in so far as they have found that *p*-mercuribromobenzoate (a specific inhibitor of SH groups) is a powerful inhibitor of gastric acid secretion.

V. Further Observations on Active Transport in Plants

1. Active Transport of Calcium during Photosynthesis

The attention of the writer was drawn to the significance of such active transport and its relevance to the redox pump mechanism by a thesis of Lowenhaupt (1954). The thesis gives an extended account of such a process, and of the literature relating thereto and also of a number of experiments conducted by its author on the leaves of *Potamogeton crispus*, a species of pond weed. The description and results of these experiments is followed by a detailed description of the redox pump theory in its application to the facts elucidated.

The theory developed is a modification of that already published by the present writer, and would appear to correlate very well the various facts of the active calcium assimilation.

Lowenhaupt points out in his introduction that the existence of two physiologically different groups of plants, and the ability of one of these groups to assimilate bicarbonate as a carbon source in the photosynthesis process, was shown by the researches of Ruttner, Avens, and Steeman Nielsen (a complete review of the literature being given by Österlind, 1949).

In a series of papers Avens (1930–1938) concluded that calcium (and some other cations) moves through certain aquatic leaves when in the light, entering the ventral side in amounts equivalent to the bicarbonate ions which enter there, and subsequently leaving the dorsal side along with hydroxyl or carbonate ions.

Such observations were confirmed and extended by Steeman Nielsen (1944, 1947, 1952) and Österlind (1952) who showed the process to be cyanide sensitive.

In this photosynthetic assimilation of bicarbonate, malate is formed as the organic synthetic product. Lowenhaupt (1954) conducted an extended investigation of the process with respect to active calcium transport and the effect thereon of various factors, light, aeration, etc. using the leaves of *Potamogeton crispus.*

The experiments of Avens (1938) for the cytochemical localization of calcium binding were repeated with the leaves of *Potamogeton crispus.* These leaves, consisting of a three-cell layer, were immersed in 0.5%

sodium oleate, or in ammonium oxalate solution, and the location of the resulting precipitate observed under the microscope. The location of the precipitate was in the external faces of the dorsal and ventral layers of cells, and never on the inner cell walls.

In the redox theory put forward by Lowenhaupt to explain the results it is considered that the Ca^{++} ions are bound to the reduced form of a respiratory ferment, the complex is then oriented internally and oxidized by a substance *Z*, liberating Ca^{++} ions. The reduced form of *Z* then enters the respiratory chain ending in oxygen.

As indicated by Lowenhaupt this is essentially similar to the redox pump theory (Conway, 1951, 1953) but differs in certain details, important for the special circumstances considered.

When the Ca^{++} ions are released after absorption they pass to the outer membrane of the dorsal cell layer and the process is repeated, the Ca^{++} ions being extruded along with OH^- or CO_3^{--} ions.

2. *Active Transport of Na^+ Ions in* Ulva lactuca

Very recently Scott and Hayward (1954) have suggested that the sodium pump in *Ulva lactuca* is associated with a redox mechanism. When poisoned with iodoacetate, there is usually a loss of potassium and an uptake of sodium. Whereas arsenate in the dark after iodoacetate treatment affords no protection against the sodium uptake caused by the iodoacetate alone, in the light, under the same conditions, sodium is rapidly secreted to the control level. With regard to potassium, arsenate (5×10^{-3} *M*) completely protects *Ulva* against the potassium loss usually observed with iodoacetate in the dark and in the light gives rise to a small reaccumulation of potassium (presumably through photosynthetic supply of carbohydrate intermediates).

The authors consider the effect of light (as given above) on the sodium pump to be quite apart from such a formation of carbohydrate intermediates and suggest "since the reducing properties of light are well known, that it is associated with a redox mechanism of some sort."

VI. References

Avens, K. (1930) *Planta* **10**, 814.
Avens, K. (1933) *Planta* **20**, 621.
Avens, K. (1936) *Jahrb. wiss. Botan.* **83**, 513, 561.
Avens, K. (1938) *Protoplasma* **30**, 104; **31**, 508; **33**, 295.
Conway, E. J. (1943) *Proc. Roy. Irish Acad.* **B48**, 161.
Conway, E. J. (1947) *Irish J. Med. Sci.* **262**, 593, 654.
Conway, E. J. (1951) *Science* **113**, 270.
Conway, E. J. (1952) "The Biochemistry of Gastric Acid Secretion." Thomas, Springfield, Illinois.
Conway, E. J. (1952) *8th Symposium Soc. Exptl. Biol.*, Bangor.

Conway, E. J. (1953) *Intern. Rev. Cytol.* **2**, 419.
Conway, E. J., and Brady, T. (1947) *Nature* **160**, 506.
Conway, E. J., and Brady, T. G. ((1950) *Biochem. J.* **47**, 360.
Conway, E. J., Brady, T. G., and Carton, E. (1950) *Biochem. J.* **47**, 369.
Conway, E. J., and Downey, M. (1950) *Biochem. J.* **47**, 347.
Conway, E. J., and Kernan, R. (1955) *Biochem. J.* in press.
Conway, E. J., and Moore, P. T. (1954) *Biochem. J.* **57**, 523.
Conway, E. J., and O'Malley, E. (1946) *Biochem. J.* **40**, 59.
Conway, E. J., and Ryan, H. (1954) *Biochem. J.* in press.
Conway, E. J., Ryan, H., and Carton E. (1954) *Biochem. J.* **58**, 158.
Davenport, H. W. (1946) *Physiol. Revs.* **26**, 560.
Davenport, H. W. (1952) *Federation Proc.* **11**, No. 3.
Davenport, H. W., and Chavre, V. J. (1953) *Am. J. Physiol.* **174**, 203.
Davenport, H. W., and Chavre, V. J. (1954) *Am. J. Physiol.* in press.
Davies, R. E. (1948) *Biochem. J.* **42**, 609.
Davies, R. E., and Ogston, A. G. (1950) *Biochem. J.* **46**, 324.
Erdman, R., and Schmerl, E. (1926) *Arch. Exptl. Zellforsch. Gewebezücht.* **2**, 280.
Fisher, R. B., and Hunt, J. N. (1950) *J. Physiol. (London).*
Gray, J. S. (1943) *Gastreonterology* **1**, 390.
Hogben, C. A. M. (1951) *Proc. Natl. Acad. Sci. (U.S.)* **37**, 393.
Ihre, J. E. (1939) "Human Gastric Secretion." Oxford, New York.
Koefoed-Johnsen, V., Ussing, H. H., and Zerahn, K. (1952) *Acta Physiol. Scand.* **27**, 38.
Kleinzeller, A. (1941) *Biochem. J.* **35**, 495.
Linderholm, H. (1952) *Acta Physiol. Scand.* **27**, Suppl. 97.
Linderholm, H. (1953) *Acta Physiol. Scand.* **31**, 36.
Lipmann, F. (1941) *Advances in Enzymol.* **1**, 99.
Lipmann, F. (1946) *Advances in Enzymol.* **6**, 231.
Lowenhaupt, B. (1954) Dissertation, University of California, Berkeley.
Lund, E. J. (1928) *J. Exptl. Zool.* **51**, 265, 291, 327
Lundegardh, H. (1940) *Ann. Agr. Coll. Sweden* **8**, 233.
Lundegardh, H. (1947) *Ann. Rev. Biochem.* **16**, 503.
Lundegardh, H. (1948) *Discussions Faraday Soc.* **3**, 139.
Maizels, M. (1954) *Symposia Soc. Exptl. Biol.* **8**, in press.
Österlind, S. (1949) *Symbolae Botan. Upsalienses* **X**, No. 3, 1.
Österlind, S. (1952) *Physiol. Plantarum* **5**, 372.
Ottoson, D., Sjöstrand, F., Stenstrom, S., and Svaetichin, G. (1953) *Acta Physiol. Scand.* **29**, Suppl. 106, 611.
Rothstein, A., and Enns, G. H. (1946) *J. Cellular Comp. Physiol.* **28**, 231.
Scott, G. T., and Hayward, H. R. (1954) *J. Gen. Physiol.* **37**, 601.
Steeman Nielsen, E. (1944) *Dansk. Botan. Ark.* **11**, No. 8, 1.
Steeman Nielsen, E. (1947) *Dansk. Botan. Ark.* **12**, No. 8, 1.
Steeman Nielsen, E. (1952) *Physiol. Plantarum* **5**, 145.
Stiehler, R. D., and Flexner, L. B. (1938) *J. Biol. Chem.* **126**, 603.
Ussing, H. H. (1948) *Cold Spring Harbor Symposia Quant. Biol.* **8**, 193.
Ussing, H. H. (1949a) *Physiol. Revs.* **29**, 127.
Ussing, H. H. (1949b) *Acta Physiol. Scand.* **17**, 1.
Ussing, H. H. (1951) *Z. Elektrochem.* **55**, 470.
Ussing, H. H. (1954) *Symposia Soc. Exptl. Biol.* **8**, in press.
Ussing, H. H., and Zerahn, K. (1951) *Acta Physiol. Scand.* **23**, 110.

Author Index

Numbers in italics indicate pages on which references are listed.

A

Abbati, M. J., 16, *26*
Abelson, P. H., 168, *194*
Ackermann, W. W., 177, *192*
Adams, D. H., 362, *373*
Adrian, E. D., 362, *373*
Ai, T. V., 176, *190*
Ajl, S. J., 168, *196*
Albaum, H. G., 174, 184, *188*
Albuquerque, R. M., *275*, 288, *295*
Aldridge, W. N., 364, *373*
Alexander, J. H., 135, *140*
Alexander, J. T., 15, *26*
Alexander, L. M., 360, 371, *375*
Alexander, W. F., 248, *262*
Alfert, M., 124, *141*, 243, *262*
Algera, L., 207, *217*
Allen, G. A., 366, *373*
Allen, M. B., 174, 178, *189*
Allfrey, V., 177, *188*
Alvarado, S., 215, *217*
Amberger, C., *101*
Anderson, D. G., 147, 152, 164, 167, 177, *188*
Anderson, H. D., 85, *99*
Anderson, N. G., 3, *26*, 277, 293, *295*
Anderson, T. F., 147, *188*
Angevine, D. M., 256, *263*
Antopol, W., 132, *138*
Aquilonius, L., 283, 286, 291, *297*
Ardao, M. I., *278*, *295*
Arisz, W. H., 183, *189*
Arkush, A. S., 247, *267*
Arnon, D. I., 161, 167, 172, 174, 177, 178, *189*
Ashbel, R., 82, *99*
Ashwell, G., 187, *193*
Astbury, W. T., 109, *113*
Atkinson, W. B., 232, *262*
Atwood, L., *138*
Austin, C. R., 275, 276, 290, *295*, *296*
Austin, L., 364, *373*
Avens, K., 394, *395*
Avery, O. T., 221, *262*
Axelrod, B., 156, 157, 161, 162, 174, 175, *189*, *193*

