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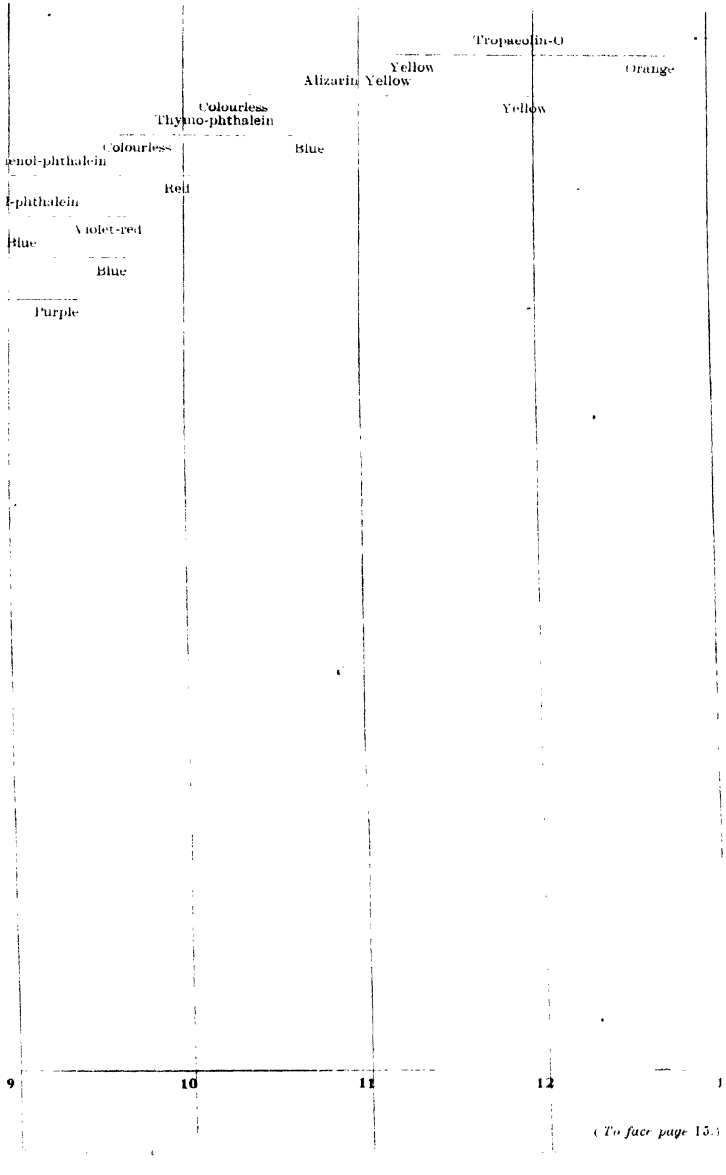
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AN INTRODUCTION TO

BACTERIOLOGICAL CHEMISTRY

BY

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Second Edition
Revised Reprint

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PREFACE TO THE SECOND EDITION

WHEN the first edition of this book appeared in 1938 it was considered that the subject of Chemotherapy of bacterial infections was in too primitive a state to warrant inclusion. The advance in our knowledge since then has made it possible and desirable to consider the topic in some detail. The ideas on which the explanation of chemotherapeutic action is based, and which form the foundation for planning further investigation, involve the chemistry of metabolic processes and naturally come within the scope of this book. Similar considerations lead to the inclusion of a chapter on Antibiotics and to a fresh presentation of the facts known about Growth Factors. These chapters constitute the main difference between the first and the present editions, but new material has been added to a number of other chapters in order to keep level, as far as possible, with the changes involved in such a rapidly growing branch of Biochemistry.

Some criticism has been expressed that bibliographical references to the original literature were not given in the first edition. It is the opinion of the author that a detailed bibliography is out of place in a small textbook intended primarily for students, but in order to extend the usefulness of the volume an endeavour has been made to give, at the end of each chapter, some references, mainly to monographs and reviews, which will serve as a guide to the original papers.

Once more it is a pleasure to acknowledge my debt to the authors whose works have supplied the material presented. Especially are my thanks due to colleagues and friends for much helpful criticism and advice.

C. G. ANDERSON.

WELLCOME PHYSIOLOGICAL RESEARCH LABORATORIES
LANGLEY COURT, BECKENHAM, KENT
July, 1946

PREFACE TO FIRST EDITION

THIS text-book is the outcome of lectures on Bacteriological Chemistry presented as part of the course for the University of London Academic Diploma in Bacteriology, and recently to students taking Bacteriology as an Honours subject in the University of Edinburgh. During the period over which these courses have extended the need has been increasingly felt for a text-book of reasonable size yet covering a sufficiently wide range of topics. Whilst many excellent monographs on various aspects of the subject exist, there seems to be no single book giving a survey of the whole field in a form suitable for students of such courses as those mentioned.

The present volume makes no claim to be encyclopaedic, but an endeavour has been made to cover the requirements of students, and perhaps of those research workers whose interests may not be primarily chemical but who feel the need for some understanding of the metabolic behaviour and chemical nature of the organisms which they are handling. In order to keep its size within reasonable limits it has been necessary to assume a knowledge of elementary organic chemistry and of a certain amount of bacteriology. In view of the rapid expansion of the subject within the past ten to fifteen years, and the consequently ever-changing views and opinions expressed concerning the various reactions involved, the selection of the appropriate material has not been easy. Without doubt much has been omitted which should have been included, and certain matters admitted which the future will show to be of only passing importance. Only certain aspects of immunochemistry have been considered and no attempt has been made to deal with the subjects of disinfection and chemotherapy,

nor with the chemistry of culture media and staining reactions. Their treatment here could be little more than a catalogue of substances and organisms, for although a large body of empirical data concerning them is available, we have as yet but little exact chemical knowledge of the mechanisms involved.

In the hope that the interest of students reading the following pages will have been stimulated, sources of further information have been indicated at the ends of the chapters.

Acknowledgment is gratefully made to the authors of the many monographs, standard works and papers which have been drawn upon freely for the material collected here.

It is a pleasure to record my deep indebtedness to Professor H. Raistrick, F.R.S., of the London School of Hygiene and Tropical Medicine, who awakened my interest in this subject, and to Professor T. J. Mackie, to whose keenness and encouragement the course in this University owes its inception.

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DEPARTMENT OF BACTERIOLOGY,
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December, 1937.

CONTENTS

PART I

GENERAL CONSIDERATIONS

CHAP.		PAGE
I.	INTRODUCTION	1
II.	HYDROGEN ION CONCENTRATION AND *p*H; OXIDATION-REDUCTION POTENTIALS	4
III.	COLLOIDS AND ADSORPTION	26
IV.	ENZYMES	36
V.	THE CHEMICAL COMPOSITION OF BACTERIA, YEASTS AND THE LOWER FUNGI	56

PART II

METABOLISM

VI.	THE NUTRITION OF THE AUTOTROPHIC BACTERIA	67
VII.	THE NUTRITION OF THE HETEROTROPHIC BACTERIA	80
VIII.	ADAPTIVE AND CONSTITUTIVE ENZYMES	89
IX.	GROWTH FACTORS	98
X.	CHEMOTHERAPY	122
XI.	ANTIBIOTICS	156
XII.	BACTERIAL RESPIRATION	187
XIII.	NITROGEN METABOLISM	214
XIV.	CARBON METABOLISM	240
XV.	ALCOHOLIC FERMENTATION	262
XVI.	THE FERMENTATION PRODUCTS OF THE LOWER FUNGI	282
XVII.	INDUSTRIAL FERMENTATIONS	310
XVIII.	THE PROTEINS OF MICRO-ORGANISMS	328
XIX.	THE POLYSACCHARIDES OF MICRO-ORGANISMS	345
XX.	THE LIPOIDS OF MICRO-ORGANISMS	369
XXI.	THE PIGMENTS OF MICRO-ORGANISMS	385

PART III

SOME ASPECTS OF IMMUNOCHEMISTRY

CHAP. PAGE

XXII. Antigens, Haptens, Antibodies and Complement - - - - - - - - - 398

XXIII. The Mechanism of Antigen-antibody Reactions 441

APPENDICES

I. The Isolation and Identification of Metabolic Products - - - - - - - - 462

II. Synonyms - - - - - - - - - 469

INDEX - - - - - - - - - - 477

BACTERIOLOGICAL CHEMISTRY

CHAPTER I

INTRODUCTION

THE subject of Bacteriological Chemistry has been steadily growing in scope and importance since the days when Pasteur studied the fermentation reactions, normal and abnormal, until, during the past one or two decades, it has expanded with such rapidity that it has now almost acquired the dignity of a special branch of Biochemistry, and is even in danger of itself becoming subdivided with production of such offshoots as Immunochemistry. This rapid growth is in part due to the ever-increasing utilisation of microbiological methods and products in industry, and in part to purely academic investigations into the mechanisms by which the bacteria, yeasts and fungi gain the energy for their growth and reproduction and synthesise the multitudinous products which they build into their cell structures or excrete into the medium in which they develop. The combination of utilitarian and academic motives has resulted in the accumulation of a vast number of facts which are only just beginning to be shaped into an ordered whole in which it is possible to see the relationships between apparently quite different modes of metabolism, and the bewildering variety of substances elaborated during such processes.

A great deal of the information which we possess is still only of an empirical nature and we have not yet found out how to fit these facts into the general picture. The present stage of development of microbiological

chemistry is somewhat like that of a half-finished jig-saw puzzle. Some areas are nearly complete ; in others only a few of the pieces have so far been fitted into place. Quite recently the filling in of the area which includes chemotherapy has been progressing rapidly as a result of the development of the sulphonamide drugs, and a reasoned account of their action can now be presented. The subject of disinfection, however, is still in a nebulous state ; we know a great deal about the necessary concentrations of disinfectants and the conditions for their action, but very little as to how the observed results are brought about. It seems probable that the present views on the mechanism of chemotherapy may well be applied to the action of disinfectants and antiseptics.

The position of Immunochemistry, in this respect, has improved considerably in the past decade and we are at last able to understand something of what is really happening during immunological reactions, although there is much detail still to be filled in and much expansion of our knowledge necessary.

Our knowledge of the metabolic processes of micro-organisms is perhaps the best developed part of the whole structure and enables us to see the connections between the modes of life of many different bacterial types. Even here, though, we still know comparatively little of the mechanism of the synthetic assimilation processes.

The scheme of treatment adopted in the following chapters has been first of all to deal with the general conditions which influence and determine the behaviour or micro-organisms. The importance of the hydrogen ion concentration and of colloidal phenomena is obvious, as also is the part played by the enzymes on which almost every stage of the life of the bacteria, yeasts and moulds depends. Then follows an account of the different ways in which micro-organisms obtain the energy and starting materials for their growth and reproduction. The

discussion of chemotherapeutic action falls logically into place here since it is held to depend on interference with the metabolic activities of the bacteria. The consideration of the by-products of metabolic and respiratory processes, the fermentation products in which man is mainly interested, follows naturally. Then the substances synthesised by the organisms for their own use are detailed. Finally a brief outline of the chemistry of antigens, antibodies and their reactions is presented.

The usual difficulty with regard to the consistent naming of bacteria has been encountered. In the absence of any standardised procedure in this country it has been considered desirable to adopt the nomenclature sanctioned by the Society of American Bacteriologists as exemplified in Bergey's "Manual of Determinative Bacteriology" (Fifth Edition). The common (as opposed to the scientific) names of certain organisms have been used in some instances. A list of synonyms covering cases which may cause confusion has been added as Appendix II.

CHAPTER II

HYDROGEN ION CONCENTRATION AND *p*H; OXIDATION-REDUCTION POTENTIALS

Hydrogen Ion Concentration.—The course of all biological processes is profoundly influenced by the degree of acidity or alkalinity of the fluid in which they take place—whether the fluid be the cell contents, or a circumambient fluid like blood, or a culture medium in which micro-organisms are growing, or a solution in which enzymes are acting. As a notable example one may consider the blood. Should it become very slightly acid death in coma will result, with the heart muscle relaxed ; on the other hand, if it should become but slightly alkaline tetany results and the heart will cease to function with the muscle contracted. The heart will only function properly when the blood is within a very narrow range between acidity and alkalinity. Similar, though as a rule not such dramatic, changes follow alteration of the normal state of other biologically concerned fluids. Some bacteria, for instance, thrive in quite strongly acid solutions but rapidly die out in alkaline conditions ; others, like the cholera vibrio, develop in alkaline but not in acid media. The same applies to enzymes ; the gastric enzyme, pepsin, is only active in breaking down proteins when in acid solution, whilst trypsin, in the pancreatic juice, requires an alkaline medium for its activity.

According to modern views an acid is defined as a substance which tends to lose a proton (hydrogen nucleus

or H^+) and a base is a substance which tends to acquire a proton. This may be expressed by the equation

$$A \rightleftharpoons H^+ + B$$

where A represents an acid and B a base. This means that every acid must be associated with a corresponding or "conjugate" base and *vice versa*. Generally the conjugate acid or base is the solvent in which the substance is dissolved. In aqueous solutions water can act as the conjugate base of an acid or as the conjugate acid of a base since it is capable of either taking up or giving up a proton. For an acid in water the equilibrium is :—

$$\underset{\text{(acid)}}{HA} + H.OH \rightleftharpoons H.OH_2^+ + \underset{\text{(base)}}{A^-}$$

$H.OH_2^+$ is what is usually known as the hydrogen ion for which the symbol H^+ is commonly used.