B

Baas-Becking, L. G. M., 214, *217*
Babkin, B. P., 331, *333*
Backler, B. S., 248, *262*
Baddar, F. G., 85, *99*
Baker, J. R., 37, *75*, 82, 85, 91, *99*, 133, *138*
Baker, R. F., 128, *140*, 230, *267*
Balbiani, E. G., 241, *262*
Ball, E. G., 146, 147, 158, 169, 170, *189*, *195*
Bandurski, R. S., 156, 157, 161, *162*, 174, 175, 176, 177, 182, 184, *189*, *193*
Bang, F. B., *272*, *295*
Bangle, R., Jr., 82, *101*
Barber, H. N., 235, 238, *262*
Barer, R., 9, 12, *26*, 34, 36, 37, 38, 39, 42, 43, 44, 69, 73, *75*, 91, *100*, 257, 258, *262*
Barets, A., 353, 354, 357, *373*
Barigozzi, C., 252, *262*
Barnett, R. J., 363, *373*
Barnum, C. P., 158, *192*, 282, 285, *295*
Barron, E. S. G., 164, 172, *189*, *192*, 278, *295*
Bartholomew, J. W., 119, 120, 128, *138*, 252, *266*
Bartley, W., 176, 183, 184, *189*
Bath, J. D., 145, 147, 152, *195*
Bauer, H., 83, *99*, 124, *138*, 236, *262*, 281, *295*
Bayliss, M., 133, *138*
Beauverie, J., 201, *217*
Becker, B., 367, *374*
Beevers, H., 161, 162, 172, 173, 176, 184, *189*, *191*, *192*
Beguin, F., 320, *333*
Behar, A., 99, *102*
Behrens, O. K., 137, *140*
Beijer, J. J., 207, *217*
Beinert, H., 181, *194*
Bell, L. G. E., 86, 97, *99*, 229, *262*
Belling, J., 249, *262*
Belozersky, A. N., 130, *138*
Benedetti-Pichler, A. A., 15, *26*
Benser, P., 47, 51, *75*

Bensley, R. R., 87, 88, *100*, 301, *333*
Benson, A. A., 177, *194*
Bentley, L. E., 168, *189*
Berenbom, M., 99, *100*
Berg, W., 82, 83, 84, *100*
Bergemann, T., *329*, *333*
Berger, R. E., 230, 255, *266*
Bergersen, F. J., 106, 107, *113*
Bergman, B., 215, *218*
Bergner, A. D., *372*, *374*
Berkley, C., *260*, *266*
Bernhard, W., *272*, *295*
Bernheim, F., 366, *373*
Bernheim, M. C. L., 366, *373*
Berrill, N. J., 65, *75*
Berry, W. K., 364, *373*
Bersin, T., 97, *100*
Bethell, F. H., 188, *194*
Beutner, E. H., 132, 133, 137, *140*, *141*
Bevelander, G., 44, 47, 50, 51, 52, *75*
Bhagvat, K., 148, 166, 170, 171, *189*, *191*
Bhargava, K. S., 153, *189*
Biale, J. B., 163, 175, 176, 185, *189*, *193*
Biedermann, W., 46, *75*
Bielig, H. J., 132, *139*
Bird, G. R., 16, *26*
Biss, R., 210, *218*
Bisset, K. A., 105, 107, *113*, 116, 117, 118, 124, 126, 127, 134, *139*
Bizzozero, G., 301, 302, 305, 310, 311, 318, 319, 320, *333*
Bjorksten, I., 96, *100*
Blinks, L. R., 151, *192*
Blout, E. R., 16, *26*, *28*, 257, *262*
Blum, F., 80, *100*
Boddy, R. H. G., *227*, *263*
Bodenstein, D., *277*, *278*, *295*
Boeke, J., 340, 341, *373*
Bogen, H. J., 149, *189*
Bogoroch, R., *260*, *265*
Bohuslav, P., 70, *75*
Boivin, A., 121, 123, 128, 129, *139*, 221, *222*, *232*, *262*
Bolten, E., 168, *194*
Boni, M., 330, *333*
Bonner, J., 144, 156, 157, 162, 163, 164, 174, 175, 176, 179, 182, 184, 185, *189*, *192*, *193*
Bonner, W. D., Jr., 170, *189*
Boricious, J. K., 234, *264*
Borysko, E., *272*, *295*
Bose, S. R., *205*, *217*
Bot, G. M., 200, *213*, *217*
Bourne, G. H., 38, 44, 68, 69, 70, 71, 73, *75*
Boutan, L., 50, *75*
Bowes, J. H., 82, *100*
Bowler, E., 207, 208, 211, *218*
Boyce, R., 60, *76*
Brachet, J., 136, *139*, 176, 177, 181, *189*, 221, 231, 233, 234, 238, 241, 245, *262*, 276, 280, 281, 285, 294, 295, *295*, *296*
Braden, A. W. H., 290, *295*, *296*
Bradfield, J. R. G., 24, *26*, 128, *139*, 254, 255, *262*, 288, *296*
Brady, T. G., 379, 388, *396*
Brakke, M. K., 160, *189*
Brandt, K., 130, *139*
Brante, G., 82, *100*
Brattgard, S. O., 256, 257, *262*
Brenner, S., 133, *139*, 249, *262*
Bressaud, R., 365, *375*
Bretschneider, L. H., 91, *100*, 146, *189*
Bringmann, G., 128, *139*
Britten, R., 168, *194*
Brodie, A. F., 132, 133, 137, *139*, *140*, *141*
Brody, T. M., 184, *189*
Brooks, V. B., 360, *373*
Brown, A. H., 178, *189*
Brown, G. L., 145, 147, 157, 158, 181, *189*, *293*, *296*, 355, *373*
Brown, R., 145, 157, 158, 160, 181, *189*, *194*
Brumberg, E. M., 251, 254, 255, *262*, *265*
Brummond, D. O., 157, 163, 164, 165, 166, 168, 179, *189*
Bryan, J. H. D., *229*, *262*
Bryan, W. H., 184, *189*
Bucher, N. L. R., 3, *29*
Buchholz, J. T., 147, 151, *189*
Buchsbaum, R., 91, 92, *100*
Buck, J. B., 230, *262*
Bull, H. B., 88, *100*
Bullough, W. S., 329, *333*
Bungenborg de Jong, P. J., 279, *296*
Burch, C. R., 254, *262*
Burdon, K. L., 118, *139*

Burian, R., 251, *262*
Burkhardt, B., 109, *113*
Burns, L., 137, *139*, *140*
Burris, R. H., 157, 163, 164, 167, 168, 180, *189*, *192*, *196*
Burström, H., 182, *189*
Bush, V., 12, *26*
Bustraan, M., 210, *220*
Butler, G. W., 171, 173, 184, *189*
Buvat, R., 152, 153, 155, 156, *189*

C

Cailloux, M., 12, *26*
Cain, A. J., 98, *100*
Caldwell, P. C., 129, *139*
Callan, H. G., 238, *262*, *276*, *292*, *293*, *296*
Calvet, F., 285, *297*
Calvin, M., 177, *189*, *194*
Cameron, Gladys, 4, *26*
Campbell, J. J. R., 168, *189*
Campbell, R. M., *222*, *262*
Cantino, E. C., 186, *190*
Canzanelli, A., 254, *262*, *267*
Caprino, G., 330, *333*
Carlson, J. G., 277, 278, *296*
Carpenter, D. C, 81, *100*
Carpenter, F. H., 125, *140*, 233, *266*
Carr, J. G., 238, *239*, *262*
Carriker, M. R., 59, *75*
Carter, C. E., 129, *140*
Carton, E., 379, 384, 387, 388, *396*
Caspari, E., 185, 186, *190*
Caspersson, T., 130, *139*, 221, 222, 223, 227, 228, 229, 237, 239, 252, 253, 254, 255, *262*, *263*, 273, 275, 281, 283, 284, 286, 287, 288, 291, 292, 294, *296*, *297*
Cassel, W. A., 105, *113*, 127, *139*
Castel, P., 248, *268*
Catchpole, H, 254, *261*
Chabaud, A., 123, *139*
Chalkley, H. W., 7, *26*
Chamberlain, C. J., 244, *263*
Chambers, R., 12, 14, 15, 16, *26*, 279, *296*
Chance, B., 170, *190*
Chance, H. L., 109, *113*, 117, *139*
Chantrenne, H., 136, *139*, 181, *189*
Chapman, G. B., 107, 108, 110, *113*
Chargaff, E., 121, 136, *139*, *141*, 233, 237, 239, *263*, *268*, 283, 285, 291, *296*
Chavré, V. J., 392, 393, 394, *396*
Chappell, J. B., 156, 183, *190*
Chayen, J., 146, 147, 158, 159, *190*, 230, 238, 241, 255, 257, *263*, *264*, *272*, *296*
Chen, Y. T., 150, 170, 172, *193*
Chèvremont M., 155, *191*
Chibnall, A. C., 200, *217*
Chlopkow, A., 309, 311, *333*
Choudhuri, H. C., 238, 250, *263*
Churney, L., 277, *296*
Claff, C. L., 17, *26*, *29*
Clapp, S. H., 251, *264*
Clara, M., 301, 302, 308, 309, 310, 311, 320, *333*
Clark, J. B., 109, *113*, 125, *139*
Clarke, H. T., 81, *101*
Claude, A, 86, *100*, *217*
Cleland, K. W., 145, 156, 157, 165, 171, 183, 184, *190*, *195*
Cleland, W. W., 168, *190*
Clemmons, J. J., 256, *263*
Clendenning, K. A., 177, *190*
Cleverdon, R. C., 127, *141*
Clifton, C. E., 125, *139*
Cloetta, M., 311, *333*
Coates, V J., 16, *26*
Coe, W. R., 57, *75*
Coers, C., 341, 350, 367, 369, 371, *373*
Cohan, M. S., 173, *195*
Cohen, B., 14, *26*
Cohen, B. S., 372, *374*
Cohen, M., 207, 208, 211, *218*
Cohn, E. J., 86, *100*
Cole, A. R. H., 16, *26*, 257, *262*
Comar, C. L., 200, *218*
Commoner, B., 228, 230, *263*
Conn, E. E., 147, 151, 152, 164, 167, 177, *188*, *194*, *195*
Conway, E. J., 377, 379, 380, 381, 384, 387, 388, 389, 390, 395, *395*, *396*
Coons, A. H., 90, *100*
Cooper, M., 81, *100*
Cooperstein, S. J., 133, *140*, 146, 157, 159, *192*
Costello, D. P., 5, *26*
Courtice, F. C., 70, *75*
Courtois, J. E., 176, *190*

Couteaux, R., 340, 341, 342, 347, 348, 350, 353, 354, 358, 359, 361, 363, 366, 367, 368, 369, 370, 371, 372, 373, 374
Cowie, D. B., 168, 194
Crafts, A. S., 182, 190
Craig, B. R., 330, 334
Crane, R. K., 161, 190
Crawford, G. N. C., 34, 36, 37, 38, 39, 42, 43, 44, 69, 75, 91, 100
Criegee, R., 84, 100
Crocker, C. G., 109, 113
Crofts, D. R., 50, 75
Currier, H. B., 182, 190
Cuypers, A., 353, 374
Czurda, V., 205, 212, 218