Applying the Law of Mass Action

$$K'_A = \frac{(a\,.\,H.OH_2^+)\ (a\,.\,A^-)}{(a\,.\,H.OH)\ (a\,.\,HA)} \quad . \quad . \quad . \quad (1)$$

where K'_A is the dissociation constant of the acid and $(a\,.\,H.OH_2^+)$, $(a\,.\,A^-)$, $(a\,.\,H.OH)$ and $(a\,.\,HA)$ are the "activities" of the hydrogen ion, conjugate base, un-ionised water and un-ionised acid respectively. The "activity" of an ion is the product of its concentration and its "activity coefficient" which is a measure of the influence of surrounding ions upon it and which accordingly depends upon the dilution of the solution. Since the activity of un-ionised water may be regarded as constant, because its amount is virtually unaffected by the very small degree of ionisation which it undergoes, equation (1) can be rewritten with the new constant K_A

$$K_A = \frac{(a\,.\,H.OH_2^+)\ (a\,.\,A^-)}{(a\,.\,HA)} \quad . \quad . \quad . \quad (2)$$

Using the more familiar symbol, H^+, for the hydrogen ion this becomes

$$K_A = \frac{(a.H^+)\ (a.A^-)}{(a.HA)} \quad . \quad . \quad . \quad . \quad (3)$$

For a base dissolved in water the equilibrium is expressed by the equation

$$\underset{\text{(Base)}}{B} + H.OH \rightleftharpoons BH^+ + OH^-$$

The dissociation constant, K'_B, for the base is then given by the expression

$$K'_B = \frac{(a.BH^+)\ (a.OH^-)}{(a.B)\ (a.HOH)}$$

Since in this case, too, the activity of the water can be regarded as constant this becomes, with the new constant, K_B,

$$K_B = \frac{(a.BH^+)\ (a.OH^-)}{(a.B)} \quad . \quad . \quad . \quad (4)$$

Water is capable of either giving up or accepting a proton. Therefore in pure water and in all aqueous solutions the equilibrium

$$H.OH + H.OH \rightleftharpoons H.OH_2^+ + OH^-$$

must exist and the dissociation constant is given by

$$K'_w = \frac{(a.H.OH_2^+)\ (a.OH^-)}{(a.H.OH)^2}$$

The activity, (a . H.OH), of un-ionised water can be regarded as constant and therefore

$$K_w = (a.H.OH_2^+)\ (a.OH^-) \quad . \quad . \quad . \quad (5)$$

The product of the activities of the acidic and basic ions of a solvent is also known as the ionic product of the solvent. Replacing $H.OH_2^+$ by the familiar symbol for the hydrogen ion, equation (5) becomes

$$K_w = (a.H^+)\ (a.OH^-) \quad . \quad . \quad . \quad . \quad (6)$$

In dilute solutions, that is when the ionic strength is small, the activity coefficients are very nearly unity and the activities ($a.H^+$) and ($a.OH^-$) of the hydrogen and hydroxyl ions can be replaced by their concentrations ($C.H^+$) and ($C.OH^-$) respectively. Equation (6) can now be written

$$K_W = (C.H^+)\,(C.OH^-) \quad . \quad . \quad . \quad . \quad (7)$$

In an exactly neutral solution, obviously, the concentration of the acidic (hydrogen) ions must be equal to the concentration of the basic (hydroxyl) ions. That is $(C.A^+) = (C.OH^-)$ and, from equation (7), each must be equal to $\sqrt{K_W}$.

The ionic product, K_W, can be determined experimentally from conductivity measurements since the conduction of electricity through a liquid depends on the number of ions available to carry the current. The value for pure water at room temperature has been found to be 10^{-14}. Consequently in neutral solution the concentration of both hydrogen and hydroxyl ions must be $\sqrt{10^{-14}}$ or 10^{-7} gram ions per litre. If more hydrogen ions than this amount are present the solution is acid and if there are less hydrogen ions the solution is alkaline, but whatever the state of the solution the ionic product is constant and equal to 10^{-14}. In other words a greater amount of hydrogen ions means a smaller amount of hydroxyl ions and *vice versa*. Consequently the strength of an alkali, as well as that of an acid, can be expressed in terms of hydrogen ion concentration.

A normal solution of a strong acid will contain about 1 gram ion per litre of hydrogen ions, the exact amount depending upon the degree of dissociation of the particular acid and the activity of the ions. A strong solution of an alkali will contain about 10^{-13} gram ions per litre of hydrogen ions, derived from the ionisation of the water. The degree of ionisation of a solution can be measured by the conductivity of the solution, which depends on the

number of ions present to carry the current, or by the depression of the freezing point of the solution, which depends on the total number of ions and molecules present. A tenth normal solution of hydrochloric acid is 91 per cent. ionised so that the concentration of hydrogen ions in such a solution will be

$$\frac{0{\cdot}1 \times 91}{100} = 0{\cdot}091 = 9{\cdot}1 \times 10^{-2} \text{ grams per litre.}$$

Acetic acid is a weak acid and is only 1·3 per cent. ionised in a tenth normal solution, and such a solution will, therefore, contain only

$$\frac{0{\cdot}1 \times 1{\cdot}3}{100} = 0{\cdot}0013 = 1{\cdot}3 \times 10^{-3} \text{ grams per litre of hydrogen ions.}$$

This method of expressing the hydrogen ion concentration and correspondingly the acidity or alkalinity of a solution is somewhat cumbersome particularly in the range of values near neutrality, from about 10^{-6} to 10^{-8} grams of hydrogen ions per litre, in which the great majority of biological phenomena occur. The adoption of the exponential or *p*H method of expression suggested by Sϕrensen in 1909, and which is now universally used, has greatly simplified the statement and comprehension of such values. Sϕrensen defined the *p*H value of a solution as the negative logarithm of its hydrogen ion concentration. That is

$$p\text{H} = -\log (\text{C.H}^+) \quad \text{or} \quad (\text{C.H}^+) = 10^{-p\text{H}}$$

For neutral water containing 10^{-7} grams per litre of hydrogen ions

$$p\text{H} = -\log 10^{-7} = \log 10^{7} = 7$$

For the example quoted above of 0·1N-hydrochloric acid containing $9{\cdot}1 \times 10^{-2}$ grams per litre of hydrogen ions,

$$p\text{H} = -\log (9{\cdot}1 \times 10^{-2}) = 2 - \log 9{\cdot}1 = 2 - 0{\cdot}959 = 1{\cdot}041$$

and for 0·1N-acetic acid,

$$p\text{H} = -\log (1{\cdot}3 \times 10^{-3}) = 3 - \log 1{\cdot}3 = 3 - 0{\cdot}114 = 2{\cdot}886$$

Blood, which contains $4{\cdot}7 \times 10^{-8}$ grams per litre of hydrogen ions, will have

$$p\text{H} = -\log(4{\cdot}7 \times 10^{-8}) = 8 - \log 4{\cdot}7 = 8 - 0{\cdot}672 = 7{\cdot}328$$

Since, as we have seen, even strongly alkaline solutions contain some hydrogen ions, and the amount of hydrogen ions is inversely proportional to the amount of hydroxyl ions (on which the alkalinity depends), the alkalinity of a solution can also be expressed in terms of its hydrogen ion concentration and the *p*H scale. Thus an alkaline solution containing $3{\cdot}7 \times 10^{-9}$ grams of hydrogen ions per litre has

$$p\text{H} = -\log(3{\cdot}7 \times 10^{-9}) = 9 - \log 3{\cdot}7 = 9 - 0{\cdot}568 = 8{\cdot}432$$

As the product of hydrogen ion concentration and hydroxyl ion concentration is constant at 10^{-14}, it is obvious that the more hydrogen ions there are present, that is, the more acid the solution, the lower will be the *p*H, tending to the value $p\text{H}=0$, which is the theoretical limit when there are no hydroxyl ions present. On the other hand, the more hydroxyl ions there are present, that is, the more alkaline the solution, the higher will be the *p*H value, tending to the hypothetical limit $p\text{H} = 14$ when there are no hydrogen ions present. Since neutrality occurs at *p*H 7, all values lower than this refer to acid solutions, whilst higher values than 7 indicate alkaline solutions.

It must be remembered that the *p*H scale is logarithmic, and that accordingly a change in *p*H value of 1 unit means a tenfold change in acidity or alkalinity. Thus a solution of *p*H 5 will be ten times as acid (will contain ten times as many hydrogen ions) as one at *p*H 6, and a hundred times as acid as one at *p*H 7. Similarly a solution at *p*H 10 will be ten times as alkaline (will contain ten times less hydrogen ions) than one at *p*H 9, and be one hundred times as alkaline as a solution at *p*H 8.

The titratable acidity of a solution must not be confused with its *p*H value. The titratable acidity depends

only on the amount of acid present ; thus, for example, all tenth-normal acid solutions will have the same titratable acidity whatever the particular acid present may be, whether it is a strong acid like hydrochloric acid or a weak acid like acetic acid. But the *p*H values of different tenth-normal acid solutions will vary widely, depending on the acid concerned. This variation in *p*H value of acid solutions containing the same equivalent weight of acid results from the different degrees of dissociation of the acids with the consequent production of different concentrations of hydrogen ions. Thus we saw that 0·1N-hydrochloric acid is 91 per cent. dissociated and has a *p*H value of approximately 1, whilst 0·1N-acetic acid, which is 1·3 per cent. dissociated, has *p*H 2·9 ; that is hydrochloric acid is nearly one hundred times as strong an acid as acetic acid, although the two solutions have the same titratable acidity. In other words, merely knowing the amount of titratable acid in a solution does not tell us enough about its properties and probable effect on a culture of bacteria or on a fermentation reaction. We must know also the *p*H value of the solution, which will give us a much better idea of the magnitude of effect to expect.

The logarithmic or exponential mode of expression is also used for the statement of dissociation constants, which are referred to as *p*K values where

$$pK = -\log K$$

K being the dissociation constant under consideration. Thus the dissociation constant of acetic acid at 25°C. is $1{\cdot}8 \times 10^{-5}$ and its *p*K value is, therefore,

$$pK = -\log (1{\cdot}8 \times 10^{-5}) = 5 - \log 1{\cdot}8 = 5 - 0{\cdot}255 = 4{\cdot}745$$

An acid like phosphoric acid, H_3PO_4, which has three ionisable hydrogen atoms, has three *p*K values, one corresponding to the dissociation of each hydrogen ion. The three dissociation constants and *p*K values of

phosphoric acid are—

For the first hydrogen ion	$K = 1{\cdot}1 \times 10^{-2}$,	$pK = 1{\cdot}959$
For the second hydrogen ion	$K = 2 \times 10^{-7}$,	$pK = 6{\cdot}699$
For the third hydrogen ion	$K = 3{\cdot}6 \times 10^{-13}$,	$pK = 12{\cdot}444$

Obviously the higher the *p*K value, the smaller the dissociation constant and the weaker the acid.

The Measurement of Hydrogen Ion Concentration.—In general there are two main methods of measuring hydrogen ion concentration, electrical and colorimetric. The electrical methods are by far the more accurate, but require the use of costly and delicate apparatus not available in many ordinary laboratories. The colorimetric methods, while not so accurate, are much cheaper, quicker and simpler to perform.

The electrical methods depend on balancing the potential difference set up between the solution of unknown hydrogen ion concentration and an electrode immersed in it against the potential of a standard cell. If a rod of metal is immersed in a solution of one of its salts, the metal will tend to dissolve to a degree depending on its "electrolytic solution pressure," producing positively charged ions of the metal, and consequently leaving a negatively charged mass of undissolved metal. On the other hand, the salt in solution ionises, and the metallic ions will exert a definite osmotic pressure, depending on the concentration of the salt and its degree of dissociation. If the ionic osmotic pressure is less than the "solution pressure" of the metal, as is the case with zinc, the latter will dissolve in order to establish equilibrium. If, however, the "solution pressure" is less than the ionic osmotic pressure, as is the case with copper or mercury, ions will be deposited on the metal and the rod will acquire a positive charge relatively to the solution. Each metal in contact with a solution of one of its salts acquires in this way a characteristic "electrode potential," the strength of which will depend on the concentration

of the salt solution and the particular metal in question. If two such electrodes, for instance, copper in copper sulphate and zinc in zinc sulphate, are joined together in an electrical circuit by connecting the two metals with a wire and the two solutions either with another wire or with a ∩-tube containing a solution (Fig. 1), then a

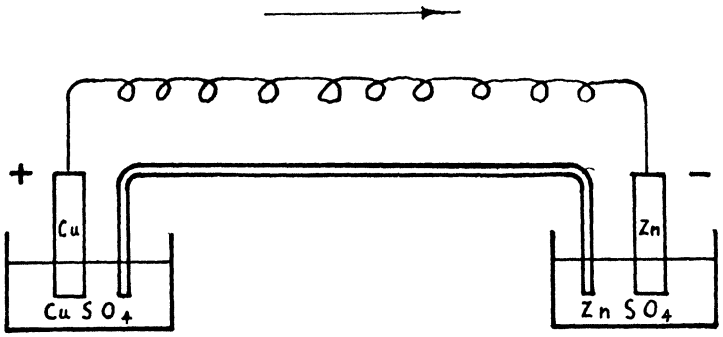


FIG. 1

current will flow in the direction of the higher to the lower potential. An electrode of this type which is very frequently employed for purposes of measurement is the standard calomel electrode in which mercury is in contact with saturated mercurous chloride and N-potassium chloride, and which has a potential of +0·56 volt at 18° C. Another standard electrode is the hydrogen electrode in which an electrode of platinum black saturated with hydrogen gas is immersed in an acid solution normal in respect to the hydrogen ions. For reasons of convenience this potential is arbitrarily taken as being zero. If the concentration of hydrogen ions is different from normal the potential will also be different from the standard, and will be a measure of the hydrogen ion concentration.

The potential differences between a standard half-cell, such as the calomel electrode, and an electrode immersed in the unknown solution are measured by balancing them

on a potentiometer until no current flows, as determined by a galvanometer (Fig. 2). Hence, knowing the electrode potential of the standard cell, that of the unknown may be calculated, and from this the hydrogen ion concentration and the *p*H determined. A third simple and convenient standard electrode is the quinhydrone electrode

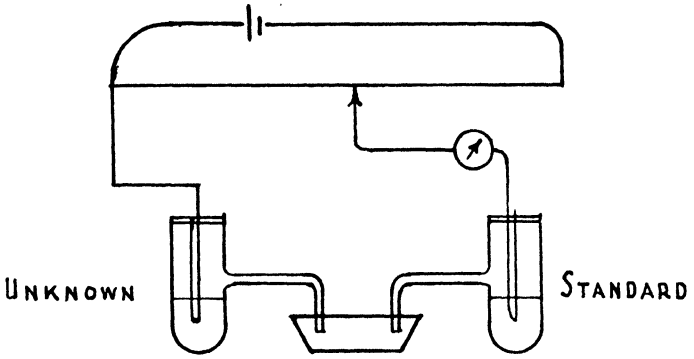


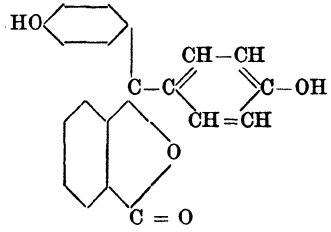
FIG. 2

in which quinhydrone is dissolved in a standard buffer solution of known *p*H, the potential of a platinum electrode immersed in such a solution depending on the *p*H.

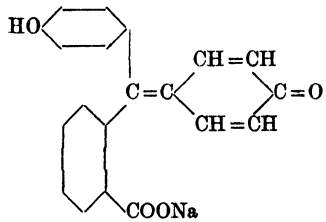
A system very commonly used in the laboratory for measurement of *p*H is the glass electrode in which a glass membrane separates a solution of normal hydrochloric acid, into which dips a calomel electrode, from the solution of unknown *p*H which is in contact with another calomel electrode immersed in 3·5 normal potassium chloride solution. The potential which is set up across the membrane depends on the *p*H of the solution and is measured by determining the null point on a potentiometer and electronic valve circuit.

Colorimetric methods depend on the use of various dyes which show colour changes over characteristic

ranges of *p*H values of the solution into which they are introduced. The colour change may be due to the conversion of an almost non-ionised weak acid or a weak base of one colour to a strongly ionised salt, where the ion has a characteristic colour. For example, methyl orange exists as the yellow non-ionised acid in acid solution, but on addition of alkali the corresponding salt is formed which ionises to give the red anion. Or the colour change may be associated with a change in the internal structure of the molecule producing a coloured quinonoid form, as is held to be the case with phenolphthalein. In acid solution the compound exists in the colourless form :—



In alkaline solution this is converted into the sodium salt of the tautomeric quinonoid form :—



which ionises to give the red anion characteristic of phenolphthalein in alkaline solution. Some of these indicators, for example, Congo red, phenolphthalein, litmus, change colour sharply over a narrow range of *p*H value, that is, with a comparatively small change in hydrogen ion concentration, and consequently are of use

in determining the end point in titration of acid and alkalies. It must be remembered that the end points which they indicate are not identical but occur at different *p*H values, about *p*H 4 for Congo red, about 7 for litmus and about 9 for phenolphthalein. Other indicators change colour gradually over a range of two to three units of *p*H value, the actual colour observed being determined by the *p*H of the solution. By matching the colour of a suitable indicator in a solution of unknown *p*H against the range of colours which it gives in solutions of known *p*H value, the hydrogen ion concentration of the unknown solution can be estimated. Indicators covering *p*H values from 1 to 13 are available, and each indicator is put up in an appropriate series of standard buffer solutions of known *p*H values in sealed tubes against which the comparisons are made. An even more convenient method is the use of the Lovibond comparator in which the standard tubes containing the indicator are replaced by a disc carrying tinted glasses of colours which match the standards, at intervals of *p*H of 0·2 unit. Besides convenience and speed in use these glass colour standards possess the advantage of not fading, a fault to which the buffer solution indicator standards are subject, particularly if they are exposed to light.

A chart showing the colour changes and *p*H range of the more useful indicators is given in Fig. 3.

Buffer Solutions.—In a great deal of biological work, particularly when dealing with enzyme systems or reactions of a similar nature, it is important and often even essential to maintain the *p*H of the solution constant in spite of the fact that an acid may be produced or used up during the course of the reaction. If such a reaction were carried out in simple aqueous solution the *p*H value would obviously change progressively with time, but by making use of buffer solutions these changes can be eliminated, the buffer solution offering a reserve of acid or alkali capable of taking up or supplying alkali or acid as occasion

demands and thus preventing any considerable change of *p*H. As the name implies, the buffer solution acts as a shock absorber, or like a sponge, to take up excess acid or alkali.

Buffer solutions are, in general, mixtures of a weak acid with one of its salts, the particular acid being chosen to suit the *p*H range desired. A weak acid, such as acetic acid, in solution is only ionised or dissociated to a small extent, so that most of it is present as undissociated neutral acetic acid molecules :—

$$HAc \rightleftharpoons H^+ + Ac^-. \qquad (1)$$

The Law of Mass Action applies to this dissociation, giving the equation

$$[H^+]\times[Ac^-] = k[HAc], \text{ or } [H^+] = \frac{k[HAc]}{[Ac^-]} \quad . \quad . \quad . \quad (2)$$

On the other hand, the salt. sodium acetate, is strongly dissociated to give sodium and acetate ions. Now in a mixture of the acid and the salt practically all the acetate ions will be derived from the highly dissociated salt and only very few from the acetic acid, and consequently, since the number of hydrogen ions cannot be greater than the number of acetate ions derived from acetic acid, the hydrogen ion concentration will be lowered. As the acetate ions in such a mixture come almost entirely from the salt their concentration will be $\alpha \times$ [NaAc], where α is the fraction of the salt which is ionised. So that we can write the last equation as

$$[H^+] = \frac{k[HAc]}{\alpha \times [NaAc]} \quad . \quad . \quad . \quad . \quad . \quad (3)$$

That is, the hydrogen ion concentration in such a mixture depends on the ratio of free acid to salt ; the higher the salt content the lower the hydrogen ion concentration. It will be seen that dilution of such a solution will have very little effect on the *p*H value, as both [HAc] and [NaAc] are altered to the same extent, the ratio being the

same. The only effect of dilution is to increase the value of α slightly as there is slightly more dissociation in dilute solutions than in concentrated ones ; hence dilution of a buffer solution will decrease the hydrogen ion concentration very slightly, that is the *p*H value will become very slightly higher.

If a strong acid such as hydrochloric acid is added to a buffer solution the hydrogen ions to which it gives rise will immediately combine with acetate ions to give undissociated acetic acid, and consequently there will be but a slight change of hydrogen ion concentration. Similarly, if an alkali such as sodium hydroxide is added, the hydroxyl ions will combine with the hydrogen ions to form water, and once more there will be very little change in hydrogen ion concentration. This perhaps becomes more clear in terms of the acid and conjugate base view. Hydrogen ions resulting from addition of acid to the systems are " neutralised " by the acetate ions acting as a weak base :—

$$H.OH_2^+ + \underset{\text{(acetate ion)}}{A^-} \rightleftharpoons \underset{\text{(acetic acid)}}{HA} + H_2O$$

Whilst hydroxyl ions are " neutralised " by the reaction

$$OH^- + \underset{\text{(acetic acid)}}{HA} \rightleftharpoons H_2O + \underset{\text{(acetate on)}}{A^-}$$

When the buffer is a weak base and one of its salts such as ammonia and ammonium acetate the corresponding reactions are

$$H.OH_2^+ + \underset{\text{(ammonia)}}{NH_3} \rightleftharpoons \underset{\text{(ammonium ion)}}{NH_4^+} + H_2O$$

and

$$OH^- + \underset{\text{(ammonium ion)}}{NH_4^+} \rightleftharpoons H_2O + \underset{\text{(ammonia)}}{NH_3}$$

The better the buffer the more acid or alkali is required to alter the *p*H of the solution by a given amount.

Usually the maximum effect is obtained when equivalent amounts of acid (or base) and its salt are present in solution, at which point the *p*H is equal to the *p*K of the acid or *p*OH (which is the exponential expression for hydroxyl ion concentration exactly analogous to *p*H) is equal to the *p*K of the base. Buffer action is usually restricted to *p*H or *p*OH ranges about one unit above and below the *p*K value. By making mixtures of appropriate quantities of the acid and salt, solutions having various *p*H values within the range can be prepared, according to the formula

$$p\mathrm{H} = p\mathrm{K} + \log \frac{\text{concentration of salt}}{\text{concentration of acid}}$$

The buffer capacity will obviously depend on the concentration of the mixture and must be selected appropriately for the purpose required. Some of the more useful buffer mixtures are shown in Table 1.

TABLE 1

Acid	Salt	*p*H Range
Phthalic acid	Potassium hydrogen phthalate	2·2 — 3·8
Phenylacetic acid	Sodium phenylacetate	3·2 — 4·9
Acetic acid	Sodium acetate	3·6 — 5·6
Potassium hydrogen phthalate.	Dipotassium phthalate	4·0 — 6·2
Sodium dihydrogen phosphate.	Disodium hydrogen phosphate	5·9 — 8·0
Boric acid	Sodium borate (borax)	6·8 — 9·2
Diethylbarbituric acid (Veronal).	Sodium diethylbarbiturate	7·0 — 9·2
Sodium borate	Sodium hydroxide	9·2 — 11·0
Disodium hydrogen phosphate.	Trisodium phosphate	11·0 — 12·0

The phosphate buffers make use of the second and third hydrogen atoms as the acids with the corresponding salts so that phosphate buffer solutions can be made covering a wide range of *p*H values.

So-called "Universal buffer mixtures" consist of a mixture of acids, with *p*K values covering a wide range, to which the calculated amounts of alkali are added to give solutions buffered at the required *p*H. Such a mixture covering the range *p*H 2 to *p*H 12 comprises phosphoric acid, citric acid, boric acid and hydrochloric acid. The same range is also covered by the mixture boric acid, citric acid, potassium dihydrogen phosphate and veronal.

Proteins and amino-acids have a considerable buffering effect since they may function as weak acids or weak bases in virtue of their carboxyl and amino groups, according to the conditions.

Oxidation-Reduction Potentials

It will be seen later that oxidation and reduction play an extremely important part in the respiratory and metabolic processes of micro-organisms. In fact it is not too much to say that their whole existence depends on such reactions, which not only supply the energy for their growth and reproduction but are also involved in the production of the intermediate compounds or "building stones" out of which are synthesised all the complex proteins, fats, carbohydrates, pigments, and so on, making up the body of the organism. As a result of the introduction of means of measuring the intensity of the oxidising or reducing power of substances in recent years the study of oxidation-reduction systems in connection with bacterial metabolism, growth and development has increased rapidly and afforded considerable knowledge of previously obscure processes.

Our first ideas of oxidation naturally involve the addition of oxygen to an atom or a compound, a typical

oxidation being such a reaction as the combustion of carbon to yield carbon dioxide,

$$C + O_2 \longrightarrow CO_2,$$

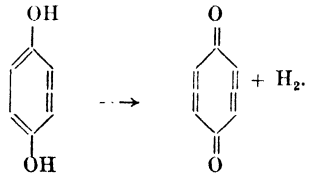
or the combustion of methane to form carbon dioxide and water,

$$CH_4 + 2O_2 \longrightarrow CO_2 + 2H_2O.$$

Besides this type of oxidation by direct addition of oxygen, there is another in which the proportion of oxygen in a compound is increased by removal of some other element such as hydrogen, a typical example being the oxidation of ethyl alcohol to acetaldehyde,

$$CH_3.CH_2OH \longrightarrow CH_3CHO + H_2,$$

or the conversion of hydroquinone to quinone,



Even this does not go far enough, however, and we now recognise that certain reactions in which no oxygen at all is involved are still of the nature of oxidations. This is particularly true where such metals as copper or iron, which have more than one valency, are involved. Thus we regard trivalent ferric salts as more highly oxidised than divalent ferrous salts, even though they may contain no oxygen at all, as is the case with the chlorides. The conversion of ferrous chloride to ferric chloride is an oxidation :—

$$FeCl_2 + Cl \rightleftharpoons FeCl_3.$$

Now these reactions, like all chemical reactions, involve the transfer of electrons from one atom to another. When hydrogen is oxidised to water the single electron which

each atom carried is handed over to the oxygen atom to help it complete its stable octet of electrons. Thus the oxidation of hydrogen means the loss of electrons to oxygen. It will be seen that oxidation of ferrous to ferric chloride also involves loss of an electron from the iron to a chlorine atom. The same thing applies to all oxidations : in every case the oxidised atom loses one or more electrons to some other atom or atoms. It has become evident that every oxidation (or loss of electrons) must necessarily be associated with a gain of those electrons by the other partner in the reactions, that is, every oxidation is accompanied by an equivalent reduction (which is a gain in electrons), and conversely every reduction must have its counterpart in a simultaneous oxidation. The one reaction cannot occur without the other.

This transfer of electrons, which is the inevitable accompaniment of all oxidation - reduction reactions, affords a means whereby the process may be measured by electrical means, since a transfer of the charged electron alters the electrical state of the parts of the system or, in other words, sets up a potential difference between the reactants. The magnitude of this potential difference depends on the ease with which the electrons are lost or gained, the greater the tendency for a movement of electrons (that is, the greater oxidising or reducing power of a substance) the greater will be the potential on one or other side of zero. The more highly oxidised a substance is (that is, the more ready it is to take up electrons) the more positive will be its potential, and the more highly reduced a substance is (that is, the more ready it is to part with electrons) the more negative will be the potential.

Here, too, as in all other reversible reactions, the Law of Mass Action applies, and in general for the reaction :—

$$\text{Reductant} \rightleftharpoons \text{Oxidant} + ne$$

(where "e" represents an electron and "n" the number of

them concerned in the particular reaction) the equilibrium will be expressed by :—

$$\frac{[\text{Oxidant}] \times [e]^n}{[\text{Reductant}]} = k, \text{ or } [e]^n = \frac{[\text{Reductant}] \times k}{[\text{Oxidant}]} \quad . \quad . \quad (4)$$

Obviously the direction in which the reaction will proceed is influenced by the free electrons ; if their number is increased the system will tend to produce more of the reductant ; if they become fewer more oxidant will be formed. Hence if we know the electronic state of the system, we have a measure of its reducing or oxidising power. The electronic state manifests itself in the electrode potentials set up when non-reacting electrodes are introduced into the system, and these potentials can be measured by comparison with standard half-cells. The electrode potentials depend on the transfer of electrons from the solution, in which they are present in concentration [e], to the electrode which can be regarded as having a constant concentration of $[e_m]$. Now the work W required to move an electron from concentration [e] to the metal electrode at concentration $[e_m]$ is equal to that required to transfer a charge of 1 faraday (F) through the potential difference E concerned. That is,

$$W = EF = RT \log \frac{[e_m]}{[e]} \quad . \quad . \quad . \quad . \quad (5)$$

where " log " indicates logarithms to the natural base, R is the gas constant, and T the absolute temperature. Rewriting the equation we get

$$E = \frac{RT}{F} \log [e_m] - \frac{RT}{F} \log [e] \quad . \quad . \quad . \quad (6$$

But since $[e_m]$ is a constant this expression becomes

$$E = K - \frac{RT}{F} \log [e] \quad . \quad . \quad . \quad . \quad (7)$$

and substituting the value of [e] from equation (4) above we get

$$E = \mathrm{K} - \frac{RT}{nF} \log k - \frac{RT}{nF} \log \frac{[\text{Reductant}]}{[\text{Oxidant}]}$$

or

$$E = k_1 - \frac{RT}{nF} \log \frac{[\text{Reductant}]}{[\text{Oxidant}]} \quad . \quad . \quad . \quad . \quad (8)$$

where k_1 is another constant, since $\mathrm{K} - \frac{RT}{nF} \log k$ is a constant for any given temperature and reaction. The electrode potential E can only be measured if it forms one element of a cell of which the other is a standard electrode, the hydrogen electrode being used as such in these cases. The potential referred to the hydrogen electrode as standard is denoted by E_h, and is given by $E_h = E - k_2$ where k_2 is the potential of the standard hydrogen electrode.