D

Dakin, W. J., 50, 59, 75
Dalcq, A., 276, 296
Dale, H. H., 355, 373, 374
Dalton, A. J., 147, 190, 309, 333
Damodaran, M., 148, 166, 190
Dangeard, P. A., 152, 153, 190, 205, 218
D'Angelo, E. G., 293, 296
Danielli, J. F., 2, 3, 26, 28, 52, 76, 91, 96, 100, 222, 223, 232, 238, 250, 251, 263, 280, 288, 295, 296, 297, 365, 374
Darboux, G., 59, 76
Darby, R. T., 171, 173, 190
Dauvillier, A., 256, 263
Davenport, C., 360, 371, 375
Davenport, H. A., 235, 236, 266
Davenport, H. E., 178, 190
Davenport, H. W., 388, 391, 392, 393, 394, 396
Davidson, J. N., 222, 233, 246, 247, 263, 264, 282, 296
Davies, D. D., 151, 156, 157, 162, 163, 164, 165, 166, 167, 169, 180, 190
Davies, H. G., 9, 26, 222, 223, 227, 232, 254, 255, 258, 263, 268, 272, 296
Davies, R. E., 176, 183, 184, 189, 388, 391, 393, 396
Davis, J. C., 132, 139, 141
Davis, J. R. A., 50, 76
Davison, A. N., 364, 374
Davison, D. C., 169, 190
Dawson, I. M., 135, 139, 140
DeBruyn, P. P. H., 250, 263
de Bruyne, C., 59, 75
De Deken-Grenson, M., 154, 158, 181, 190
Deeley, E. M., 223, 263
de Fonbrune, P., 12, 15, 16, 27
DeLamater, E. D., 105, 107, 113, 125, 126, 128, 132, 133, 139, 140, 141, 236, 239, 250, 263
Delaporte, B., 125, 130, 139
Delauney, A., 221, 262
De Ley, J., 119, 128, 139
Delmotte, N., 128, 139
Dempsey, E. W., 232, 263
Denham, S. W., 98, 100
Denning, N. P., 98, 101
Denz, F. A., 363, 367, 370, 374
de Rezende-Pinto, M. C., 203, 219
Deriaz, R. E., 124, 141
De Robertis, E., 270, 274, 296
Dervichian, D. G., 279, 296
de Sipin, M., 117, 118, 141
DeSmul, A., 228, 237, 240, 263
de Villepoix, M. R., 46, 50, 78
Devreux, S., 241, 263
de Waele, A., 50, 78
Deysson, G., 146, 190
Diacumakos, E., 14, 26
Dianzani, M. U., 119, 139, 158, 190
Dickens, F., 162, 190
Diefenbach, H., 249, 267
Dischendorfer, O., 210, 218
Di Stefano, H. S., 124, 139, 235, 236, 237, 263
Dobson, E. O., 238, 263
Dodson, O. E., 276, 292, 296
Dolschansky, E., 70, 76
Dornesco, G. Th., 308, 333
Dornfeld, E. J., 273, 296
Dotterwech, H., 50, 76
Dounce, A. L., 148, 187, 190, 242, 264 282, 297
Doutreligne, S. J., 198, 212, 218
Dow, W. A., Jr., 163, 184, 190
Downey, M., 379, 396
Doyle, W. L., 230, 263
Drabkin, D. L., 245, 263
Drawert, H., 145, 146, 147, 190
Drew, G. H., 45, 76
Dreyer, G., 119, 139

Dubois, F., 62, *78*
DuBuy, H. G., 148, 156, 161, 171, 185, *190, 191, 196*
Ducet, G., 169, 171, 172, 173, *190, 194*
Düvel, D., 202, *219*
Dufrenoy, J., 119, *140*
Dulbecco, R., 4, *27*
Duncan, R. E., 278, *296*
Duryee, W. R., 12, *26*, 273, 276, 278, 279, *292, 296*
Dustin, P., 251, *263*
Duthie, E. S., 43, 44, 69, 70, *76*, 308, *333*
Duvick, D. N., 160, *190*
Dyar, M. T., 159, 160, *190*

E

Eagles, B. A., 168, *189*
Earle, W. R., 4, 5, *27, 28, 29*
Easson, L. H., 366, *374*
Ebel, J. P., 131, *140*
Ebeling, H. H., 253, *268*
Edsall, J. T., 81, 88, *100*
Edstrom, J. E., 14, 17, *27*
Eggleston, L. V., 168, *192*
Ehrenberg, L., 276, 279, *296*
Ehrhard, H. B., 125, *139*
Ehrhart, O, 83, 84, *101*
Ehrich, W. E, 245, *263, 264*
Eichenberger, M., 154, *190*
Einarson, L., 247, 249, *263*
Eisenberg, P., 116, *140*
Eklöf, H., 301, *333*
Elford, J., 135, *140*
Elliott, W. H., 180, *190*
Elowe, D. G., 169, *193*
Elson, D., 283, 285, 291, *296*
Elssner, E., 50, *76*
Ely, J. O., 236, 237, 254, *263*
Emerson, J. H., 12, *27*
Emmel, V. M., 99, *100*
Engstrom, A., 9, *27*, 223, 227, 252, 256, 257, 258, 260, *262, 263, 264, 265*, 274, 282, 286, 287, *297*
Enns, G. H., 388, *396*
Enriques, P., 57, *76*
Ephrussi, B., 132, *140, 141*, 186, 188, *190*
Epstein, G. W., 104, *113*
Erdman, R., 386, *396*
Erickson, J., 182, *192*
Erickson, R. O., *232, 263*, 281, *296*
Ernster, L., 165, *192*
Errera, M., 241, 242, 254, 255, *262, 263*
Estable, C., *272*, 274, *292, 296*
Evans H. J., 169, *191*
Evans, V. J., 4, *27*

F

Fabricant, C., 125, *140*
Fager, E. W., 177, 178, *191*
Faller, J. L., 85, *101*
Farr, P. S., 250, *263*
Farrant, J. L., 147, 152, 156, 183, *191*
Fauré-Fremiet, E., 42, 71, *76*
Feigelson, M., 150, 153, 156, 175, *191*
Feingold, D., 82, *102*
Feldberg, W., 355, 362, *373, 374*
Felix, M., 3, *27*
Feng, T. P., 357, 359, *374*
Ferry, J. D., 88, *100*
Fessard, A., 353, *374*
Fetzer, S., 82, *100*
Feulgen, R., 97, *100*, 104, *113*, 235, 240, *263*
Ficq, A., 284, *296*
Finean, J. B., 84, *100*, 206, 208, 209, 211, *218*
Fischer, A., 4, *27*
Fischer, M. H., 329, *333*
Fisher, A., 70, *76*
Fisher, H., 112, *113*
Fisher, R. B., 389, *396*
Fitton Jackson, S., 145, 147, 157, 158, 181, *189*
Fitz-James, P. C., 124, 127, *140*
Fitzgerald, P. J., 2, 9, *27*, 252, 256, 260, *264*
Flax, M. H., 249, 250, *264*
Fleure, H. J., 50, *76*
Flexner, L. B., 378, *396*
Flick, E. W., 232, *267*
Florey, H. W., 301, 302, 308, 310, 311, 312, 329, 330, 331, 332, *333, 334*
Flory, L. E., 20, *27, 29*
Flower, M. S., 16, *28*
Folch, J., 87, *100*
Folkes, J. P., 181, *191*
Foraker, A. G., 98, *100*
Fordham, M. G. C., 59, *76*
Forman, C., 245, *263*
Foster, C. L., 232, *264*

Foster, M., 241, 249, *265*
Fowler, C., *7*, *28*
Fox, D. L., *57*, *75*
Fraenkel-Conrat, H., 81, *100*
Franz, F., *232*, *264*
Fraser, R. D. B., *257*, *258*, *264*
Frazer, S. C., *229*, *246*, *264*, *268*
Frederic, J., 155, *191*
French, D., 81, *100*
French, R. C., 184, *191*
Frenkel, A. W., 178, *189*
Fretter, V., 32, 34, 39, 40, 42, *52*, 57, 59, 60, 70, 71, *76*
Frey-Wyssling, A., 87, *100*, 198, 201, 203, 207, 208, 209, 211, 212, 213, *218*, *293*, *296*
Friedenwald, J. S., 99, *101*, *362*, 367, 368, *374*
Friedman, H. P., Jr., *259*, *264*
Friedman, N. B., 330, *333*, *334*
Frisch, A. W., 4, *27*
Fritz, G. J., 173, *191*
Fukuda, M., *236*, 237, *267*
Fuld, M., 181, *194*
Fuller, R. C., 177, *194*

G

Gaffron, 134, *141*
Gale, E. F., 181, *191*
Galeotti, G., 308, *333*
Gall, J. G, *292*, *293*, *296*
Galstoff, P. S., 41, *76*
Galston, A. W., 200, *218*
Gatenby, J. B., 38, 39, 43, 44, 69, 70, *76*
Gates, R. R., *273*, *276*, 288, *296*
Gay, H., 231, 233, 234, *264*
Geitler, L., 198, 201, 205, *218*
Geneves, L., 152, 153, *191*
George, W. C., 33, 53, 54, 55, 57, 64, 65, 71, 73, *76*
Georgi, C. E., 137, *140*
Gerard, R. W., 353, 373, *374*
Gerber, I., 251, 259, *267*
Gerola, F. M., 244, 250, *264*
Gersch, M., *276*, 289, *296*
Gersh, I., 254, *264*, 301, *333*
Giacomini, E., *352*, *374*
Giardina, G., 181, *191*
Gibbs, M., 161, 162, 177, *189*, *191*
Gilbert, P. T., Jr., 15, *27*
Giroud, A., 210, *218*
Glassman, A., 259, *266*
Glaubach, S., 132, *138*
Glick, D., 2, 16, 24, *27*, 133, *138*, 147, 148, *191*, 223, 227, *264*, 280, *296*, 304, *333*, 366, *374*
Glimstedt, G., 228, 257, *264*
Goddard, D. R., 144, 145, 149, 156, 170, 171, 172, 173, *190*, *191*
Godnev, T. N., 215, *218*
Goldie, H., 3, *27*
Gollub, M. C., 177, *191*
Golman, L., 132, *138*
Gomirato, G., 257, *264*
Gomori, G., 2, *27*, 98, *100*, 222, 250, *264*, 280, 288, *296*, 304, *333*, 361, 362, 363, 364, 365, 370, *374*
Goodrich, E. S., 39, 71, *76*
Goodspeed, T. H., *252*, *268*
Gorham, P. R, 177, *190*
Gossner, W., 247, *264*
Gots, J. S., 132, *139*
Graham, A., 55, 56, 57, 59, *76*
Granick, S., 198, 200, 203, 207, *218*
Gray, J., *273*, *297*
Gray, J. E., 46, *76*
Gray, J. S., 389, *396*
Greco, J., 82, *102*
Green, D. E., 148, 170, 179, 180, 181, *191*, *194*
Green, R. W., 82, 83, *100*
Greenspan, E., 251, 259, *267*
Greiner, C. M., 161, 177, *189*
Gresson, R. A. R., 39, 41, 69, 70, 71, *76*
Grey, D. S., 16, *28*, 254, *264*
Grimson, H., 250, *266*
Gros, C., 187, *191*
Grossman, M. I., 99, *102*, 331, *333*
Gueft, B., 244, 246, 247, 249, 250, *265*
Günther, P., 353, *374*
Guernsey, F. S., 149, 150, 184, *192*
Guex-Holtzer, S., 108, *113*
Guild, R., 254, *262*
Guilliermond, A., 144, 146, 152, 154, 156, *191*, 197, 215, 216, *218*
Gunter, J. M., 364, *374*
Gurin, S., 168, *192*
Gustavson, K. H., 81, 82, 83, *100*
Gutheil, F., 57, 69, *76*

Guthrie, M. J., 289, *297*
Gutmann, E., 347, *374*
Gutstein, M., 120, *140*
György, P., 117, 118, *141*

H

Haardick, H., 132, *139*
Haberlandt, G., 199, *218*
Hack, M. H., 82, *100*
Hackett, D. P., 163, 165, 169, 170, 173, 175, 182, 184, *191*, *196*
Hadorn, H., 81, *101*
Haege, 388, *396*
Hammerling, J., 284, 295, *297*, *298*
Haenel, U., 250, *264*
Hagenau, F., *272*, *295*
Hakansson, R., 228, 257, *264*
Hale, C. M. F., 117, 124, *139*, *140*
Hallen, O., 257, *262*, *264*
Halliday, N, 82, *100*
Hammarsten, E., 234, *264*
Hammarsten, G., 234, *264*
Hammick, D. L., 85, *99*
Hammond, M. R., 155, 159, *195*
Hamperl, H., 321, *333*
Hanson, E. A., 200, 214, *217*, *218*
Harding, C., *273*, *297*
Harding, J., 70, *75*
Harkins, D. M, 229, *266*
Harman, J. W., 150, 153, 156, 175, *191*
Harris, C., 372, *374*
Harris, J., 10, 13, *28*
Harris, S., 247, *264*
Harris, T. N., 247, *264*
Harrington, N. J., 244, *264*
Harrison, M. F., 3, *27*
Hartman, P. E, 132, 133, 137, *139*, *140*, *141*
Hartree, E. F., 171, 173, 178, *191*
Harvey, E. B., 5, *27*
Harvey, T. S., 117, 118, *141*
Hashim, S. A., 237, *264*
Hastings, J. A., 12, *26*
Haughton, I., 34, 44, 55, 61, 69, *76*
Hauschka, T. S., 3, *27*
Haurowitz, F., 86, 89, *100*
Hawes, R. C., 366, *373*
Hawkins, R. D., 362, 364, *374*
Hayward, H. R., 395, *396*
Hazan, S. J., 146, *191*
Hazeltine, P., 152, 153, 155, *194*
Hayasi, K., 46, 48, 49, 51, 52, *76*
Heather, A. H., 250, *264*
Heaysman, J. E. M., 52, *76*
Heden, C. G., 121, 135, *140*
Heidelberger, C., 282, *298*
Heidenhain, M., *322*, *333*
Heisenberg, W., 229, *264*
Heitz, E., 198, 199, 200, 202, 211, 215, 216, *218*, 270, *275*, *297*
Held H., 341, *374*
Hellström, H., 215, *218*
Henderson, H. J., 105, 107, *113*, 132, 133, *140*
Henry, H., 119, *140*, 232, 251, *252*, *264*
Heotis, J. P., 137, *140*
Herdman, W. A., 60, *76*
Herrick, F. H, *273*, *297*
Herrmann, H., 234, *264*
Herskowitz, I. H., 223, 226, 227, 230, *265*
Hesselbach, M. L., 161, 172, *191*
Higinbotham, N, 187, *191*
Hill, J. C., 38, 39, 44, 69, 70, *76*
Hill, R., 148, 166, 170, 171, 173, 178, *189*, *190*, *191*
Hillary, B. B., 235, 236, *264*
Hillier, J., 107, 108, 110, *113*, 125, 137, *140*
Hilson, G. R. F., 15, *27*
Himes, M. H., 250, *264*
Hinsey, J. C., 350, *374*
Hinshelwood, Sir Cyril, 129, *139*
Hintner, H., 357, *375*
Hirata, A. A., 44, 51, *76*
Hirshfeld, A., 174, *188*
Hisako, O., *277*, *297*
Hitchcock, C. H., 245, *264*
Hodes, R., 8, *28*, 227, *266*
Hodges, M. E., 237, *268*
Höber, R., 88, *101*
Hoff, E. C., 372, *375*
Hoffman, H., 125, *140*
Hofmann, K. A., 83, 84, *101*
Hogben, C. A. M., 390, *396*
Hogeboom, G. H., 24, *29*, 145, 146, 155, 158, 164, *191*, *222*, *264*
Holden, C., 170, 171, 172, *191*
Holleman, J. W., 181, *191*

Hollender, A., 9, *28*, 258, 259, *266*
Hollinshead, W. H., *372*, *375*
Holt, S. J., 341, 364, 367, 368, 371, *374*
Holter, H., 8, 17, 24, *27*, 171, *191*
Holton, F. A., 175, *195*
Horne, R. W., 135, 136, *141*
Horning, E. S., 69, *76*
Horstadius, S., 3, *28*
Hotchin, J. E., 135, *140*
Hotchkiss, R. D., 117, *140*, 304, *333*
Howard, A., 147, 182, *191*, 260, *264*
Huber, P., 308, 311, 312, 331, 332, *333*
Hubert, B., 198, 212, 213, *218*
Hughes, A., 273, *277*, *297*
Hughes, W. L., Jr., 85, 86, *100*, *101*
Hunt, J. N., 389, *396*
Hunter, E. F., Jr., 174, 175, 176, *192*
Hunter, M. E., 126, *140*
Hurst, H., 119, *140*
Huseby, R. A., 282, 285, *295*
Husek, H., 16, *28*
Huxley, A. H., 274, 286, 287, *298*
Hyatt, M. T., 186, *190*
Hyden, H., 256, 257, *262*, 279, *297*
Hygen, G., 201, *218*

I

Ihre, J. E., 388, *396*
Immers, J., 288, 289, *297*
Irvine, W., 244, *265*
Irving, A. A., 203, *219*
Isherwood, F. A., 150, 170, 172, *193*
Itikawa, O., 240, *264*

J

Jacob, M., 222, *266*
Jacobson, W., 283, *297*
Jacobsson, F., 223, 254, *263*
Jacoby, F., 365, *374*
Jagendorf, A. T., 157, 172, 182, *192*
Jakob, E., 207, *219*
Jakovlev, V. A., 369, *375*
James, W. O., 172, 173, *192*
Jang, R., 161, *189*
Janssens, F., 59, *76*
Jarvi, O., 118, *140*
Jeener, R., 136, *139*, 285, *297*
Jennings, M. A., 331, *334*
Jentoft, V., 4, *27*
Jodrey, L. H., 51, *76*
Jones, A. S., 120, *140*, 252, *264*
Johannson, M., 241, *263*
Johnson, B. K., 254, *264*
Johnson, F. P., 321, *333*
Johnson, M. J., 168, *190*
Johnson, R. B., 177, *192*
Johnson, T. B., 251, *264*
Johnston, M. H., 98, *100*
Jones, R. C., 16, *28*
Jones, R. N., 16, *26*
Jordan, H. E., 62, 66, 71, *76*, *77*
Jordan, J. H., 250, *264*
Jorgenson, M., 272, 281, *297*
Just, E. E., 3, *27*

K

Kahler, H., 309, *333*
Kaja, H., 202, 215, 216, *218*
Kalishevich, S. V., 215, *218*
Kallman, B. J., 252, *266*
Kallman, F., 84, *101*
Kammeraad, A., 330, *333*
Kanajew, J., 61, *77*
Kaplan, E. H., 161, *192*
Kaplan, M. H., 90, *100*
Kaplan, N. O., 169, *195*
Karasek, H., 311, *333*
Karstens, W. K., 207, *217*
Kassel, R., 2, 25, *27*
Katz, B., 353, *374*
Kauffmann, C., 83, *101*
Kaufmann, B. P., 231, 233, 234, *264*
Kauko, I., 320, *333*
Kausche, G. A., 132, *139*, 207, *218*
Kay, E. R. M., 242, *264*
Kaye, M. A. G., 81, *102*
Keilin, D., 184, *192*
Keiser-Nielsen, H., 299, *333*
Kellenberger, E., 106, *113*
Kelly, E. G., 249, *264*
Kendall, F. E., 86, *101*
Kenten, R. H., 82, *100*
Kern, M., 172, *192*
Kern, R. A., 137, *140*
Kern, S. F., 137, *140*
Kernan, R., 379, 380, 381, *396*
Kessel, E., 50, *77*
Kielley, R. K., 176, *192*

Kielley, W. W., 176, *192*
Kilby, B. A., 362, *373*
Kimmelstiel, P., 82, *101*
Kindred, J. E., 62, 63, 64, *77*
King, E. D., 187, *192*
King, H. K., 135, *140*
King, K. W., 180, *192*, *196*
Kingkade, M. J., 137, *140*
Kingsbury, B. F., 159, *192*
Kirchner, H. A., 62, *77*
Kirk, P. L., 2, 5, *27*, *29*
Kirkpatrick, P., 256, *265*
Klebs, G., 212, *218*
Klein, E., 251, 254, 259, 260, *265*
Klein, G., 3, *28*, 251, 254, 259, 260, *265*
Klein, R. M., 164, 172, *189*, *192*
Kleinzeller, A., 388, *396*
Klemperer, P., 244, 246, 247, 249, 250, *265*
Klotz, I. M., 85, *101*
Knaysi, G., 117, 118, 125, 128, 133, *140*, 236, *265*
Koefoed-Johnsen, V., 385, *396*
Koelle, G. B., 362, 363, 364, 365, 366, 368, 369, *374*
Koenig, H., 232, *265*
Kohler, A., 253, *265*
Kohout, P. M., 170, *191*
Kollmann, M., 41, 69, 71, *77*
Koníček, H., 44, *77*
Kopac, M. J., 2, 3, 6, 10, 12, 13, 14, 15, 16, 18, 19, 20, *22*, *23*, *24*, *25*, *26*, *27*, *28*, *29*
Kopech, G., 4, *28*
Kopsch, F., 301, *333*
Kornberg, A., 164, *192*
Korson, R., 224, 228, 245, *265*
Kosterlitz, H. W., 222, *262*
Koydl, F., 201, *219*
Koza, R. W., 244, *264*
Krantz, F., 249, *267*
Krause, W., 337, *374*
Krebs, H. A., 168, *192*
Krech, E., 160, *193*
Kretchmer, N., 158, *192*
Krieg, A., 131, *140*
Krimsky, I., 52, *75*
Krossing, G., 200, *218*
Kuhne, W., 337, 348, 349, *375*
Kuffler, S. W., 353, 354, 373, *374*, *375*
Kunitz, M., 233, 247, 255, *265*
Kuntz, A., 232, *268*
Kupfer, A., 9, *28*, 258, 259, *266*
Kurnick, N. B., 8, *28*, 221, 222, 223, 226, 230, 231, 233, 236, 241, 242, 243, 244, 245, 246, 247, 249, 251, 253, 254, 255, 259, 260, 261, *265*, *266*, *267*
Kuster, E., 197, 199, 201, 205, *218*
Kutz, R. L., 88, *100*

L

Lack, C. H., 109, *113*
Lackey, M. D., 148, 156, 171, 185, *190*
La Cour, L., 249, *265*
Larz, H., 211, *218*
Lagerstedt, S., 249, *265*, 273, 275, 283, 284, *297*
Lajtha, L. G., 260, *265*
Lalich, J. J., 256, *263*
Lamargene, P., 256, *265*
Land, E. H., 16, *28*
Lange, P. W., 227, *265*
Lanz, H., Jr., 17, *28*
LaPorte, Y., 353, 373, *374*
Larionov, L. T., 251, 254, *262*, *265*
Lassek, A. M., 85, *101*
Laties, G. G., 150, 151, 163, 164, 165, 166, 167, 175, 176, *192*
Laves, W., 234, 244, *265*
Lavin, G. I., 231, *266*
Lazarow, A., 133, *140*, 146, 157, 159, *192*
Laznitski, I., 277, *297*
Leaf, G., 293, *296*
Leblond, C. P., 210, *218*, 260, *265*, 305, 320, *334*
Lecomte, C., 228, 237, 240, *263*
Leduc, E. H., 90, *100*
Lee, S. L., 244, 246, 247, 249, 250, *265*
Lees, M., 87, *100*
Lehmann, F. E., 83, *101*, 210, *218*
Lehoult, Y., 221, *262*
Leibson, R., 359, *375*
Lemberg, R., 65, *77*
Lembke, A., 128, *140*
Lenhoff, H. M., 169, *195*
Lenormant, H., 16, *26*
Leonardi, G., 121, *140*
Leopold, A. C., 149, 150, 184, *192*