Hence

$$E_h = k_1 - \frac{RT}{nF} \log \frac{[\text{Reductant}]}{[\text{Oxidant}]} - k_2$$

or

$$E_h = E_o - \frac{RT}{nF} \log \frac{[\text{Reductant}]}{[\text{Oxidant}]} \quad . \quad . \quad . \quad (9)$$

where $E_0 = k_1 - k_2$, which is a constant for the system.

It follows from a consideration of this equation that the observed oxidation-reduction potential, E_h, depends on E_o, which is a constant for the particular system under consideration, and on the ratio of the concentrations of the reduced and oxidised constituents of the system. The more reduced substance there is present the lower will be the E_h value, and the greater the proportion of oxidised substance the higher will be the potential. When the concentration of the reductant equals that of the oxidant, that is, when the system is half oxidised, it is obvious that $E_h = E_o$, since the ratio $\frac{[\text{Reductant}]}{[\text{Oxidant}]} = 1$ and its logarithm $= 0$. Thus if the potentials of different

systems are compared at half complete oxidation or reduction they can be arranged in order of their oxidising or reducing intensities. A system having a certain value of E_o will oxidise (and be reduced by) all systems having a negative or less positive potential, and in its turn will reduce (or be oxidised by) all systems having a more positive or less negative potential. Alternatively, if we know the value of E_h and of E_o for any system it is possible to calculate its state of oxidation or reduction, *i.e.*, the proportion of oxidised and reduced constituents in it.

Besides the direct electrical method of measuring the electrode potential use is often made of the simpler but less accurate method of employing oxidation-reduction indicators. A number of organic dyes are capable of existence in the oxidised and reduced conditions, the two states being characterised by different colours. The obvious example is methylene blue, which in the oxidised state has a blue colour, but which in the reduced state, known as leuco-methylene blue, is colourless. A considerable number of other dyes, mostly of the indophenol series, are known which undergo a colour change on conversion from the oxidised to the reduced form. These dyes, like other oxidation-reduction systems, have a characteristic range of E_h from the oxidised to the reduced state, the particular E_h value depending on the proportion of oxidised to reduced dye present. The colour, too, will depend on the ratio of oxidised and reduced components, so that an observation of the colour of such a dye will give information of the degree of oxidation or reduction which it has undergone, and consequently of the E_h value of the mixture. The addition of a small quantity of an appropriate dye to an oxidation-reduction system will serve as an indicator of the E_h value obtaining in the system. A range of dyes which cover E_h changes from about $+0{\cdot}4$ volts to $-0{\cdot}01$ volts is available, so that by using a suitable dye an estimate of the oxidising or reducing intensity of a system can be made very readily.

It must be remembered that many organic oxidation-reduction systems, particularly the indicator dye systems, involve weak bases or acids, and accordingly their behaviour and the potentials to which they give rise will depend on the *p*H of the solutions in which they are active. As a result, it is necessary to maintain the *p*H value of the solutions constant by the use of buffers and to record the *p*H value at the time of the measurement in order that the values of E_h may be of significance.

Another important fact which must be borne in mind when considering oxidation-reduction potentials is that E_h is purely a measure of intensity of effect and not of capacity. It gives information as to whether a given substance will oxidise or reduce another substance but not as to how much of the second compound can be oxidised or reduced. In this respect it is analogous to *p*H, which describes the intensity of acidity or alkalinity but gives no indication of how much alkali or acid may be required to alter the *p*H by a given amount. Or, again, E_h is analogous to temperature, which indicates the intensity of heat or cold but gives no clue as to how much heat must be added to or subtracted from a given body in order to alter its temperature to some other value. We know that a body with a high temperature will lose heat to one of lower temperature or *vice versa*, but from a knowledge of the temperatures alone we cannot predict what the final temperature of the pair will be.

The application of oxidation-reduction potentials will be considered in their appropriate places in connection with bacterial respiration and metabolism.

For further information :—

W. M. Clark, "The Determination of Hydrogen Ions," Third Edition London, 1928.

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CHAPTER III

COLLOIDS AND ADSORPTION

AS in all biological happenings, the behaviour of colloids and the phenomena of adsorption play a dominant role in the chemistry of micro-organisms. Not only is this true in the actual life processes occurring within the cell and in the action of enzymes isolated from various bacteria, for instance, and bringing about reactions outside the cell, but the colloidal nature of the substances taking part in all the numerous reactions grouped under the heading immunochemistry is obvious. It suffices to mention that all antigens are colloidal and that the antibodies which they provoke are, if not themselves serum proteins, always carried in the serum and associated with proteins which are colloids. For this reason the properties of colloidal solutions, and particularly those of lyophilic colloids, to which class the proteins and complex carbohydrates belong, are of the greatest importance.

Colloid systems are composed of at least two phases : the **disperse phase,** consisting of very small particles ranging in size from 10 to 200 mμ (μ=one micron=one-thousandth of a millimetre and mμ or milli-micron, sometimes erroneously written $\mu\mu$, equals one-thousandth of μ or one-millionth of a millimetre), which are distributed through the dispersion medium or **continuous phase.** If both continuous and disperse phases are liquids the system is an emulsion ; if a solid is dispersed throughout a liquid the resulting system is known as a suspension. These are the important systems from our point of view,

though others, gas in liquid (foams), liquid in gas (fogs), solid in gas (smokes) and solid in solid are equally important in other fields of work. The particles which constitute the disperse phase may be collections of large numbers of atoms or molecules, as is the case with gold sols and cadmium sulphide sols, or they may consist of single very large molecules or of a comparatively few molecules, which is usually the case with the proteins and polysaccharides (of molecular weight of the order of 15,000 to 100,000 or higher) whose molecules are so large that they fall within the colloidal range of sizes. If the particles of the disperse phase are much larger than 200 mμ they tend to settle out comparatively rapidly under the influence of gravity, whilst if they are much smaller than 10 mμ they cease to behave as colloids and show the properties of crystalloid solutions.

In a stable colloid system the minute particles are prevented from cohering and coagulating by two processes. In the first place they are constantly bombarded by the molecules of the continuous phase which keeps them in the ceaseless zig-zag motion known as Brownian movement, and secondly the particles, as a rule, carry an electric charge which may be positive or negative according to the system. Since all the particles in any one system carry a like charge they tend to repel one another, and so remain uniformly distributed throughout the continuous phase. The charge carried by the particles may be due to the adsorption of ions from the solution, or it may be due to ionisation of the particles themselves or to a combination of both factors.

Colloids can be divided into two large classes, the **lyophobic** (solvent-hating) sols and the **lyophilic** (solvent-loving) sols. The first group contains those systems in which the disperse phase has little if any attraction for the continuous phase. To this group belong most inorganic sols, like gold and the sulphides, and also emulsions of oil in water. They show no tendency

to gelatinise. The lyophilic colloids comprise systems in which the disperse phase has a considerable attraction for the continuous phase. The outstanding examples of this class are the protein and polysaccharide colloids, which are characterised by a tendency to gelatinise or form gels under appropriate conditions.

The lyophobic systems are much less stable than the lyophilic systems ; in other words, lyophobic sols depend almost entirely on their surface charge for stability. This surface charge can be measured by observation of the direction and speed of migration of the particles when submitted to a known potential gradient. It has been found for most lyophobe systems that if the charge or the surface potential of the particles is greater than about $\pm$ 15 millivolts the sol is stable. If the charge is reduced by any means below this critical value the particles tend to aggregate into larger and larger masses until finally they settle out completely. One of the easiest ways of altering the surface charge on the particles is to add an electrolyte to the sol. The electrolyte will dissociate into positively and negatively charged ions ; a negatively charged sol like gold or collodion will adsorb the positively charged metallic ions with a progressive neutralisation of the charge on the sol particles. If sufficient electrolyte is added to reduce the surface charge below the critical value the sol will coagulate. Since the ions of monovalent metals, like sodium, carry a less charge than those of divalent metals, like calcium, and these less than the ions of trivalent metals, such as lanthanum, it is necessary to add more of a sodium salt than of a calcium salt and more of a calcium salt than of a lanthanum salt to have the same coagulating effect on a given sol. For example, a certain collodion sol was found to be equally effectively coagulated by N/2-NaCl, by N/16-$CaCl_2$ and by N/680-$LaCl_3$. In the case of positively charged sols, like ferric hydroxide, it is the negatively charged anion which is adsorbed on to the sol particles and is important in

bringing about coagulation by the same mechanism of lowering the surface charge below the critical value. Again the same valency rule applies, the higher the valency of the ion the greater is its effect, except in the cases of hydrogen and hydroxyl ions which for some reason are much more effective than other ions.

Similar coagulation can be brought about by adding a positively charged colloid to a negatively charged one. When the positively charged colloid is present in sufficient quantity to lower the surface charge on the particles of

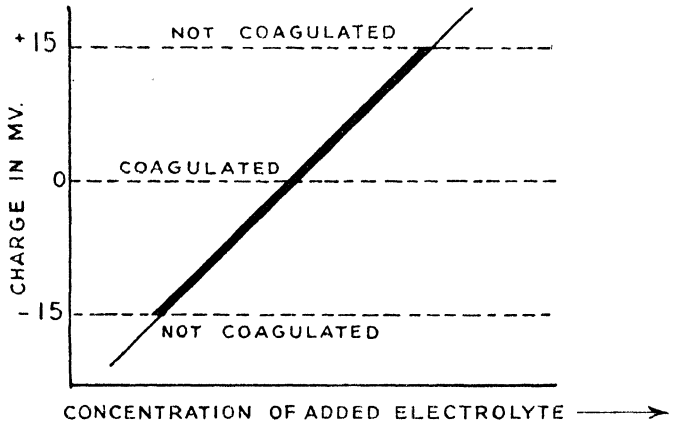


FIG. 4

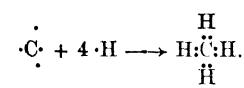
the other below the critical value coagulation occurs. If addition of the positively charged colloid is continued a time will come when the charge on the particles becomes greater than the critical value on the positive side, and the system will no longer coagulate but will remain dispersed; that is coagulation will only occur within a zone of concentration of the added colloid, the limits of the zone being between the concentrations of added colloid necessary to keep the surface charge between the positive and negative critical values (see Fig. 4). The

same effect is also shown when electrolytes are added to colloids, the coagulation only taking place within a zone of concentration, the critical value being overshot by addition of an excess of the electrolyte with consequent adsorption of the oppositely charged ion.

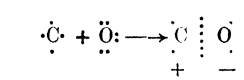
Lyophilic colloids are much less sensitive to the action of electrolytes than are lyophobic sols. This is due to the fact that the particles of a lyophilic colloid have a strong attraction for the continuous phase, usually water in systems of bacteriological interest, in which they are suspended. As a result of this attraction the particles of the colloid become covered by a layer of water which acts as a buffer between them and hinders their collision and consequent aggregation even when the surface charge is well below the critical value for a lyophobe system. In other words, lyophilic colloids have a lower critical surface potential than the lyophobes. It varies from ± 2 to ± 8 millivolts, according to the particular colloid concerned. Accordingly, considerably more electrolyte (or oppositely charged colloid) is needed to alter the charge on a lyophilic sol sufficiently to cause coagulation.

Polar Groups.—The lyophilic character of proteins and polysaccharides and similar substances is due to the presence of polar groups. As is well known, the combination of atoms to form molecules is brought about through the agency of the valency electrons. In the case of such ionisable compounds as sodium chloride the bond is effected by the complete transfer of an electron from the sodium atom, which has one more electron than its stable octet, to the chlorine atom, which has one electron less than its stable octet, whereby both atoms attain the stable octet structure. But in the process the sodium atom acquires a positive charge by the loss of the negative electron, whilst the chlorine atom becomes negatively charged by the gain of the

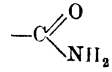
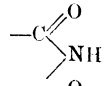
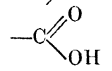
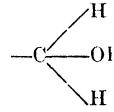
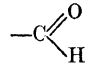
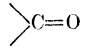
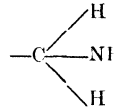
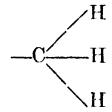
same electron, and the two ionised atoms are held together in sodium chloride by their opposite charges. In the case of non-ionised compounds, such as the majority of organic compounds, the bond is formed by the sharing of a pair of electrons between the atoms, one electron of each pair being supplied by each atom to give a co-valent bond :—



By this sharing of electrons the carbon atom acquires, in effect, the stable octet structure and the hydrogen atoms have two electrons each, as in the inert gas, helium. A double bond is formed by the sharing of two pairs of electrons and a triple bond by the sharing of three pairs. Since there is no actual transfer of electrons, the molecules are neutral and uncharged ; but one of the atoms may have a stronger pull on the electron pair than its neighbour, and accordingly the electrons will be displaced to some extent from the equilibrium position so that the atom to which they are more strongly attracted will have a relatively greater negative charge than the atom from which they tend to be pulled away. As a result, a group of atoms in which this occurs will act as if it were a minute magnet with two poles, e.g.

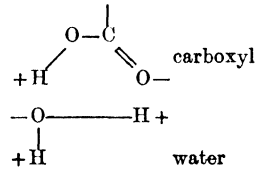


Such groups are known as polar groups. The most commonly occurring of such polar groups are those which involve oxygen, especially doubly linked oxygen, nitrogen or halogens. The polar strength of the groups varies considerably from group to group, but is approximately constant for any one group. As examples of such groups, in order of their strength, may be mentioned

Amide		13,200
Peptide		10,600
Carboxyl		9,000
Alcohol		7,250
Aldehyde		4,700
Ketone		4,300
Amine		3,500
Methyl		1,800

The nitro group,—NO_2, is also polar, and is of approximately the same strength as the hydroxyl group as it occurs in alcohols. The numerical values given, which indicate the comparative strength of the groups, represent the energy (in calories per gram molecule) required to separate the molecules from one another and are calculated from the heat of evaporation of compounds containing them. The more strongly polar the group the greater is the attraction between molecules containing the group, and in consequence the greater the energy required to separate the molecules from one another on conversion from the liquid to the vapour state.

Water is a polar molecule, the oxygen atom being negatively and the hydrogen atoms positively charged. When a compound containing a polar group, such as a carboxyl group, is introduced into it, the polar group of the water will be attracted to that of the compound and water will associate itself with the compound :—



If the polar group of the compound is strong compared with the rest of the molecule (the non-polar part) the substance will be soluble in water, as is acetic acid ; but if the non-polar " tail " is long the polar " heads " will be attracted to and held in the water, leaving the tail projecting out from the surface and making a film arranged in an orderly manner on the surface of the water. If a compound has only very weak polar groups, or no polar groups at all, as is the case with the paraffins, it will be insoluble in water, and its molecules will lie higgledy-piggledy on the surface without forming an orderly film.

The proteins which make up bacterial protoplasm, or which form the colloidal carriers of enzymes, or which occur in serum and take part in the various immunological reactions, possess considerable numbers of strong polar groups, principally the peptide linkages, —CO.NH—, the carboxyl group, —COOH, and the amino groups, —NH_2. In virtue of these strong polar groups they have a strong affinity for water, and when they are in solution they are surrounded by films of adsorbed water, which lowers their critical surface potential and so renders them much more stable and less liable to be coagulated than the lyophobic colloids. The polysaccharides are

lyophilic because they contain a high proportion of polar hydroxyl groups, —OH, and oxygen links, —O—.

Adsorption.—It is the attractive forces between these polar groups in neighbouring molecules which are largely responsible for holding groups of molecules together, as, for example, in protein or cellulose fibres. They also account in very large measure for adsorption phenomena, and particularly for specific adsorption. Large molecules, like those of proteins, will have definite distribution "patterns" of polar groups according to the amino-acids of which they are built up, and depending on the order in which the amino-acids are arranged in the molecule. Other molecules which may have a distribution of polar groups giving a "pattern" corresponding to that on the protein molecule will be adsorbed; if the polar patterns do not correspond there will be less and less adsorption or weaker and weaker adsorption as the patterns differ more and more from one another. This offers a physical explanation for the well-known "lock and key" simile which Emil Fischer suggested to explain enzyme specificity and which Ehrlich used of antibody specificity.

Now if the polar groups on a protein are in close proximity to those on the second adsorbed molecule, which may be represented diagrammatically thus:—

$$\begin{matrix} - & + & - & - & + \\ + & - & + & + & - \end{matrix}$$

, the charges due to the polar groups will neutralise one another, to a certain extent at least, if not completely, and the complex of the two molecules will no longer be polar in effect, and as a result will no longer have such an attraction for water molecules. If enough of the polar groups are masked in this way the system will more and more lose its lyophilic character and more and more come to resemble a lyophobic colloid. In this state it will be readily aggregated by metallic ions. If the protein is an antibody and the adsorbed molecule an antigen we get a picture of the sort of thing

that happens in serological reactions such as agglutination or precipitation.

The strength of these polar forces falls off rapidly with the distance from the polar groups, so that they are effectively exerted only if the groups come into close proximity. Unless the distribution of the groups in different molecules corresponds closely, then, there will be little tendency to adsorption. The application of this idea to account for the sharp specificity of many enzyme reactions and of the serological reactions will become obvious when these subjects are developed (Chapters IV and XXIII).

For further reading :—

N. K. Adam, "The Physics and Chemistry of Surfaces." 3rd Edition. Oxford University Press. London, 1941.

E. F. Burton, "The Physical Properties of Colloidal Solutions." Monographs on Physics. Longmans, Green & Co. London, 1916.

H. Freundlich, "Colloid and Capillary Chemistry." Methuen & Co. London, 1926.

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CHAPTER IV

ENZYMES

IT is becoming more and more recognised that enzymes play a predominant part in the life processes of all organisms, large and small. The results of innumerable investigations all go to show that enzymes are concerned in and control the metabolic and respiratory reactions of all living things. The supply of foodstuffs in appropriate form, the supply of energy required for their utilisation, and the synthesis of simple units into the complex compounds characteristic of living entities all fall within the province of enzyme action. It is to the action of enzymes that living cells and tissues owe their ability to perform at low temperatures and with the mildest of reagents a vast number of complicated reactions which so far are beyond the powers of the organic chemist with all the resources of the modern laboratory at his disposal.

In this chapter we shall consider some of the general properties of enzymes before studying the particular effects of certain of them when we come to deal with the respiratory and metabolic activities which they initiate and maintain.

Such fermentations as the production of alcohol from sugar solution, the production of vinegar from alcohol and the formation of lactic acid from lactose in the souring of milk, and such putrefactive processes as the breakdown of plant and animal materials are among the oldest reactions recognised and used by mankind. A long and

bitter controversy raged during the late eighteenth and the nineteenth centuries as to whether or not such fermentations were due to living entities. Berzelius in 1837 put forward the suggestion that the processes were due to catalysts, whilst Liebig considered that putrefaction and similar processes were due to vibrations set up by the disintegration of living cells. It fell to Pasteur, about 1870-75, to show that all these processes were associated with the vital processes of minute living organisms, yeasts, bacteria and fungi, and that if the life of the micro-organism was destroyed (by heating, for instance) then the fermentations were brought to a standstill. Pasteur described the responsible micro-organisms as " organised ferments."

Parallel with this controversy various digestion and breakdown reactions by plant and animal juices and extracts were being described. Substances were isolated from such extracts which could bring about the same reactions in the test-tube. For instance, Planche in 1810 had observed that solutions of guaiacum were turned blue by extracts of certain roots (this is perhaps the first recorded isolation of an enzyme) ; amygdalin had been shown to be hydrolysed by an extract of bitter almonds from which Liebig and Wohler prepared the enzyme emulsin ; Payen and Persoz showed in 1832 that starch was hydrolysed by the enzyme diastase, which they obtained by precipitation of barley malt extracts with alcohol ; the protein degrading preparations pepsin and trypsin had been obtained from the gastric juice and from the pancreatic juice respectively. The behaviour of these and other substances like them was recognised as being similar in many respects (such as ready destruction by heat) to the action of the " organised ferments," and they came to be known as " soluble " or " unorganised ferments." Then followed a long discussion as to whether there was any essential difference between the organised

and the unorganised ferments. The discussion was virtually brought to an end when, in 1897, Buchner ground up yeast with sand, submitted the mixture to high pressure, and obtained a non-living cell-free juice which was capable of converting glucose into alcohol and carbon dioxide in just the same way as the living yeast with which he started. It is as a result of this investigation that the term enzyme (Greek: *en*=in; *zyme*=yeast) has come into being as the general term for all such substances. Since that time many other similar active preparations have been obtained from diverse biological systems, usually by the maceration of the organism or tissue in water and precipitation of the enzyme as an amorphous powder by addition of alcohol or acetone. As a result of this and later work it has become an accepted fact that although enzymes are only produced by the living cell, once they have been so produced the cell is no longer necessary for their action, which can occur quite independently of the life of the cell that brought them into being. Both Pasteur, who maintained that fermentation only occurred if a living organism was concerned, and Liebig, who argued that fermentation could occur in the absence of life, were correct up to a point, but neither went far enough to complete the story. Life is necessary to bring about the formation of the enzyme, but the enzyme may remain active after the death of its parent cell and still cause fermentation.

Enzymes as Catalysts.—Enzymes can be regarded as biochemical, organic catalysts which are produced by living organisms. All living cells contain enzymes of one sort or another, often a large variety of them. A catalyst is a substance which changes the rate of a chemical reaction, usually, although not always, accelerating it; in some cases a catalyst may act by removing some inhibiting factor, thus enabling a reaction to proceed

which otherwise would not do so. The catalyst will, as a rule, influence a reaction between amounts of reagents many thousand times its own weight, and it can generally be recovered unchanged in quantity and constitution at the end of the reaction.

Enzymes are not only catalytic in their action but are often much more active than inorganic catalysts which bring about the same reaction. The enzyme lactase, for example, hydrolyses the disaccharide, lactose, to glucose and galactose many hundreds of times more rapidly than does twice normal hydrochloric acid where the hydrogen ions act as the catalyst. Inorganic catalysts, such as acid in the instance just described or in the hydrolysis of esters,

$$CH_3CO.OC_2H_5 + H_2O \rightleftharpoons CH_3COOH + C_2H_5OH,$$

do not alter the equilibrium point of the reactions which they catalyse but only shorten the time required to attain the equilibrium condition. That is, the catalyst accelerates both directions of a reversible reaction to an equal extent. Likewise the amount of catalyst has no influence on the final equilibrium quantities of the reagents, but the velocity with which the equilibrium state is reached is proportional to the amount of catalyst. Under strictly controlled conditions this is also generally true of enzymes, although in certain cases enzymes may alter the equilibrium value as well as the velocity of a reaction. This is in all probability due to their colloidal nature and a complete irreversible adsorption of a part of the reactants, with consequent alteration of the active concentrations on which the equilibrium depends. During a reaction enzymes often become partially destroyed or lose some of their activity as a result of side reactions, with a consequent slowing down of the velocity of the reaction, although the final equilibrium reached is unaltered.

Since catalysts and enzymes do not, in general, affect the equilibrium of a reversible reaction they should be able to bring about the formation of, say, an ester from the constituent acid and alcohol as well as influence the hydrolysis of the ester. This is found in practice to be the case. For instance, lipase hydrolyses esters, such as ethyl butyrate, with production of the alcohol and the acid, but if it is allowed to react with a mixture of ethyl alcohol and butyric acid it will catalyse the production of the ester. Similarly the disaccharides maltose and cellobiose have been obtained by the action of maltase and emulsin respectively on glucose solutions ; but the yields of disaccharide are very low, as the equilibrium state is far over on the side of hydrolysis. Polypeptides have also been built up by the action of pepsin on mixtures of peptides.

The Chemical Nature of Enzymes.—The chemical nature of most enzymes is still a mystery. They are all regarded as proteins, or as being protein like, although for a time there was doubt about some of them, such as invertase, peroxidase and lipase, which were very highly purified by Willstätter and his colleagues and then failed to exhibit the biuret, Millon and ninhydrin reactions typical of proteins, although possessing 500 to 20,000 times the activity of the original crude preparations. This failure is very probably due to the fact that the enzyme solutions contained so little of the very highly active enzyme that positive chemical reactions could not be elicited. It may be noted that, generally, these highly purified enzymes are considerably less stable than cruder preparations, in which the impurities seem to have a protective effect. All the crystalline enzymes which have been prepared, listed in Table 2, are either protein in character or contain a protein fraction combined with a prosthetic group.

TABLE 2

Enzyme	Crystallised By
Urease	Sumner
Pepsin	Northrop
Trypsin	Kunitz and Northrop
Chymotrypsin	Kunitz and Northrop
Carboxypeptidase	Anson
Ficin	Watti
Papain	Balls, Lineweaver and Thompson
Ribonuclease	Kunitz
Acetaldehyde reductase	Negelein and Wulff
Catalase	Sumner and Dounce
Amylase	Caldwell, Booker and Sherman
Lysozyme	Abraham and Robinson
"Yellow Enzyme"	Warburg and Theorell
Peroxidase	Theorell

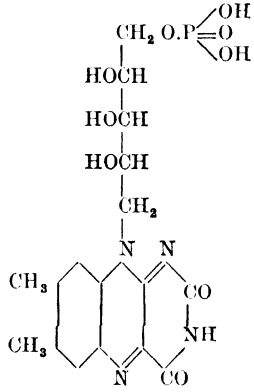
It has been held by many workers that certain metals, notably iron and copper, are essential constituents of enzymes; some enzymes, such as catalase, peroxidase, tyrosinase and ascorbic dehydrogenase, certainly contain these metals, but very active preparations of others have been obtained which are quite free from them.

Catalase can be split, by treatment with dilute acid, to give two inactive fragments, a colloidal protein carrier and the prosthetic group on which the activity of the intact enzyme depends. The prosthetic group is identical with the hæm of hæmoglobin, since if it is coupled with globin from the animal from which the enzyme was derived it yields the hæmoglobin characteristic of that species.

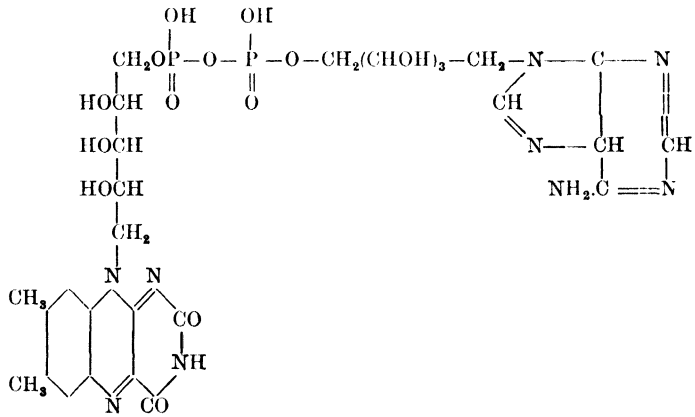
Tyrosinase and ascorbic dehydrogenase contain copper in the form of hæmocyanin, analogous to the iron porphyrin compound hæmoglobin.

The flavo-protein enzymes such as Warburg's "yellow respiratory enzyme," which occurs in bottom yeast and in *Lactobacillus delbrückii* for instance, *d*-amino-oxidase, occurring in liver or kidney, and diaphorase present in

animal tissues and micro-organisms, can also be split by dilute acid to give a specific protein carrier and either riboflavin-5-phosphoric acid from Warburg's enzyme



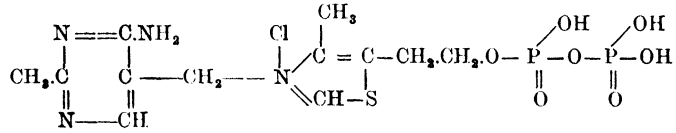
or flavine adenine dinucleotide,



from the others as prosthetic groups. Riboflavin, it should be remembered, is vitamin B_2, and a growth factor for many organisms (see p. 113). The " yellow " enzyme can be reconstituted by allowing synthetic

riboflavin-5-phosphoric acid (or lactoflavin-5-phosphoric acid as it was originally called because of its isolation from milk) to react in neutral solution with the carrier.

The enzyme carboxylase, occurring in yeast, and causing the breakdown of pyruvic acid in alcoholic fermentation (see p. 277) appears to be a complex of aneurin diphosphate, magnesium and a protein carrier. Aneurin diphosphate,



also occurs as the prosthetic group in pyruvic oxidases occurring in *Lactobacillus delbrückii*, *Streptococcus haemolyticus* and gonococci and possibly in the acetic acid bacteria.

So far prosthetic groups have only been detected in the endo-enzymes concerned in respiration and not in the hydrolytic enzymes.

The mechanism by which these enzymes bring about their specific activities will be discussed in connection with bacterial respiration in Chapter XII.

To sum up, we may say that although we know a little about the chemical nature of a very few of the multitudinous enzymes, of the vast majority we know nothing beyond the effects they have and the conditions under which those effects are brought about.