LePage, G. A., 161, *192*, 282, *298*
Lepardy, J., 281, *298*
Leslie, J., 233, *263*
Lessler, M. A., 235, *265*
Leuchtenberger, C., 224, 225, 241, 244, *246*, 247, 249, 250, 253, 261, *265*, *267*, 284, 286, *297*
Leuchtenberger, R., 253, 261, *265*
Levan, A., 3, *27*
Levanto, A., 118, *140*
Levene, D., 231, *266*
Levinthal, C., 112, *113*
Levitt, J., 149, 158, 159, 183, 185, *192*
Lewis, W. H., 67, 70, *77*, 230, *266*, *272*, *297*
Lewitzky, G., 215, *219*
Leydig, F., 46, *77*
Leyon, H., 154, *192*, 205, 207, 208, 211, 216, *219*
Lhotka, J. F., 235, 236, *266*
Li, C. F., 235, *266*
Lieb, H., 82, *101*
Liebaldt, E., 197, *219*
Lieben, F., 251, 259, *267*
Liebman, E., 32, 33, 61, 62, 64, 65, 67, 68, 70, 71, *77*
Likely G. D., 5, *28*, *29*
Lillie, R. D., 82, *101*, 236, 250, *266*, *267*, 330, *334*
Lindberg, O., 165, *192*
Linderholm, H., 385, 386, *396*
Linderstrøm-Lang, K., 17, 18, 24, *27*, *28*, 230, *266*
Lindström, B., 9, *27*, 256, 257, 258, *262*, *263*, 274, 282, 286, 287, *297*
Link, G. K. K., 164, 172, *189*, *192*
Lipardy, J., 122, 125, *142*
Lipman, F., 383, *396*
Lison, L., 82, 83, 90, 91, *101*, 365, *375*
List, T., 57, *77*
Litt, M., 282, *297*
Littlejohn, J. M., 146, *191*
Lloyd, B. J., 309, *333*
Lockhart, E. E., 169, *192*
Loeser, C. N., 260, *266*
Loewi, O., 355, *375*
Lomakka, G., 223, 254, *263*
Long, W. G., 183, *192*
Longe, R., 46, *77*
Longley, J., 236, *266*
Lorch, I. J., 2, 3, *28*, 295, *297*
Loring, H. S., 125, *140*, 233, *260*
Lorrain-Smith, J., 82, 83, *101*
Lovelace, F. E., 81, *100*
Low, F. N., 84, *101*
Lowe, C. U., 229, *266*
Lowe, J. S., 160, *195*
Lowenhaupt, B., 394, *396*
Lowry, O. S., 8, *28*
Luck, J. M., 67, *77*, 85, *102*
Ludford, R. J., 92, *101*, 254, *266*
Lumb, E. S., 222, *266*
Lumsden, C. E., 8, *28*
Lund, E. J., 378, *396*
Lundegardh, H., 170, 171, 173, 178, 184, *192*, 378, *396*

M

McAllister, F., 212, *219*
Maccari, A., 119, *139*
Macary, J, 131, *140*
McCarty, M., 221, *262*
McClendon, J. H., 147, 149, 150, 151, 152, 155, 156, 157, 158, 159, 171, 172, 185, *192*
McClintock, B., 270, 272, 275, 294, *297*
McDonald, M. R., 231, 233, 234, *264*, *266*
MacDougald, T. J., 38, 39, 44, 69, 70, *76*
Macfarlane, M. G., 183, *193*
MacGee-Russell, G., 34, *77*
McIndoe, W. M., 222, *263*
McKinney, R. E., 118, *140*
Macklin, C. C., 310, 318, 320, *334*
Macklin, M. T., 310, 318, 320, *334*
McLean, R. A., 132, 133, 137, *141*
MacLeod, C. M., 221, *262*
McManus, J. F. A., 117, *140*, 304, *334*
McMaster, R. D., 277, 278, *296*
MacMunn, C. A., 41, *77*
Maengwyn-Davies, G. D., 99, *101*
Mahler, H. R., 161, 169, *192*, *193*
Mair, W., 83, *101*
Maizels, M., 387, *397*
Makower, R. V., 151, *193*
Malmgren, B., 121, *140*
Malmstrom, B. G., 223, 227, *264*
Maly, R., 215, 216, *218*

Mamelock, H. L., 82, *101*
Mancini, R. E., 90, *101*
Mandel, L., 222, *266*
Mandel, P., 187, *191* 222, *266*
Mandels, G. R., 172, *193*
Mangenot, G., 197, *218*
Manigault, P., 46, 48, 49, 50, *77*
Mann, G., 91, *101*
Mansour-Bek, J. J., 53, 55, 57, 72, 73, *77*
Mansour, K., 53, 55, 56, 57, 72, 73, *77*
Mapson, L. W., 150, 170, 172, *193*
Marinone, G., 222, *266*
Markert, C. L., 171, 172, 173, *195*
Markley, K. SE., 83, *101*
Marnay, A., 357, 358, *375*
Marshak, A., 104, 109, 111, *113*, 136, *140*, 285, *297*
Martin, B. F., 365, *374*
Martin, C., *2*, *27*
Martin, F. P., 301, 302, 320, 329, *334*
Martin, J., 44, *75*
Martini, F., 358, 359, *375*
Marquardt, H., 186, *193*, 217, *219*
Marza, E. V., 289, *297*
Marza, V. D., 289, *297*
Masacré, M., 162, *193*
Massini, P., 177, *189*
Mast, S. O., 7, *28*
Mateyko, G. M., 3, 5, 6, *28*
Mathews, L. H., 67, *77*
Mathews, M. B., 172, *193*
Matsuda, H., 247, *267*
Matz, C. H., 16, *28*
Maxwell, E., 187, *193*
May, K. R., 12, *28*
Mazia, D., 294, 295, *297*
Mecham, D. K., 81, *100*
Meckel, H., 46, *77*
Medawar, P. B., 81, 91, *101*
Melander, Y., 250, *266*
Mellanby, K., 64, *77*
Mellors, R. C., 9, 16, *28*, 230, 255, 257, 258, 259, *262*, *266*
Melnitzky, L., 2, *27*
Mendel, B., 362, 364, *374*
Meng, C. W., 360, *375*
Menke, W., 198, 200, 201, 207, 208, 213, *219*
Mer, E., 46, *77*
Merrill, D. P., 16, *28*
Metz, C. W., 230, *263*
Metzner, H., 200, 210, *219*
Metzner, P., 198, *219*
Meves, F., 215, *219*
Mevius, W., 202, *219*
Meyer, A., 197, 205, 209, 211, 215, *219*
Meyer-Arendt, E., 227, *266*
Meyer-Arendt, J., 227, 232, *264*, *266*
Meyer, K., 299, *334*
Meyer, K. H., 81, *101*
Michaelis, L., 83, 84, *101*, 231, *266*
Michel, B. E., 164, *189*
Mickelwright, H. L., 8, *28*, 227, *266*
Middlebrook, W. R., 81, *101*
Mii, S., 170, *191*
Mika, E. S., 187, *191*
Mikhail, H., 85, *99*
Miles, U. J., 146, *190*
Militzer, W., 137, *139*, *140*
Miller, W. A., 87, *101*
Millerd, A., 144, 150, 156, 157, 162, 163, 164, 165, 166, 167, 174, 175, 176, 179, 182, 185, *189*, *192*, *193*
Millott, N., 34, 54, 55, 56, 57, 58, 60, 69, 72, *77*
Minck, A., 131, *140*
Minck, R., 119, 123, 131, *139*, *140*, *142*, 232, *262*
Mirsky, A. E., 87, 88, 92, *101*, 161, 177, 186, *188*, *195*, 222, 223, 226, 230, 231, 239, 242, 244, 246, 253, 254, *265*, *266*, *267*, 282, 293, *297*
Mitchell, H. K., 186, *193*
Mitchell, J. S., 250, *266*
Mitchell, M. B., 186, *193*
Mitchell, P., 120, *140*, 252, *266*
Mitchison, J. M., 3, *28*
Mittler, T., 43, *78*
Mittwer, T., 119, 120, 128, *138*, 252, *266*
Mladenovic, M., 82, *101*
Moe, H., 311, 313, 314, 321, 325, *334*
Möbius, M., 199, *219*
Mogensen, K. R., 230, *266*
Molisch, H., 209, *219*
Mommaerts, W. F. H., 200, *219*

Monné, L., 210, *219*
Montgomery, T. H., *270*, *272*, *273*, 280, *293*, *297*
Monty, K., 282, *297*
Mookerjee, S., 4, *28*
Moore, A. R., 87, *101*
Moore, J., 61, *77*
Moore, P. T., 379, *396*
Morgan, J. F., 4, *28*
Morpeth, E., 360, *375*
Morrione, T. G., 82, *101*
Morrison, P. R., 88, 97, *100*, *102*
Morse, M. L., 129, *140*
Morton, H. E., 56, 57, 58, *77*, 138, *141*
Moscona, A., 4, *28*
Moses, M. J., *226*, *236*, *266*
Mowry, R. W., 82, *101*
Moyer, L. S., 88, *101*
Moyle, J., 120, *140*, *252*, *266*
Mudd, S., 105, 107, 110, *113*, *114*, 126, 128, 132, 133, 134, 137, *139*, *140*, *141*, *142*
Mudge, G. H., 183, *195*
Muhlethaler, K., 207, 208, 211, 212, *218*, *219*
Muggleton, P. M., 120, *140*, *252*, *264*
Mullins, L. T., 183, *193*
Mulnard, J., *273*, *297*
Munro-Fox, H., 67, *77*
Muntwyler, E., *229*, *266*
Murray, M. R., 4, *28*
Murray, P. D. F., 354, *375*
Murray, R. G. E., 108, *113*, 116, 117, 118, *141*
Myers, D. K., 360, *373*

N

Nachlas, M. M., 363, 370, *375*
Nachmansohn, D., 357, 358, 359, 361, 365, 371, *372*, *374*, *375*
Nadson, G. A., 187, *193*
Nageotte, J., 367, *375*
Naora, H., 226, 228, 229, 237, *266*, *267*
Nason, A., 161, 169, 172, 173, *193*, *196*
Nasset, E., 331, *334*
Nassonov, D. N., 301, 308, 310, *334*
Nastuk, W. L., 15, *26*
Neish, A. C., 200, *219*
Nelson, L., 278, *295*
Nelson, T. C., 57, *77*
Nermut, M., 127, *141*
Neumann, G., 46, *77*
Newcomb, E. H., 146, 157, 162, 163, 172, 173, 176, 179, 184, *189*, *193*
Newcomer, E. H., 127, *141*, 144, 146, 149, 154, 159, 183, 185, *193*
Nicholas, J. S., 234, *264*
Nickell, L. G., 160, *189*
Nicloux, M., 67, *77*
Nihoul, E., 127, *142*
Nissl, F., 96, *101*
Nitschmann, H., 81, *101*
Noack, K., 212, *219*
Noel, R., 341, 365, *373*, *375*
Norberg, B., 252, 260, *266*
Norris, K. P., 230, 238, 241, 255, *263*
Norris, R. F., 117, 118, *141*
Northcroft, R. D., 4, *28*
Northrop, J. H., 4, *28*
Nossal, P. M., 168, *193*
Novelli, A., 248, *266*
Novelli, G. D., 164, *193*
Nowinski, W. W., 270, 274, *296*
Nurnberger, J., 274, 282, 286, 287, *297*
Nyman, M. A., 136, *141*

O

Oberdorfer, W., 234, 244, *265*
Oberling, C., *272*, *295*
O'Brien, J. A., Jr., 154, 160, *193*
Ochoa, S., 164, 173, 178, *193*, *195*, *196*
Oeding, P., 130, *141*
Österlind, S., 394, *396*
Offner, A., 16, *26*
Ogston, A. G., 391, 393, *396*
Ogur, M., 127, *141*, 174, *188*, 231, 232, *263*, *266*, 281, *296*
Ogura, Y., 240, *264*
Okunuki, K., 148, 170, 173, *193*
Olcott, H. S., 81, *100*
Oldewurtel, H. A., 161, 169, 172, 173, *193*
O'Malley, E., 379, 387, 388, *396*
Oomen, H. A. P. C., 64, *76*
Oparin, A. I., 185, 186, *193*
Opie, E. L., 231, *266*
Orinstein, L., *138*
Ornstein, L., *226*, 258, *266*

Orton, J. H., 60, *77*
Oster, G., 250, *266*
Ottoson, D., 385, *396*
Overend, W. G., 235, *266*