Physical Properties of Enzymes.—Enzymes, in general, are soluble in water and in dilute alcohol, but are precipitated from solution by ammonium sulphate or by high concentrations of alcohol or of acetone. Chemically and physically they are very unstable substances, one or two being so sensitive that even mechanical shaking is sufficient to destroy their activity.

All enzymes appear to be colloidal when in solution in so far as they are unable to diffuse through semi-permeable membranes, and in showing the Tyndall effect when a beam of light is passed through such a solution. Like the proteins, which in fact many enzymes may be, most of them are amphoteric in nature, that is, they may behave either as weak acids or weak bases depending on the acidity or alkalinity of the medium in which they are dissolved. As a result of their colloidal and amphoteric character enzymes are usually active adsorbing agents, and also display their maximum activity at an optimum *p*H value.

Separation of Enzymes.—The majority of enzymes do not diffuse out of intact cells into the surrounding fluid but are held within the cell structure probably by adsorption to various cell constituents. Hence, in order that they may be isolated, the cell system has to be broken down by mild means in order not to destroy the enzyme at the same time. This may be effected by a mechanical process, such as grinding with sand, as did Buchner when he obtained zymase from yeast cells, or by such chemical action as the use of weak alkali or acid ; or the cells may be disintegrated by using solvents like ether, chloroform, toluene, or acetone, but these also remove fatty constituents. The treated cells may then be extracted with water, salt solutions, dilute acids or alkalies, dilute alcohol, glycerol, or some similar agent, depending on circumstances. Having obtained a crude enzyme solution in this way, it may be purified in a variety of ways, the appropriate method depending on the particular enzyme in question. Thus salts, acids and alkalies may be removed by dialysis. The enzyme may be precipitated from solution, usually along with considerable quantities of inactive protein, by alcohol, acetone, ammonium sulphate, or other protein precipitants. A method which has been particularly valuable in the separation and purification of enzymes, especially in the hands of Willstätter and his

school, is that of selective adsorption. It has been found that enzymes, in virtue of their colloidal and amphoteric nature, are readily adsorbed by such materials as kaolin, kieselguhr, charcoal and alumina. By carrying out these adsorptions, using appropriate adsorbants and appropriate conditions of acidity or alkalinity, it has been found possible to adsorb one enzyme and leave others in solution, and then, by altering the *p*H value of the solution in which the adsorbed complex is suspended, to wash out or " elute " the enzyme again and so obtain it free from other enzymes or inactive accompanying substances. Kaolin is negatively charged and will adsorb positively charged basic substances, whilst alumina is positively charged and adsorbs negatively charged acidic substances. It is obvious that by altering the *p*H of the solution the charge on the adsorbant, the enzyme, and the complex of the two can be altered and the adsorption or elution of the enzyme controlled. Thus the enzyme peroxidase, which has basic properties, is adsorbed on kaolin from dilute acid solution, and can then be eluted from the adsorbate (the complex of adsorbant and enzyme) by dilute ammonia. Invertase which, together with maltase, is adsorbed on alumina from acid solution can be selectively eluted by a solution of acid phosphate which does not remove the maltase. Or invertase can be adsorbed on kaolin in acid solution and eluted with dilute sodium hydroxide solution.

The methods adopted for the crystallisation of enzymes usually involve the use of fairly concentrated enzyme in an appropriately buffered solution of a salt at fairly low temperatures. For example, pepsin crystallises when an alkaline solution is brought to *p*H 3 with sulphuric acid. Pepsinogen can be crystallised, after preliminary purification, from 0·4 saturated ammonium sulphate solution at *p*H 6·5. After fractionation of a pancreatic extract by ammonium sulphate, chymotrypsinogen can be obtained as long needles by crystallisation at *p*H 5

from 0·25 saturated ammonium sulphate ; chymotrypsin crystallises from 0·01 N sulphuric acid, whilst trypsin crystallises from solution in 0·5 saturated magnesium sulphate solution in borate buffer at *p*H 9.

The course of purification procedures can only be followed by measurement of the activity of samples of the enzyme at various stages, since no chemical methods of estimating the enzyme as such are available. The enzyme activity is expressed in terms of enzyme units, which define the amount of change which the enzyme can bring about under standardised conditions of concentration of substance acted on, *p*H, temperature, concentration of activators, salt concentration and amount of change. As an example may be quoted Willstätter's invertase unit, which is the amount of enzyme which will reduce the rotation (after addition of alkali to bring about mutarotation of α-glucose) of 4·0 g. of sucrose in 25 ml. of a 1 per cent. solution of NaH_2PO_4 at 15·5° C. to 0° in one minute. The activity of an enzyme preparation is usually quoted as the number of units per gram of dry weight. It is clear that the unit and the necessary set of conditions will differ for each enzyme, but must be rigidly adhered to for any one enzyme.

The Effect of Conditions on Enzyme Action.—(*a*) *Concentration of Enzyme.*—In general the rate of reaction is proportional to the enzyme concentration as long as other conditions such as *p*H are maintained constant by the use of buffer solutions. In cases where deviations from this rule have been observed they have usually been traced to the gradual destruction of the enzyme, or the production of inhibitors or other substances which combine irreversibly with the enzyme and so alter its effective concentration. It is to be emphasized that the equilibrium condition of the reaction is not altered by the presence or by the amount of the enzyme unless the products of the reaction are involved in side reactions resulting in a

change in their effective concentration. It is only the velocity of the reaction which is altered.

(*b*) *Substrate Concentration.*—The term "substrate" is applied to the substance on which the enzyme exercises its catalytic properties. Invertase catalyses the conversion of the substrate sucrose into glucose and fructose. Hydrogen peroxide is the substrate which under the action of the enzyme catalase breaks down into water and oxygen.

With low concentrations of substrate the reaction velocity is in many cases proportional to the substrate concentration for a given concentration of enzyme, but at higher concentrations the rate of reaction rises less rapidly than the concentration. This is very probably due to the saturation of the enzyme surface by adsorption of the substrate to form the hypothetical intermediate compounds which have been postulated in most theories of enzyme action. In some cases, too, the enzyme adsorbs a part of the products of the reaction with a consequent slowing of the rate of reaction. It seems to be a general rule that the oxidising and reducing enzymes are saturated by the substrates at considerably lower concentrations than are the hydrolytic enzymes.

(*c*) *Heat.*—Rise of temperature at first increases the rate of reaction of enzymes in the ordinary way common to all chemical reactions; but at comparatively low temperatures an optimum is reached, and then the activity falls off with further increase of temperature until at about 70° C. the action of most enzymes is stopped, whilst 100° C. is sufficient to inhibit all known enzyme action. It is seen that the effect of temperature is a result of the competition between the acceleration of chemical reactions by rise of temperature and the gradual destruction of the enzyme at higher temperatures. The optimum temperature varies with the particular enzyme concerned, but the majority of enzymes have temperature optima falling between 35° and 45° C. Freezing has no permanent

effect on most enzymes, their activity being merely temporarily inhibited or greatly reduced at low temperatures with recovery to the normal rate on warming up again.

(*d*) *pH.*—The acid or alkaline reaction of the medium in which an enzyme operates has a profound effect on its activity. There is an optimum *p*H value for each enzyme, and any considerable departure from that value results in inactivation of the enzyme. Thus pepsin, the proteolytic enzyme of the stomach, is only active in acid solution with an optimum at *p*H 1·4, whilst the pancreatic enzyme, trypsin, will only hydrolyse proteins in alkaline solution with an optimum between *p*H 8·2 and 8·7. The salivary amylase hydrolyses starch in slightly acid conditions, its optimum being at *p*H 6·7 ; it is completely inhibited by the acid conditions of the stomach in which pepsin is most active. The variation of the activity of proteolytic enzymes with change in *p*H value in many cases appears to run parallel with the calculated dissociation curve of the protein substrate if it is assumed, for instance, that pepsin reacts with the acid cation, and that trypsin reacts with the basic anion of the protein. Papain, with an optimum at *p*H 7·0, appears to attack the undissociated protein molecule. It is for this reason that the *p*H for optimum activity of an enzyme varies somewhat from substrate to substrate, the optimum corresponding with the *p*H required for maximum ionisation in the right direction. In these cases the active part of the enzyme appears to be the un-ionised part.

(*e*) *Activators.*—Often an inorganic or organic activator is necessary before an enzyme can bring about its normal effect. Thus papain, a proteolytic enzyme found in melon seeds, must be activated by hydrocyanic acid or hydrogen sulphide before it will attack peptones, although more complex proteins are hydrolysed by it in the absence of the activator. The animal amylase, ptyalin of saliva, will only hydrolyse starch when chlorine ions are present ;

if all the mineral constituents of the enzyme preparation and of the starch are removed by dialysis no hydrolysis will occur when the solutions are mixed, but immediately a certain amount of sodium chloride is added to the mixture breakdown of the starch commences. Many oxidation enzymes, or oxidases, require manganese ions as activator. Trypsin, as it is obtained from the pancreas, will only hydrolyse the partially degraded proteins, protamines and peptones ; in order that it may attack the complex parent proteins it must be activated by another enzyme, enterokinase, which is to be found in the intestinal juice. Enterokinase probably owes its activating effect to the conversion of the pro-enzyme, trypsinogen, into trypsin. A number of activators, for example, the heat stable co-enzyme in yeast juice (about which we shall say more when we deal with alcoholic fermentation) function by forming an essential link in a chain of reactions (see p. 267).

(*f*) *Inhibition.*—Many heavy metals have the power of inhibiting enzyme activity. Thus mercury salts "paralyse" the hydrolysis of sucrose by invertase, and barium salts inhibit the breakdown of urea by urease. This action is probably the result of the adsorption of the metal on to the enzyme with a consequent blocking of the adsorption of the substrate. Removal of the metal by dialysis, for instance, or by appropriate chemical means restores the activity of the enzyme, no permanent harm having been done to it. Anæsthetics like chloroform and urethane inhibit dehydrogenase activity, whilst cyanides, carbon monoxide and sulphides inhibit oxidases, a fact which has had an important bearing in the sorting out of respiratory mechanisms. Certain organic bases, such as amines, will inhibit the action of invertase, but their effect can be annulled by the action of aldehydes.

It is possible that a closer study of the inhibiting action of compounds or chemical groups on different types of enzymes may throw considerable light on the

nature of the groups in enzymes which are responsible for their activity.

Some substances of known or unknown constitution may act as specific inhibitors of certain enzymes, and such substances have been called anti-enzymes. Thus normal serum contains an anti-trypsin which prevents its activity; this anti-trypsin is possibly a polypeptide which combines with trypsin to the exclusion of its normal substrate. The intestinal wall contains an anti-pepsin by which pepsin is prevented from digesting the tissue proteins. Heparin, a preparation obtained from liver and used to prevent the clotting of blood, is an anti-prothrombin which hinders the clotting of blood in the veins by preventing the interaction of prothrombin and calcium ions to form thrombin. Anti-enzymes in the immunological sense are also known; for instance, if the enzyme amylase is injected into rabbits it produces an anti-amylase which specifically inhibits the action of the enzyme. If malt-amlyase is injected the anti-enzyme inhibits only malt amylase and not the salivary or pancreatic amylases. Similar antibodies specific for urease and ribonuclease have also been prepared. An interesting example is afforded by the α-toxin of *Clostridium welchii* which has been shown to be essentially a lecithinase whose action is specifically inhibited by *Cl. welchii* antitoxin.

The Specificity of Enzymes.—Enzymes differ from the majority of inorganic catalysts in being highly specific in their action, and this is particularly true of the hydrolytic enzymes. Enzymes which hydrolyse proteins will not have any effect on fats or on carbohydrates, nor will carbohyrdate splitting enzymes hydrolyse proteins or fats. The specificity goes even deeper than this; maltase, for instance, will hydrolyse only those sugars which have the same type of linkage between the glucose molecules as occurs in maltose, that is, it will only attack the α-glucose bond. Emulsin, on the other hand, will

only hydrolyse sugars like cellobiose which have β-linkages, or glucosides like amygdalin which also have β-glucose bonds. Invertase attacks only sucrose or the trisaccharides, raffinose or gentianose, which contain the same glucose-fructose unit as sucrose. It has not the slightest effect on maltose, cellobiose or lactose. The acids (inorganic catalysts), on the other hand, attack all these sugars at approximately the same rate, and, moreover, they also catalyse other hydrolyses such as the breakdown of protein or of esters and fats with equal facility. The proteolytic enzymes besides being specific as a class also show a certain amount of "internal" specificity, although this is not so sharp. Pepsin and trypsin, for instance, are capable of hydrolysing a whole series of proteins, but the peptidases which attack poly- and lower peptides are much more specific in their action, generally speaking only hydrolysing compounds which have common structures or arrangements of amino-acids. The specificity is often sharp enough to distinguish between optical isomers, one isomer (usually the naturally occurring one) being attacked, while the other is not attacked at all or only very slowly.

The lipases, or fat-splitting enzymes, while completely specific as a group, that is, capable of hydrolysing only fats and esters, show a relatively low degree of "internal" specificity. Thus any lipase will hydrolyse almost any fat or ester, but there is a certain amount of relative specificity. For example, liver lipase hydrolyses esters readily but fats only slowly, whilst the pancreatic lipase behaves conversely, hydrolysing fats readily and esters slowly.

Enzymes which bring about other types of reaction than hydrolysis are, in general, less specific than the hydrolytic enzymes. They usually catalyse the same type of reaction, oxidation, dehydrogenation, and so on, for a whole range of substrates, which only need to have

in common the possibility of undergoing the change which the particular enzyme effects.

The Classification of Enzymes.—Since, for the most part, enzymes are not well-defined chemical entities they cannot be named as compounds in accordance with the normal chemical usage, but they are named by what they do, their specificity being made the basis of the usual classification and nomenclature. An enzyme is normally named by affixing the syllable " -ase " to the root of the name of the substrate on which it acts or to the type of reaction which it catalyses. For example, the enzyme which hydrolyses invert sugar is called invertase ; enzymes which break up esters are known as esterases, those acting on proteins are proteases, those on carbohydrates are carbohydrases. Enzymes influencing oxidation and reduction reactions are called oxidases and reductases respectively. Some names, such as pepsin and trypsin, given to enzymes in the past and which have become generally accepted are still retained, although they do not conform to the general system.

For a number of enzymes, particularly those involved in respiratory processes, the nature of the prosthetic group is known, and they are sometimes classified on this basis. Thus catalase and peroxidases are porphyrin-protein enzymes because their prosthetic groups contain iron porphyrin complexes. The pyridino-protein enzymes are those which involve the di- or tri-phosphopyridine nucleotides, co-enzymes I and II, attached to specific protein carriers. The flavoprotein enzymes, in which the prosthetic group is riboflavin (see p. 42) are concerned with the oxidation and reduction cycles of the co-enzymes I and II. The copper-protein enzymes, such as tyrosinase and ascorbic oxidase, contain hæmocyanin. Carboxylase, which carries aneurin as its prosthetic group, is a thiamino-protein enzyme.