P

Paal, C., *101*
Packer, D. M., 2, *29*
Paech, K., 160, *193*
Paigen, K., 145, *193*
Painter, T. S., 286, *297*
Palade, G. E., 107, *113*, 147, 151, 152, 154, 155, 156, 178, *191*, *193*, *196*. 199, 203, 205, *219*, *220*
Paneth, J., 301, 302, 307, 311, 320, *334*
Panijel, J., 231, *266*, 273, 285, 286, 287, 288, *297*
Papanicolaou, G. M., 259, *266*
Pappenheim, A., 241, *266*
Pappenheimer, A. M., Jr., 170, *193*
Paris, C. H., 210, *220*
Paris, R., 162, *193*
Pariser, S., 244, *265*
Parker, R. C., 4, *28*
Parriaud, H., 152, 153, *190*
Parrish, M., Jr., 16, *26*
Patau, K., 226, *266*
Patterson, W. B., 88, *101*
Patzelt, V., 304, 311, 320, *334*
Pearse, A. G. E., 2, *29*, 222, 232, 235, 236, 238, 239, 247, 248, 251, 252, *267*, 280, *297*, 304, *334*
Pearson, J. A., 150, 164, 185, *193*
Pease, D. C., 230, *267*
Peczennik, O., 57, *77*
Pelc, S. R., 147, 182, *191*, 260, *264*
Pelseneer, P., 52, *77*
Pennington, D., 117, *141*
Perner, E. S., 145, 146, 147, 149, 151, 155, 156, 157, 159, *193*, *194*
Perry S. V., 156, 183, *190*
Peshkov, M. A., 128, *141*
Peterfi, T., 12, *29*
Peters, D., 123, *141*
Petry, G., 96, *101*
Phillips, H., 81, *101*
Piekarski, G., 104, *113*, 122, 130, *141*
Piper, A., 109, *113*
Pisano, M., 131, *142*
Pischinger, A., 92, *101*
Plantefol, L., 197, *218*
Plattner, F., 357, *375*
Plaut, W. S., 260, *267*
Pollister, A. W., 124, *141*, 224, 225, 228, 231, 234, 241, 242, 244, 246, 247, 249, 250, 253, 254, *265*, *266*, *267*, 274, 282, 284, 286, 287, *297*
Pollock, B. M., 171, *191*
Popoff, N. W., 311, *334*
Porsche, J. D., 88, *100*
Porter, K. R., 84, *101*, 207, *218*, 272, *297*
Porter, V. S., 98, *101*
Portugalov, V. V., 369, *375*
Post, L. C., 207, 209, 210, *220*
Potter, R. L., 188, *194*
Potter, V. R., 3, *29*, 169, *194*
Potts, F. A., 57, 69, 72, *78*
Powell, E. O., 15, *29*
Pratt, R., 119, *140*
Prenant, M., 48, 49, 50, 52, 62, *78*
Preuner, R., 134, *141*
Price, C. A., 149, 150, 151, 155, 156, 157, 158, 163, 165, 166, 167, 183, 184, *194*
Price, J. P., 235, *262*
Pricer, W. E., Jr., 164, *192*
Priestley, J. H., 203, *219*
Proescher, F., 247, *267*
Propst, L. M., 161, 169, 172, 173, *193*
Puckett, W. O., 62, *78*
Puytorac, P., 153, *194*

Q

Quastler, H., 187
Quayle, J. R., 177, *194*
Queiroz Lopes, A., 252, *267*, 287, *298*

R

Rabinowitch, E. I., 200, *219*
Raaflaub, J., 183, *194*
Rachele, J. R., 15, *26*
Racker, E., 161, 172, *192*, *194*
Rafalko, L. S., 236, 240, *267*
Ramon y Cajal, S., 301, *333*
Randall, J. T., 252, *267*
Ransmeier, R. E., 353, 373, *374*
Ranvier, L., 337, *375*
Ranzoli, F., 294, *297*
Rapport, D., 254, *262*, *267*

Rather, L. J., 277, *297*
Ratner, S., 81, *101*
Ratsimamanga, A. R., 210, *218*
Rattenbury, J. A., 249, *267*, 274, 275, 286, 288, 293, *297*
Ravich-Birger, E. D., 104, *113*
Ravin, H. A., 363, 368, *375*
Rawitz, B., 50, *78*
Rayleigh Lord, 228, 253, *267*
Reaumur, R., 50, *78*
Recknagel, R. O., 155, *194*
Regan, C. T., 67, *78*
Reichert, W., 46, *78*
Reif, A. E., 169, *194*
Reinert, G., 12, *29*
Rettie, T., 82, *101*
Reznikoff, P., 14, *26*
Rhodin, J., 152, 155, *195*
Richards, B. M., *223*, *263*
Richardson, K. C., 69, *76*
Ris, H., 92, *101*, *222*, *223*, 224, 226, 230, 231, 234, 239, 241, 249, 254, *265*, *266*, *267*, 274, 282, 286, 287, 293, *297*
Ritchie, D., 147, 152, 153, 155, 187, *194*
Ritter, U., *329*, *333*
Roberts, R. H., 168, *194*
Robertson, J. D., 336, *375*
Robertson, R. C., 250, *263*
Robertson, R. N., 147, 150, 152, 156, 164, 183, 185, *191*, *193*, *194*
Robinow, C. F., 106, 108, 109, *113*, 116, 117, 118, 122, 124, 127, *141*
Robinson, C. V., 8, *28*
Robinson, E., 145, 157, 158, 181, *194*
Robinson, J. R., 182, *194*
Roche, J., 147, 183, *194*
Rochlin, E. J., 187, *193*
Rodesch, J., 187, *191*
Roe, M. A., 250, *267*
Roll, P. M., 125, *140*, 233, *266*
Romeis, B., 304, *334*
Rosen, G., 127, *141*, 231, *266*
Rosenberg, A. J., 169, 171, 172, 173, *190*, *194*
Rosenberg, L. L., 178, *189*
Rosenfeld, 132, *141*
Ross, H. E., 159, *194*
Ross, M. H., 236, 237, 254, *263*
Rossenbeck, H., 104, *113*, 235, 240, *263*
Rothenberg, M. A., 365, *375*
Rothstein, A., 387, 388, *396*
Rouget, C. H., 337, 341, *375*
Roukhelman, M., 130, *139*
Rozsa, G., 147, 151, *194*
Ruch, F., 228, *268*
Rudd, J. T., 67, *78*
Ruska, H., 207, *218*
Rutenburg, A. M., 98, *102*
Ryan, H., 379, 384, 387, *396*
Ryland, A. G., 153, *194*

S

Sacerdotti, C., 319, *334*
Saez, F., 270, 274, *296*
St. Amand, G. S., 4, *29*
St. Aubin, P. M. G., 3, *29*
Saltman, P., 149, 160, 174, *194*
Salton, M. R. J., 119, 135, 136, *141*
Sanders, F. K., 247, *267*
Sandritter, W., 249, *267*
Sanford, K. K., 5, *28*, *29*
Santesson, L., 275, 283, *296*
Sarachek, A., 187, *194*
Sarciron, R., 131, *142*
Sarkar, N. K., 181, *194*
Saunders-Singer, A. E., 12, *26*
Savage, N., 109, *113*
Sawano, E., 53, *78*
Sawyer, C. H., 360, 371, *375*
Sax, K., 187, *192*
Sax, K. B., 232, *263*, 281, *296*
Scaletti, J. V., 127, *141*
Scarisbrick, R., 170, *191*
Scarth, G. W., 201, *219*
Schaechter, M., 133, *141*
Schaefer, W. B., 109, *113*
Schaffer, J., 299, 300, *334*
Scherrer, A., 215, *219*
Schertiger, A. M., 187, *194*
Scheuing, G., 235, *268*
Schiller, W., 91, *102*
Schimper, A., 197, 205, 215, *219*
Schmerl, E., 386, *396*
Schmidt, H. D., 12, *29*
Schmidt, H. H., 211, *219*
Schmitz, F., 215, *219*
Schneid, B., 251, 259, *267*
Schneider, O., 83, 84, *101*

Schneider, W. C., 3, 24, *29*, 98, *102*, 107, *113*, 145, 146, 148, 155, 158, 159, 161, 164, 184, *191*, *192*, *194*, 231, *267*
Schneiderman, H. A., 184, 187, *191*, *192*
Schofield, G., 312, *334*
Scholander, P. F., 14, 17, *29*
Schrader, H., 286, *297*
Schreiber, R., 226, *267*
Schurhoff, P. N., 197, 199, *219*
Schultz, J., 252, *263*, 281, 283, 286, 288, 291, *296*, *297*
Schulze, F. E., 299, 300, 301, 302, *334*
Schumacher, J., 117, 120, *141*
Schumann, G., 308, 310, 332, *334*
Schuster, G. P. K., 107, *113*
Schwartz, L. I., 244, *265*
Schwerdt, R. F., 162, *196*
Schwertz, F. A., 204, 213, 214, 215, *220*
Schwimmer, S., 151, *193*
Scott, E. M., 98, *101*
Scott, G. H., 2, *29*
Scott, G. T., 395, *396*
Scott, S. G, 119, *139*
Seeds, W. E, 258, *267*
Seifter, E., 159, 163, 164, *194*
Seifter, S., 229, *266*
Seifriz, W., 87, 88, *102*
Seki, M., 86, 97, *102*
Selby, C. C., 147, *194*
Seligman, A. M., 82, 98, *99*, *102*, 363, 368, 370, *373*, *375*
Semmens, C. S, 235, *267*
Senn, G., 211, *219*
Serra, J. A., 252, *267*, 275, 283, 286, 287, 288, 293, *295*, *297*, *298*
Seshachar, B. R., 222, 232, *267*
Setterfield, G., 226, *267*
Sharma, A. K., 222, 235, 237, *267*
Sharp, L. W., 197, *220*
Sharpensteen, H. H., 151, *194*
Shaver, J. R., 233, *262*
Shaw, M., 160, *194*
Shelton, E., 98, *102*, 107, *113*
Shigenaga, M., 281, *298*
Shinke, N., 281, *298*
Shrader, R. E., 20, *29*
Shurcliff, W. A., 255, *267*
Sibatani, A., 226, 228, 236, 237, 241, 247, *266*, *267*
Siegel, S. M., 160, *194*
Siegler, E. H., Jr., 16, *26*
Siekevitz, P., 181, *194*
Siem, R. A., 133, *138*
Simmel, E., 2, *27*
Simon, E. W., 163, 165, 173, 175, 176, *191*, *194*, *195*
Simmonds, W. J., 42, 70, *78*
Simmons, H. S., 242, *264*
Simpson, W. L., 86, *102*
Singal, S. A., 146, *191*
Singer, M., 88, 97, *100*, *102*, 232, *263*
Sioli, H., 47, 48, *78*
Sjöstrand, F., 385, *396*
Sjöstrand, F. S., 152, 155, *195*, 206, 208, 209, 211, *218*
Slater, E. C., 145, 156, 157, 165, 169, 171, 175, *190*, *195*
Slautterback, D. B., 146, 157, *195*
Slonimski, P. P., 132, *141*
Smiles, J., 92, *101*, 254, *266*
Smith, A. G., 110, *113*, 125, 128, *141*
Smith, F. G., 169, *195*
Smith, G. M., 212, *220*
Smith, R. A., 168, *189*
Snapper, I., 251, 259, *267*
Sols, A., 161, *190*
Somerson, N. L., 138, *141*
Sonderegger, T. B., 137, *140*
Sonnenschein, N., 24, *29*
Sorokin, H., 145, 146, 147, 153, 154, 155, 157, *195*
Sotelo, J. R., *272*, *274*, *292*, *296*
Sparrow, A. H., 155, 159, *195*
Spector, W. G., 183, *195*
Spencer, A. G., 183, *193*
Sponsler, O. L., 88, *102*, 145, 147, 152, *195*
Srivastava, D. S., *276*, *298*
Stacey, M., 119, 120, 124, *140*, *141*, 232, 235, 251, *252*, *264*, *266*, *267*, 299, *334*
Stafford, H. A., 144, 145, 147, 149, 150, 152, 156, 157, 158, 159, 160, 166, 167, 170, 171, 172, 177, 185, *188*, *191*, *195*
Stahlecker, H., 232, *265*
Stanbury, S. W., 183, *195*

Stearn, E. W., 231, *267*
Stearn, M., 231, *267*
Stedman, E., *237*, 238, *267*, *268*, 366, *374*
Stedman, Ellen, *237*, 238, *267*, *268*
Steedman, H. F., 304, *334*
Steeman Nielsen, E., 394, *396*
Steinbeck, A. W., 70, *75*
Steinecke, F., 212, *220*
Steinmann, E., 198, 201, 205, 206, 207, 208, 209, 211, 213, *218*, *220*
Stempen, H., 129, *141*
Stenlid, G., 182, *195*
Stenram, U., 249, *268*, *275*, *298*
Stenström, S., 385, *396*
Stern, H., 135, *139*, 161, 177, 186, *188*, *195*
Stern, K. G., *67*, *78*
Stetten, D., Jr., 88, *101*
Stevens, C. E., 260, *265*, 305, 320, *334*
Stewart, D. C., 5, *29*
Stewart, W. N., 187, *194*
Stich, H., 284, *298*
Stiehler, R. D., 378, *396*
Still, J. L., 161, *192*
Stille, B., 124, *141*
Stock, A., 35, 48, *78*
Stocking, C. R., 148, 149, 160, 182, 185, *190*, *195*, *196*
Stockinger, L., 81, *102*, 277, 288, 294, *298*
Stoerk, H. C., 360, *375*
Stoll, A., 213, *220*
Stolte, H. A., *62*, *78*
Stowell, R. E., 99, *100*, 231, 233, 235, 238, 240, *268*, *277*, 283, 284, *297*, *298*
Street, H. E., 160, *195*
Strehler, B. L., 178, *195*
Streim, H. G., 230, 255, *266*
Striebich, M. J., 146, *191*
Strittmatter, C. F., 146, 158, 169, 170, *195*
Strugger, S., 154, *195*, 198, 200, 201, 202, 203, 207, 209, 215, 216, 217, *220*
Stumpf P. K., 146, 157, 160, 161, 163, 179, 180, *193*, *195*
Sulkin, N. M., 231, 232, *268*
Sun, C. N., 159, *196*
Sussman, A. S., 171, 172, 173, 187, *195*
Sutcliffe, J. F., 183, *195*
Svaetichin, G., 385, *396*
Sveinsson, S. L., 17, *29*
Svensson, G., *228*, *268*
Svinkina, A.A., 104, *113*
Sweeney, B. M., 182, *195*
Swift, H., 124, *141*, 222, 226, 228, *229*, 236, 247, *267*, *268*, 282, *298*
Sydenstricker, V. P., 146, *191*
Szafarz, D., 285, *297*

T

Taft, E. B., 243, 244, *268*
Takashima, S., *213*, *220*
Takatsuki, S., 33, 34, 39, 41, 45, 53, 58, *73*, *78*
Tamm, C., 237, *268*
Tandler, C. J., 288, *298*
Tanner, F., 109, *113*
Taylor, A. N., 286, *297*
Taylor, C. V., 12, *29*
Taylor, J. H., 284, *298*
Taxi, J., 341, 342, 348, 363, 366, 367, 368, 369, *374*, *375*
Teece, E. G, 124, *141*
Tello, J. F., 347, *375*
Teorell, T., 234, *264*
Teresi, J. D., 85, *102*
Terlaw, A. L., 253, *268*
Terrell, A. J., 172, *193*, *196*
Tewfik, S., 161, *195*
Theis, E. R., 81, *102*
Thimann, K V., 150, 151, 157, 165, 166, 167, 182, *191*, *194*, *195*
Thoma, K., 234, 244, *265*, *268*
Thomas, J. B., 205, 207, 209, 210, 213, *220*
Thomas, L., 229, *266*
Thomas, L. J., 289, *298*
Thomas, R., 236, *268*
Thompson, H. W., *257*, *262*
Thompson, R. H. S., 362, *373*
Thomson, R. Y., *229*, *268*
Thorell, B., 228, 261, *265*, *268*, 283, *298*
Thung, T. H., 207, *217*
Tiegs, O. W., 348, 350, *375*
Timberlake, H. G., 212, *220*
Ting, Y. C., 357, 359, *374*
Tipton, S. R., 4, *29*
Tissières, A., 186, *193*
Todd, G. W., 172, *195*

Tolbert, N. E., 173, *195*
Tolmach, L. J., 178, *195*
Tomcsik, J., 108, *113*
Torda, K., 358, 359, *375*
Torrey, J. G., 182, *195*
Townsend, G. F., 187, *194*
Treece, E. L., 133, *141*
Trueman, E. R., 48, 49, 50, 52, *78*
Tschassownikow, S., 301, *334*
Tsou, K. G., 363, 368, *375*
Tulasne, R., 110, 111, 112, *114*, 119, 121, 123, 129, 137, 138, *139*, *141*, *142*, 232, *262*
Turchini, J., 248, 256, *268*
Turek, P., 52, *78*
Tuttle, L. C., 137, *140*
Tyner, E. P., 282, *298*

U

Uber, F. M., 252, *268*
Ullrich, H., 201, *220*
Unna, P. G., 241, *268*
Urquhart, J. M., 85, *101*
Ussing, H. H, 385, 387, *396*

V

Vallance-Owen, J., 82, *102*
Valverde, R. E., 308, *333*
van der Heyde, H. C., 64, *76*
Van der Walt, J. P., 109, *113*
Van Fleet, D. S., 147, *195*
Van Herverden, M. A., 130, *141*
van Iterson, W., 109, 112, *114*, 207, *217*
van Kien, L. K., 248, *268*
Vannini, E., 244, 250, *264*
Varner, J. E., 180, *196*
Vaughan Williams, E. M., 353, 354, 373, *374*, *375*
Vendrely, C., 129, *139*, *141*, *142*, *222*, 241, 253, 261, *262*, *265*, *268*
Vendrely-Randavel, C., *232*, *268*
Vendrely, R., 119, 121, 122, 123, 125, 127, 129, 131, 138, *139*, *141*, *142*, 221, *222*, *232*, 241, 253, 261, *262*, *265*, *268*, 281, *298*
Venkatesan, T. R., 148, 166, *190*
Vennesland, B., 147, 152, 164, 167, 177, *188*, *191*, *195*
Vercauteren, R., 233, 241, *268*
Vermund, H., 285, *295*
Verne, J., 98, *102*
Vernon, L. P., 178, *195*
Vertregt, N., 207, 209, 210, *220*
Vialli, M., *222*, *268*
Vincent, W. S., 274, 276, 277, 280, 281, 285, 286, *287*, 289, *298*
Virno, M., 330, *333*
Vishniac, W., 178, *195*
Vogel, H. J., 104, *113*
Vogt, M., 4, *27*
Voit, K., 104, *114*
von Brücke, F. T., 372, *373*
von Euler, H., 215, *218*
Vonk, H. J., 57, *78*
von Kokas, E., 321, *333*
von Mohl, H., 197, *219*
von Nageli, C., 211, *219*
von Prittwitz, J., 134, *141*
von Seillers, R. F., 302, *334*
von Tellyesniczky, K., 85, 86, 91, *102*
von Wettstein, F., 217, *220*

W

Wachstein, M., 131, *142*
Wagge, L. E., 33, 34, 37, 43, 44, 47, 49, 50, 52, 53, 55, 69, 70, *78*
Wagner, R., 336, *375*
Wakkie, J. G., 198, *220*
Walker, E. W. A., 119, *139*
Walker, J. F., 81, *102*
Walker, P. M. B., *222*, 223, 232, 254, 255, *263*, *268*
Wang, K. J., 99, *102*
Watanabe, M. I., 184, *195*
Waygood, E. R., 172, 173, *195*
Weare, J. H., 86, *100*
Webb, M., 117, 119, 129, *142*, 283, *297*
Webb, R. A., 308, 312, 331, *333*
Webb, R. B., 109, *113*, 125, *139*
Webber, B., 330, *334*
Weber, F., 200, 201, 207, 210, *220*
Weber, M. W., 169, *195*
Webster, G. C., 170, 171, 172, 176, 180, 181, 182, *196*
Weeks, D. C., 182, *194*
Weibull, C., 107, 108, 109, *113*, *114*, 132, 136, 137, *142*

Weidel, W., 135, *142*
Weiden, V., 232, *264*
Weier, E., 198, 200, 201, 207, *220*
Weier, T. E., 148, 149, 185, *196*
Weil, A., 82, *102*
Weinstein, J., 2, *27*
Wells, H. S., 321, *327*, 332, *334*
Welsch, M., 127, *142*
Welsh, F. V., 254, *266*
Welsh, J. H., 58, 73, *78*, *372*, *375*
Welshimer, H. J., 108, *114*, 118, *142*
Wenderoth, H., *232*, 233, 259, *268*
Werner, I., 299, *334*
Whatley, F. R., 174, 178, *189*, *190*
Wheat, R. W., 168, *196*
Whipple, D. V., 41, *76*
White, J. C., 231, 233, 247, *263*, *268*
Wiame, J. M., 131, *142*
Widstrom, G., *237*, *268*
Wieland, H., *235*, *268*
Wieler, A., 198, 203, 207, *220*
Wigand, R., 123, *141*
Wiggins, L. F., 124, *141*
Wilbur, K. M., *277*, *295*
Wilcox, H., 250, *267*
Wildman, S. G., 172, 182, *192*
Wilkins, M. H. F., 9, *26*, *222*, *227*, 258, *263*, *268*
Wilkins, M. J., 147, 152, 156, 182, 183, *191*, *194*
Wilkinson, J. H., *277*, *297*
Williams, C. M., 170, 184, *193*, *195*
Williams, M. A., 320, *334*
Williams, W. L., 229, *266*
Willmer, E. N., 31, *32*, *78*
Wilson, D. G., 180, *192*, *196*
Wilson, E. B., 281, *298*
Wilson, R. R., 232, *264*
Wingstrand, K. G., 250, *266*
Winterscheid, L. C., 105, 107, *113*, *114*, 132, 133, 134, 137, *139*, *140*, *141*, *142*
Wislocki, G. B., 232, *263*
Withers, R. F. J., 364, 368, 371, *374*
Wolff, E., 62, *78*
Wolff-Heidegger, G., 321, *334*
Wolken, J. J., 152, 154, 155, 178, *196*, 203, 205, 213, 214, 215, *220*
Wolman, M., 82, 99, *102*
Wood, W. A., 162, *196*
Woodard, J., 226, *267*
Woodburn, M., 126, *139*
Woods, M. W., 148, 156, 171, 185, *190*, *196*
Woods, P. S., 278, *296*
Wormell, R. L., 81, *102*
Wosilait, W. D., 172, *193*, *196*
Wright, R. C., 98, *101*
Wright, R. D., 331, *334*
Wuhrmann, K., 201, *218*
Wyckoff, R. W. G., 112, *114*, 135, *140*, 147, 151, *194*, 253, *268*

Y

Yagi, Y., 147, 183, *194*
Yakar, N., 247, *268*
Yates, H. B., 254, 255, *268*
Yin, H. C., 159, *196*
Yocum, C. S., 173, *196*
Yokochi, C., 301, 308, *334*
Yokoyama, H. O., 99, *100*
Yonge, C. M., 37, 41, 53, 54, 55, 56, 57, 58, 59, 69, 73, *78*
Yoshida, T., 3, *29*
Young, J. Z., 347, *374*
Youngner, J. S., 4, *29*