There are two large groups of enzymes important in the chemistry of micro-organisms. The **Hydrolases**

comprise all those enzymes which bring about hydrolytic reactions of various sorts. They are further divided into the carbohydrases, the proteases, the lipases (attacking fats), esterases, amidases, and so on. Their main function is the breaking down of complex food materials, proteins, polysaccharides and fats, into simpler units readily utilisable by the organism for its nutrition. Generally speaking, their action involves only very small energy changes. Since their action is on more or less non-diffusible substrates they would be virtually useless if they were retained within the cell, so Nature has decreed that the hydrolases as a class shall be secreted into the medium outside the cell; they are extra-cellular or Exo-enzymes.

The other large group of enzymes comprises those involved in the processes of respiration and metabolism. They are known as **Desmolases.** Most of the reactions with which they are concerned involve considerable energy changes and, in fact, it is their function to supply the energy requirements of the cell. To this group belong the oxidases and reductases, zymase (the system of enzymes in yeast responsible for alcoholic fermentation), catalase and other enzymes involved in anærobic fermentation. Their activity would not benefit the cell if it were carried on outside its confines, and normally such enzymes are held within the cell and are not liberated into the surrounding medium unless the cell becomes damaged. These enzymes are Endo-enzymes.

Theories of Enzyme Action.—At present our ideas as to the mode of action of enzymes are somewhat nebulous, but depend, as is to be expected, on our conception of the mechanism of catalysis in general. Catalysts may act in two ways, either reacting chemically to give unstable intermediate compounds which then break down with formation of the end product and setting free the catalyst again (as is the case with the oxides of nitrogen in the manufacture of sulphuric acid), or the catalyst may act

as a carrier, increasing the active concentration of one or more of the reactants, which is probably the mechanism of the catalytic hardening of oils by hydrogenation in presence of nickel. Most theories of enzyme action involve the formation of an intermediate complex between substrate and enzyme, but the type of compound formed and its mode of formation are as numerous as the theories. Michaelis, for instance, considers that the enzyme an substrate are in homogeneous solution, and that the union between them is an ionic reaction. Bayliss, on the other hand, believed that the substrate is specifically adsorbed on to the enzyme surface and that a chemical reaction then takes place at the surface, resulting in the conversion of the substrate into the end product. Fodor and Abderhalden regard the adsorption as being non-specific, but consider that a specific decomposition of the adsorbate occurs. Willstätter suggests that the enzyme has a specifically reactive group, the prosthetic group, which is stabilised on a colloidal (usually protein) carrier. More recently Quastel and his co-workers have developed Wieland's ideas of hydrogen activation as the cause of oxidation to account for the behaviour of bacterial oxidation and reduction enzymes. They regard an enzyme as being an active centre of high energy in a cell surface caused by the interplay of the affinities of neighbouring molecules. The active centre is believed to exert a specific power of adsorption on the substrate and to activate it by distorting its electronic system, rendering the adsorbed molecule of substrate unstable and capable of undergoing the chemical change characteristic of the enzyme. Thus an enzyme is considered to be a property of the surface, but to be specific because of the groupings involved.

For further reading :—

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CHAPTER V

THE CHEMICAL COMPOSITION OF BACTERIA, YEASTS AND THE LOWER FUNGI

THE problem of the composition of micro-organisms can be approached in two ways: either by the purely qualitative method of microscopical examination after appropriate selective staining or by chemical methods of isolation, which may be made quantitative as well as qualitative.

As examples of the microscopical method may be quoted the use of osmic acid which stains fats; the blue colour given by starch and the red-brown colour by glycogen with iodine, and the blue colour given by cellulose in presence of zinc chloriodide. Certain dyes, too, are selective in their action; Sudan III, for instance, dyes fat globules red but leaves unstained other portions of the cell; the nucleoprotein of metachromatic or volutin granules is stained selectively by such nuclear stains as polychrome methylene blue. These methods are of value in showing the distribution of the constituents in the cell, particularly in the case of the larger cells, such as those of yeasts, but their use is obviously attended with great difficulty when they are applied to such minute cells as those of bacteria in which, generally speaking, details of internal structure are not easily visible. The microscopical methods also suffer from the drawback that they only identify groups of substances, and usually do not distinguish between the members of such groups. Thus Sudan III stains all fats alike, and gives no clue as to the particular sort of fat in a given organism.

The chemical methods afford a means of separating the various constituents from one another and allow

their individual investigation. Since the organisms are so very small it is essential to grow them in large quantities in order that appreciable amounts of their constituents may be obtained.

The usual methods are (1) to wash off the growth from solid media, or (2) to separate the cells from a liquid medium by means of a centrifuge or by filtration. Most species of bacteria or yeasts grow well on the surface of appropriate nutrient media rendered solid by the addition of agar, from which the cells may be scraped or washed. The mycelia of the moulds or lower fungi, however, frequently grow into such solid media and resist removal. Centrifugalisation is the simplest and quickest means of separating the growth of yeasts or bacteria from liquid cultures, and the deposit of cells can easily be washed free from the soluble constituents of the medium. Bacteria, particularly, are not easy to recover by filtration, since they are far too small to be retained by ordinary filter papers, and if in any quantity soon clog the pores of a porcelain filter candle. The moulds are normally easy to obtain by filtration since they form a compact mass of mycelium.

The cells of the micro-organism, having been obtained free from extraneous substances derived from the medium, can be submitted to analysis in bulk to determine the water content and the amount and nature of the mineral constituents. Usually the organisms are submitted to a fractionation in order to isolate the various types of substance present. For instance, the bacterial gums may be dissolved out in water; fats and waxes may be extracted with such solvents as alcohol, ether, chloroform or acetone; nucleic acids are extracted with weakly alkaline buffer solutions; the basic proteins can be dissolved in acid buffer solutions.

The results of such analyses of micro-organisms vary very considerably, from organism to organism, with the conditions of growth and age of the organism and with

the methods used for isolation and estimation of the particular component under consideration. The values given, therefore, can be regarded only as indications of the general make up of the cells.

Water Content.—The water content of micro-organisms is usually determined by observing the loss in weight on drying at 100° to 110° C. in the air or at lower temperatures in a vacuum oven. The values for bacteria vary with the species, ranging from 73·3 per cent. in the case of *Escherichia coli* to 98·3 per cent. for *Acetobacter aceti*, the organism commonly known as "mother of vinegar." The majority of values fall between 75 and 85 per cent. A certain amount of variation is to be expected as the result of the differing amounts of water adsorbed by different bacteria; capsulated and mucilaginous organisms will naturally retain more water than such bacteria as *Esch. coli*.

The yeasts also have a varying water content, from 69·2 to 83 per cent., according to various reports. The average value lies at about 75 per cent. The moulds seem not to vary so much in the amount of water they contain, the values recorded falling between 84·3 and 88·7 per cent.

Spores seem to contain very much less water, of the order of 40 to 50 per cent.

Mineral Constituents.—The ash or mineral content of micro-organisms is usually estimated by incineration. The total ash content of bacteria, yeasts and fungi varies considerably with the species, and for any one species with the conditions under which it is grown. Different investigators give values varying from 2 to 30 per cent. for bacteria, 3·8 to 7·0 per cent. for yeasts, and 6·0 to 12·2 per cent. for fungi.

The chief constituents of the ash are phosphorus, sodium, potassium, magnesium, calcium, silicon and sulphur together with chlorine as chlorides. The outstanding feature of the ash content is the high proportion

of phosphorus, especially in the acid-fast bacteria. The ash of most bacteria contains 10 to 45 per cent. of phosphorus, that of the acid-fast bacteria 43 to 74 per cent. and of yeasts 47 to 59·4 per cent. The ash of yeasts also has a particularly high potassium content, averaging 38 per cent.

Proteins.—Estimation of the protein content of micro-organisms is usually based on the total nitrogen content as determined by the Kjeldahl method, the nitrogen value being multiplied by the factor 6·25. There are several fallacies in this method. First of all the Kjeldahl method estimates only about 85 per cent. of the total nitrogen present, since the nitrogen of certain types of compound (nitro-, nitroso-, azo- or azoxy-compounds and such ring compounds as pyrimidines and purines) is not capable of estimation by this method. Secondly, it is assumed that all the nitrogen is present as protein, which is not the case. Nor is it true that all proteins contain 16 per cent. of nitrogen, the value on which the conversion factor 6·25 is based; 16 per cent. is only an average value.

The values recorded for nitrogen are 2 to 14 per cent. for bacteria, 5 to 12 per cent. for yeasts and 2·3 to 8·3 per cent. for moulds, corresponding to approximate protein contents of 12·5 to 87·5 per cent., 32 to 75 per cent. and 14 to 52 per cent. respectively. The carbon content of each of the three groups of organism lies between 45 and 55 per cent., and assuming that all the nitrogen is present as protein, and deducting the corresponding amount of carbon, it can be seen that the bacteria are relatively rich in proteins, whilst the moulds are richer in the non-protein carbon compounds.

Most of the protein of micro-organisms is, of course, found in the protoplasm of the cells. This protoplasm is not homogeneous, however, as most cells contain granules of nuclear material; in fact, some bacterial protoplasm seems to be entirely composed of nuclein

as judged by its ability to stain with those aniline dyes which stain chromatin in the higher plants. The nitrogenous constituents of protoplasm fall into three groups : (*a*) simple proteins, (*b*) amino-acids derived by the breakdown of proteins and (*c*) the nucleoproteins.

The bacteria and yeasts appear to contain proteins of the globulin and albumin types if solubility in buffer solutions of known *p*H and the concentrations of ammonium sulphate required to precipitate the fractions are used as criteria. Globulin is precipitated from solution by the addition of ammonium sulphate to 50 per cent. of saturation. Albumin is soluble in this concentration of ammonium sulphate, but is precipitated when solutions are saturated with the salt. Globulin can be separated into euglobulin, which is insoluble in distilled water, and pseudo-globulin which is soluble. Partially degraded proteins, protamines, peptones and polypeptides are also present, as well as such conjugated proteins as glycoproteins, phosphoproteins, lecithoproteins and nucleoproteins.

The amino-acids obtained on hydrolysis of bacterial and yeast proteins include all the common ones found in proteins from other sources. The amino-acids proline, phenylalanine and tyrosine appear to be absent from the proteins of moulds.

The nucleoproteins, which are all soluble in dilute alkali and which are precipitated from such solution by acid, constitute about 2 to 3 per cent. of the dry weight of bacteria. On hydrolysis those of the tubercle bacillus give a mixture of the pyrimidine and purine types of nucleic acid, but those of most other bacteria give the purine type only. The yeast nucleoproteins are like those of the tubercle bacillus in yielding both types of nucleic acid. The nucleoproteins of the moulds appear to have escaped examination. The metachromatic granules or volutin found in many bacteria, yeasts and moulds (particularly in the diphtheria bacillus) appear to be

nucleic acids as such and not nucleoproteins, since they give nucleic acid staining reactions and are not digested by trypsin and pepsin.

The proteins and nucleoproteins will be considered in more detail in Chapter XVIII.

Carbohydrates.—Estimates of the carbohydrate content of micro-organisms are not very accurate and vary from 12 per cent. in some water bacilli to about 28 per cent. in the diphtheria and tubercle bacilli. The yeasts may contain from 27 to 63 per cent. and the moulds 7·8 to 40 per cent.

The cell membranes of bacteria have been claimed by certain workers to contain cellulose, but this has never been satisfactorily proved except in the case of *Acetobacter xylinum*, which synthesises quite large yields of cellulose from a variety of sugars. Cellulose appears not to occur in yeasts or moulds.

Less complex polysaccharides, in the sense of smaller molecules though not necessarily from a chemical point of view, are very common constituents of nearly all micro-organisms. The obvious examples are the " soluble specific substances " so characteristic of many species of bacteria, the capsules and gums of other species, the glycogen of yeast and the polysaccharides of many moulds. These polysaccharides may be built up from glucose, galactose, mannose, fructose, pentoses, glycuronic acids or mixtures of these units, as will be seen when their study is resumed in Chapter XIX.

The presence of chitin, a polysaccharide built up of glucosamine units, in bacteria and yeasts is still very doubtful, although its presence in the cell wall of many moulds seems to be established quite definitely.

Polysaccharides are found combined with protein in the mucoproteins which constitute the capsules of many bacterial species.

" Reserve carbohydrates " are found in many bacteria and yeasts and have been given various names and

ascribed various structures. The glycogen of yeast is the most well known and satisfactorily proved of these; it is also said to occur in many bacteria. There is still controversy as to whether the "granulose" of such anaerobic bacteria as *Clostridium butyricum* (*Granulobacter butylicum*) is true starch or not, in spite of giving a blue colour with iodine and being hydrolysed by amylase. The amount of these polysaccharides (starch, glycogen, granulose, iogen, etc.) varies with the age of the cell and the amount of nutrient material available; their quantity falls off if the organism is starved and immediately rises again if the cells are transferred to a rich medium.

Simpler carbohydrates also occur in micro-organisms. Thus trehalose, a non-reducing disaccharide composed of two glucose units, is to be found in yeast and many moulds, up to 4·5 per cent. of *Aspergillus niger* consisting of this substance. Pentoses occur in the nucleoproteins of micro-organisms.

The hexahydric alcohol, mannitol, is common in moulds, especially in species of *Aspergillus* and *Penicillium*, and in some bacteria.

Lipoids.—The lipoids comprise all those compounds which are soluble in the so-called "fat solvents," ether, alcohol, acetone, chloroform and light petroleum. The fats, waxes and phosphatides and certain of their breakdown products, such as the fatty acids, are thus included in this group. As mentioned earlier the fat droplets in micro-organisms can be stained in various ways, black by osmic acid, red by Sudan III, blue by α- or β-naphthol and dimethyl-*p*-phenylenediamine in weak alkaline solution, or yellow by dimethyl amido-azobenzene.

The total lipoid content is usually regarded as corresponding to the material extracted by ether, which may vary from 1·6 per cent. of the dry weight of *Corynebacterium diphtheriæ* to 41 per cent. of *Mycobacterium tuberculosis*. In yeasts the content varies from 2 to 5

per cent. for young actively growing cultures to 15 per cent. for old cultures. Certain yeasts, however, particularly *Torula lipofera* and *Endomyces vernalis* can produce up to 60 per cent. of fat under favourable conditions; they have been used as a source of fats when more usual supplies have failed due to war conditions, for instance. The fungi have lipoid contents from 4 to 41·5 per cent.