Z

Zacks, S. I., 58, 73, *78*, *372*, *375*
Zamenhof, S., 233, *268*
Zawansin, A. A., 62, *78*
Zeiger, K., 91, 92, *102*
Zelitch, I., 173, *196*
Zerahn, K., 385, *396*
Zeuthen, E., 8, 16, 17, *27*, *29*
Ziegenspeck, H., 212, *220*
Zilliken, F. W., 117, 118, *141*
Zipkin, R., 301, 311, 319, 320, *334*
Zirkle, C., 274, *298*
Zollinger, H. U., 92, *102*
Zollinger, N. V., 277, *298*
Zorzoli, 231, 233, *268*
Zworykin, V. K., 20, *29*

Subject Index

A

Acetate, activation, in plants, 179
Acetic acid, effect on tissue components, 85-86
Acetone, effect on tissue components, 86-87
Alcohol, effect on tissue components, 86-87
Algae, chloroplasts, microscopic structure, 201
Amoebae, 2
Amoebocytes, 31-78
  activity, during shell repair, 43-52, 70, 71-72
    in molluscan digestion, 53-59, 73-74
    theories of shell formation and, 49-50
  excretory function, 59-60
  molluscan, origin, 69, 74
    cytology of, 33-43
  origin of, 43, 69, 74
  phosphatases in, 50
  in phyla other than the molluscs, 61-68, 74
Ascites tumor cells, 3
Aspidistra, chloroplast, electron micrograph of, 206
Autoradiography, application in histochemistry, 260

B

Bacteria,
  capsule of, 116-120
  cell membrane of, 116-117
  cell wall of, 116-117
    chemistry of, 135-136
    isolation of, 135-136
  cytochemistry of, 117-120
  cytology of, 103-114
  cytoplasmic bodies of, 130-134
    isolation of, 136-138
  equivalents of mitochondria in, 131-134
  formation of giant and dwarf forms of, 110
  histochemistry of, 115-142
  metachromatic granules of, 130-131
  nucleus of, 104, 120-130
    cytochemical demonstration, 122-130
    structural elements of, isolation, 134-138
Balances, for microdetermination of cellular components, 8
Biosyntheses, in plants, 177-182

C

Calcium,
  active transport during photosynthesis in aquatic plants, 394-395
  in molluscan mantle, 48-49
Carotenoids,
  molecular lattices for, in algal chloroplasts, 214
Cat, intestinal goblet cells of, 314-317, 323, 324, 326, 327, 328
Cations,
  active transport, redox pumps in, 377-396
    in yeast, 378-384
Cells,
  bacterial, comparison with cells of higher forms, 110-112
  centrifugation, 5-6
  components, determination of small amounts, 6-12
    balances for, 8
    by optical methods, 9
    by X-ray absorption, 9
    effect of fixatives on, 96ff.
  handling and preparation of, 2-6
  localization of enzymes in, 22
  metabolic activity, nucleolar changes during, 275-276
  separation from solid tissues, 3-4
  volume, measurement, 7-8
Cements, intercellular, enzymatic hydrolysis, 4
Centrioles, bacterial, 105-106
Chlorophyll, molecular lattices for, in algal chloroplasts, 214
Chloroplasts,
  algal, electron microscope studies on, 205-207
    molecular lattices for chlorophylls and carotenoids in, 214
  composition, 200-201

continuity of, and heredity, 217
development of, 215-217
electron microscope studies on, 203-212
granular, 207-210
electron micrographs of disintegrated, 208
submicroscopic structure, 210
membrane of, 211
methods of investigation, 198-199
molecular structure of the grana lamellae, 212-215
shape and size, 199-200
starch formation in, 212
structure of, 197-220
microscopic, 201-203
Cholinesterases, junctional, 355-373
activity, at the "neural" and "aneural" parts of the muscle, 357-358
effect of section of motor nerve on, 358-361
biochemistry, 357-361
histochemistry, 361-373
localization of, 361-373
artifacts and, 364-368
methods for, 361-364
Chromates, effects on tissue constituents, 82-83
Chromosomes, bacterial, 106
Cytochrome c, in plants, 169-170
Cytology,
bacterial, 103-114
problems of fixation in, 79ff.

D

Deoxyribonucleic acid, nucleolar, 285-286

E

Eggs, marine invertebrate, 3
Electron microscopy, application to histochemistry, 258
Enzymes,
hydrolysis of intercellular cements, 4
localization in cells, 22
nucleolar, 288-289
tissue, effect of fixatives on, 98-99
Epithelium, intestinal, 304-306
Euglena gracilis, chloroplasts, electron micrographs of, 204

F

Fatty acids, metabolism in plants, 179
Feulgen reaction, 235-241
in bacteria, 124-126
Fixation,
criteria for good, 94-96
effect on living protoplasm, 87-96
problems in cytology, histology, and histochemistry, 79-102
Fixatives, see also individual compounds
effects on tissues, 80-87, 96-99
blocking, 97
hydrolytic, 97
mordanting, 97
oxidizing, 98
physicochemical, 96ff.
Flagella, 108-109
Flagellatae, algal, structure of chloroplasts in, 203-204
Formaldehyde, effect on tissue components, 81-82
Frog skin,
active transport of Na^+ ions through, 385-387
comparison with transport from yeast cells, 386-387
relation to oxygen consumption, 386
structure, 385-386

G

Glycolysis, in plants, 159-162
Goblet cells, 299-304
functional and pharmacological aspects of, 329-332
intestinal, 304-333
of cat, 314-317, 323, 324, 326, 327, 328
distribution and number of, 319-329
formation, 310ff.
of rat, 303, 306, 307
secretory cycle, 310ff.
secretory mechanism, 307-310
Gram stain, 119-120

H

Helix aspersa,
amoebocytes of, 69
cytology of, 33-38, 39

Histochemistry,
of bacteria, 115-142
of nucleic acids, 221-268
problems of fixation in, 79ff.
Histology, problems of fixation in, 79ff.
Historadiography, of nucleic acids, 256-257
Histospectroscopy, infrared, of nucleic acids, 257-258
Hydrogen ions,
secretion by gastric mucosa, 387-394
analogy with yeast, 387-390
oxygen consumption and, 391-394

I

Interferometry, application to histochemistry, 258

K

Krebs cycle, in higher plants, 162-169

L

Lipids, nucleolar, 288

M

Matrix, activity, comparison of microsomal activity and, 24-25
Membranes, cellular, of bacteria, 108
Mercuric chloride, effect on tissue components, 85
Methyl green, staining of nucleic acids with, 241-245
Microchromatography, 17
Microdilatometry, 17-20
Micromanipulators, 12-13
Micromanometry, 16
Microneedles, fabrication, 15
Microphotometry, 16
Micropipettes, fabrication, 15
Microrgy, 12-16
cytochemical, 1-29
applications, 22-25
microscopical equipment, 13
pipette-controlling devices, 13-14
techniques, 15-16
ultraviolet, 20-21
Minerals, in nucleolus, 289
Mitochondria,
bacterial, 107, 131-134
chemical composition, 158-159
cytology of isolated, 155-157
effect of radiation on, 187
plant, 143-196
distribution, 154-155
electron transfer and, 169
genetic, importance, 185
isolation, 148-151
methods for studies on, 146-147
origin, 154-155
physiology of, 182-185
structure, 151-154
terminology, 144-146
Molluscs,
digestion, amoebocytic activity in, 53-59, 73-74
mantle, calcium reserves in, 48-49
importance in shell repair, 43-48
tracer studies on, 51-52
shell, formation of, theories concerning, 49ff.
amoebocytic activity and, 49-50
Moist chambers, 14-15
Motor end-plate,
mammalian, 337-350
structure, 338
Mougeotia, pyrenoids, electron micrographs, 204
Mucosa, gastric,
secretion of H^+ ions by, 387-394
analogy with yeast, 387-390
oxygen consumption and, 391-30

N

Neuromuscular junctions,
general properties, 336-337
localization of cholinesterases at, 361-373
in vertebrates, 350-355, 356, 357
Nucleic acids,
bacterial, chemistry of, 121-122
determination in tissues,
physical methods for, 252-260
histochemistry of, 221-268
fixation of tissues, 229-230
physical methods in, 252-261
requirements for histochemical analysis, 222-229
sources of error, 223ff.
staining methods, 231-252

Nucleolus,
chemistry of, 280-289
deoxyribonucleic acid content, 285-286
enzymes, 288-289
lipids, 288
mineral content, 280
ribonucleic acid content, 281-285
components, origin of, 289-293
evidence from studies of nucleolar chemistry, 290-291
from studies on telophase reconstitution, 289-290
genetic evidence, 291-292
role of nuclear sap in, 293
of nucleolonema in, 292-293
experimental modification of morphology, 276-279
inclusions, 272-273
function, 293-295
interphase, morphology of, 272-280
physical nature of, 273-274
structure, theories of, 279-280
telophase, structure of, 274-275
Nucleus,
bacterial, 104, 120-130
cytochemical demonstration, 122-130
Feulgen technique, 124-126
phase contrast microscopy as control for staining techniques, 129
by Piekarski-Robinow, 122-123
use of enzymes for, 123-124
isolation, 136
cellular, estimation of enzymatic activity in, 22-24

O

Osmium tetroxide, effect on tissue components, 83-85

P

Phosphatases, in amoebocytes, 50-51
Phosphorylation, in plants, 174-177
Photofluorometry, application in histochemistry, 259-260
Photosynthesis, in plants, 177-179
active transport of calcium during, in aquatic, 394-395
Picric acid, effect on tissue components, 85
Plants,
aquatic, active transport of calcium during photosynthesis in, 394-395
biosyntheses in, 177-182
cytochrome c in, 169-170
glycolysis in, 159-162
higher, chloroplasts of, 201-203
Krebs cycle in, 162-169
mitochondria of,
see Mitochondria, plant
photosynthesis in, 177-179, 394-395
phosphorylation in, 174-177
protein metabolism in, 180-182
respiration, mitochondria and, 159-174
pathways, 171-174
role of cytochrome oxidase in, 170ff.
Polysaccharides, bacterial, staining of, 117-119
Protein metabolism, in plants, 180-182
Proteins,
nucleolar, 286-288
tissue, effect of fixatives on, 88-89, 96-97
Protoplasm,
constituents, fixation of, 89-91
effect of fixation on living, 87-96
structure, 87-88
fixation and, 91-93
Pyronin, staining of nucleic acids with, 245-248

R

Radiation, effect on plant mitochondria, 187-188
Rat, intestinal goblet cells of, 303, 306, 307
Redox pumps, in active transport of cations, 377-396
Ribonucleic acid, nucleolar, 281-285

S

Selaginella Watsonii, chloroplast, electron micrograph of, 206
Sodium ions,
active transport through frog skin, 385-387

comparison with transport from yeast cells, 386-387
active transport in *Ulva lactuca*, 395
Spectroscopy, ultraviolet, of nucleic acids, 253-256
Spores, bacterial, nucleus of, 109
Starch, formation in chloroplast, 212
Subcellular particulates, application of cytochemical micrurgy to isolated, 22-24
Submicromanipulator, volumetric, 9-11
Subneural apparatus, 342, 343, 348-349, 351

T

Tissue cultures, 4-5
Tissues,
effects of fixatives on, 80-87, 96-99
fixation for histochemical analysis of, 229-231
for nucleic acid determination, 229-230
separation of cells from solid, 3-4

U

Ulva lactuca, active transport of sodium ions in, 395

V

Vacuoles, in bacteria, 107-108

Y

Yeast,
active transport of cations in, 378-384
effects of external redox systems on, 379-382
nature of the effect, 382-384
relation of phosphate energy to active transport of Na^+ and K^+ ions, 383-384