As with other substances the lipoid content varies with the conditions of growth of the organism. Media rich in glycerol or sugar yield organisms with a higher fat content than do media containing but little sugar. Aeration usually increases the fat content.

The Fats.—The fats appear to serve the purpose of reserve material in many species, although the high fat content of old yeast cells and of partially poisoned cells of other types has led to the view that fat production may be a sign of degeneration.

Waxes and Higher Alcohols.—In view of the exceptionally high lipoid content of the tubercle bacillus this organism has naturally been the most studied as regards such products. As will be seen later (Chapter XX), this and other acid-fast organisms contain a variety of waxes and alcohols. The diphtheria bacillus also yields waxes, which have not yet been investigated chemically.

Sterols.—It has been claimed that the unsaponifiable matter of the fat of some bacteria contains mixtures of sterols in small amounts. They are quite common in the yeasts, forming up to 20 per cent. of yeast fat. Yeast is a commercial source of the sterol, ergosterol, used in the manufacture of calciferol and synthetic vitamin-D. Certain fungi also contain sterols, ergosterol being found free and as the ester with palmitic acid.

Phosphatides.—The phosphatides precipitated from the "ether extract" by acetone are widely distributed in micro-organisms, and, in fact, are probably present to more or less extent in all of them. The non-acid fast

organisms contain 0·5 to 2·0 per cent. of phosphatides, whilst the tubercle bacillus contains about 6·5 per cent. Yeasts also contain a high proportion of phosphatides.

Pigments.—Many species of bacteria, yeasts, and fungi are pigmented; the fungi nearly all contain pigment in some form, but the majority of bacteria, on the other hand, show only minimal pigmentation. Beijerinck has classified the pigment producing micro-organisms according to the site of the occurrence of the pigment: *Chromophoric* organisms contain the pigment in the protoplasm, as in the case of bacterio-purpurin, the pigment of certain sulphur bacteria. *Parachromophoric* organisms carry the pigment in some other part of the cell than the protoplasm, usually in the cell wall or in the capsule. As examples may be quoted some blue bacteria, the moulds and species of *Torula* (coloured yeasts). *Chromoparous* organisms excrete the pigment as such into the medium, as does *Serratia marcescens* (*B. prodigiosus*); or a colourless leuco-compound may be excreted and this may become oxidised in the medium to the coloured compound, which is the case with the green pigment of *Pseudomonas aeruginosa* (*B. pyocyaneus*). The mould *Helminthosporium gramineum* produces a pigment, helminthosporin, which sheathes the mycelium with crystals.

In comparatively few cases has the constitution of these pigments been worked out, but in general they are either carotenoid pigments or melanins.

The pigments will be considered further in Chapter XXI.

Coloured colonies of certain bacteria are produced for diagnostic purposes by the use of special media. This may be illustrated by the production of the pink colonies of lactose fermenting organisms on MacConkey's medium, and by the black colonies of the diphtheria bacillus on McLeod's tellurite medium, in which cases a product of the metabolism of the organism reacts with a substance

added to the medium for that purpose. Such colour production is not, of course, pigment formation in the true sense of the term, but an artificial chemical test applied by the bacteriologist.

Growth Substances.—Certain bacteria, yeasts and moulds produce substances which accelerate the growth either of themselves or of other micro-organisms. Of these "bios" is perhaps the best as well as the oldest known of such substances. Of recent years many such "growth substances" or "growth factors" have been discovered. They will be discussed in Chapter IX.

Vitamins.—Yeast is a rich source of the vitamin-B complex, and it has been claimed that *Esch. coli*, *Bacillus subtilis*, *B. mycoides*, *Torula rosea* and *Oospora lactis* can synthesise it. Bacteria and yeasts do not appear to produce vitamins -A, -C or -D to any marked extent, but certain of the lower fungi of the genera *Aspergillus* and *Penicillium* produce a strongly reducing substance which gives the chemical reactions of vitamin-C (ascorbic acid). Many of the growth factors are vitamins.

Antibiotics.—Many micro-organisms produce substances which have an inhibitory effect on the growth of other micro-organisms. The name antibiotic has been applied to such substances, among which penicillin is an outstanding example. They comprise compounds of a very wide range of constitution. They will be considered in some detail in Chapter XI.

The composition of bacteria, yeasts and fungi, as regards their main constituents, is summarised in Table 3.

So far comparatively little appears to be known about the composition of the viruses. Evidence is accumulating, however, that there is a range of viruses of increasing complexity from the crystalline proteins of the tobacco mosaic viruses to those like vaccinia and the influenza viruses which have a composition similar to that of bacteria.

TABLE 3

	Bacteria.	Yeasts.	Fungi.
	Per Cent.	Per Cent.	Per Cent.
Water	73·3 to 98·3	69·2 to 83·0	84·3 to 88·7
Ash	2·0 ,, 30·0	3·8 ,, 7·0	6·0 ,, 12·2
P (as P_2O_5 in ash)	10·0 ,, 74·0	45·0 ,, 59·4	...
Carbon	45·0 ,, 55·0	45·0 ,, 55·0	45·0 ,, 55·0
Nitrogen	2·0 ,, 14·0	5·0 ,, 12·0	2·3 ,, 8·3
Protein	12·5 ,, 87·5	32·0 ,, 75·0	14·0 ,, 52·0
Carbohydrate	12·0 ,, 28·0	27·0 ,, 63·0	7·8 ,, 40·0
Total lipoid	1·6 ,, 41·0	2·0 ,, 15·0	4·0 ,, 41·5

Much detailed information on the composition of the cell constituents of micro-organisms is collected in :—

R. E. Buchanan and E. I. Fulmer, "Physiology and Biochemistry of Bacteria," Vol. I., Chapter III. Baillière, Tindall & Cox. London, 1928.

CHAPER VI

THE NUTRITION OF THE AUTOTROPHIC BACTERIA

THE autotrophic bacteria are those which thrive on the simplest of inorganic compounds as sources of energy, carbon and nitrogen. Like plants they are independent of other organic matter for their growth. They derive their carbon from carbon dioxide and their nitrogen from ammonia, nitrates or nitrites. The energy necessary for their growth and reproduction is obtained in one of two ways. The **photosynthetic** autotrophs utilise radiant energy from the sun. The **chemosynthetic** autotrophs are able to grow in the dark and obtain the energy required for the assimilation of carbon and nitrogen by means of certain simple chemical reactions.

The autotrophs and the metabolically closely related blue-green algæ and unicellular green plants may be grouped as shown in Table 4 (p. 68).

It has been suggested that, as these organisms use very simple substances for their metabolism, they are the primitive types of bacteria which were first developed on the Earth before more complex organic nutrients were available, and that the bacteria which have more complicated requirements have been gradually evolved from them as a result of changing conditions.

The autotrophic bacteria contain protoplasm and other cell constituents very similar to those found in what we regard as being ordinary bacteria. Obviously, then, they must be capable of very complex synthetic reactions in order to build up such compounds from the simple raw materials carbon dioxide and ammonia.

TABLE 4
(*After Knight*)

	Energy Source.	Carbon Source.	Nitrogen Source.
Photosynthetic—			
Blue-green algæ	$CO_2 + H_2O \xrightarrow{\text{light}} HCHO + O_2$	CO_2	Atmospheric nitrogen
Unicellular green plants	$CO_2 + H_2O \xrightarrow{\text{light}} HCHO + O_2$	CO_2	Ammonia
Green bacteria	$CO_2 + 2H_2S \xrightarrow{\text{light}} HCHO + 2S + H_2O$	CO_2	
Anaerobic purple sulphur bacteria (*Thiorhodaceæ*)	$CO_2 + 2H_2S \xrightarrow{\text{light}} HCHO + 2S + H_2O$	CO_2	Ammonia
Anaerobic purple "non-sulphur" bacteria (*Athiorhodaceæ*)	CO_2+simple organic compounds, or $CO_2 + H_2$	CO_2	Doubtful
Chemosynthetic—			
Aerobic sulphur bacteria	Oxidation of H_2S or thiosulphate	CO_2	Ammonia
Aerobic obligate autotrophs	Oxidation of ammonia, nitrite, $FeCO_3$, $MnCO_3$	CO_2	Ammonia nitrite
Facultative autotrophs	Oxidation of thiosulphate H_2, CO, CH_4, $FeCO_3$. Can also grow on ordinary media.	CO_2	Ammonia nitrate

The autotrophs live and grow in purely mineral media which must contain the oxidisable substance characteristic of the particular organism depending for its existence on the oxidation of that compound. No organic nutrients are required; in fact the obligate autotrophs will not grow on ordinary media containing organic carbon. There are comparatively few obligate autotrophs; the most important among them are the nitrifying bacteria, the purple sulphur bacteria and some of the iron bacteria. The facultative autotrophs, of which there is a greater variety, may derive their energy and growth requirements from the oxidation of inorganic substances, with a corresponding reduction of carbon dioxide to give the starting materials for synthesis, or they may grow on already formed organic substances as a source of carbon. Some of the sulphur and iron bacteria, together with the hydrogen, carbon monoxide and methane

bacteria, are facultative autotrophs. Evidence is accumulating that those organisms which are now regarded as obligate autotrophs may all be capable of growth in the presence of organic matter if the proper conditions can be discovered.

The autotrophic bacteria show a wide variety of morphology, ranging from coccal forms and rod forms to multicellular filamentous forms. The photosynthetic autotrophs contain pigments, for example the bacteriopurpurin of the purple sulphur bacteria, which act as respiratory pigments much like the chlorophyll of green plants.

We can classify the autotrophic bacteria on the basis of their metabolic activities, as follows :—

A. Oxidise Nitrogen Compounds.

1. Ammonia to nitrite, e.g. *Nitrosomonas*, *Nitrosococcus*.
2. Nitrite to nitrate, e.g. *Nitrobacter*.

B. Oxidise Sulphur or Sulphur Compounds.

1. Simple bacteria, e.g. *Thiobacillus*.
   - (*a*) Obligate autotrophs—
     - (i) Aerobic
       - (*α*) Nearly neutral conditions, e.g. *Th. thioparus*.
       - (*β*) Acid conditions, e.g. *Th. thio-oxidans*.
     - (ii) Anaerobic, e.g., *Th. denitrificans*.
   - (*b*) Facultative autotrophs.
2. Higher bacteria (complex morphology).
   - (*a*) Colourless, e.g. *Beggiatoa*, *Thiothrix*, etc.
   - (*b*) Red or purple pigmented, e.g. *Thiocystis*, etc.

C. Oxidise Ferrous or Manganous Compounds (Iron Bacteria).

1. Simple bacteria—
   (*a*) Long sheathed filaments, e.g. *Didymohelix* (*Gallionella*).
   (*b*) Coccoid masses, e.g. *Sideromonas*.
2. Filamentous bacteria, e.g. *Leptothrix*, *Crenothrix*.

D. Hydrogen Bacteria, *Hydrogenomonas*.

We will now consider some of these in more detail.

The Nitrifying Organisms.—Winogradsky has contributed much of our knowledge of these organisms. They are divided into two groups, those which oxidise ammonia to nitrite (*Nitrosomonas*) and those which oxidise nitrite to nitrate (*Nitrobacter*). A species has been reported which can oxidise ammonia directly to nitrate and which can use nitrate as a source of nitrogen. They are strict autotrophs and must have ammonia or nitrite, as the case may be, for their continued existence. *Nitrosomonas* has an optimum *p*H of 8·3 to 8·8 for oxidation of ammonia to nitrite. *Nitrobacter* oxidises nitrite to nitrate between *p*H 8·3 and 9·3. The presence of organic substances, when tested *in vitro*, inhibits their growth and respiration. They are aerobic, non-sporing cocci and short rods which occur almost universally in soils. The nitrifying organisms have a considerable agricultural importance in that between them they are largely responsible for maintaining the supply of nitrate used in plant metabolism.

The Sulphur Bacteria.—The sulphur bacteria form a very heterogeneous group. They include obligate and facultative autotrophs and may be aerobes, facultative anaerobes or strict anaerobes. There are two important aerobic obligate autotrophs. One of these, *Th. thioparus*, was isolated from soil as small non-sporing rods by

Nathansohn. It grows under nearly neutral conditions and oxidises thiosulphate, tetrathionate, or sulphides with separation of sulphur, which is deposited outside the cells. For example, thiosulphate is oxidised as follows :—

$$2Na_2S_2O_3 + O_2 \longrightarrow 2Na_2SO_4 + 2S,$$

and tetrathionate probably by the reaction :—

$$Na_2S_4O_6 + Na_2CO_3 + O \longrightarrow 2Na_2SO_4 + CO_2 + 2S.$$

The second type, *Th. thio-oxidans*, was found by Waksman in soils in the neighbourhood of sulphur deposits, but is not ordinarily found in soils which have not been treated with sulphur. *Th. thio-oxidans* produces large quantities of sulphuric acid, and in fact only grows in somewhat strongly acid conditions at *p*H 2 to 3, corresponding to 5 to 10 per cent. of sulphuric acid. It performs this remarkable function by the direct oxidation of sulphur or thiosulphate :—

$$2S + 3O_2 + 2H_2O \longrightarrow 2H_2SO_4$$

$$Na_2S_2O_3 + H_2O + 2O_2 \longrightarrow Na_2SO_4 + H_2SO_4$$

Sulphur is taken into the cell prior to oxidation by solution in fat globules situated at the ends of the organisms. The oxidation of sulphur, by which energy is provided, can take place in the absence of carbon dioxide, and the latter can be assimilated in the absence of sulphur oxidation, either aerobically when no free sulphur is available, or anaerobically when oxidation cannot occur, provided that the products of the latter reaction are available in the cell. That is, there must be some " accumulator " mechanism in the cell by which energy is stored, to be used in the subsequent metabolism of carbon dioxide. It has been shown that the oxidation of sulphur is coupled with the conversion of inorganic phosphate from the medium into organic phosphate esters in the cells, and that when carbon dioxide is utilised,

the esters break down with liberation of inorganic phosphate. As sulphur can be oxidised for long periods in the absence of carbon dioxide and with only a limited amount of inorganic phosphate it is probable that the latter is involved in a cycle of reactions, as in alcoholic fermentation by yeasts (see Chapter XV). It is considered that the energy provided by the oxidation of sulphur is used in the synthesis of a storage carbohydrate which can subsequently be broken down again, *via* phosphorylation, during carbon dioxide utilisation. The phosphate esters which have been isolated from *Th. thio-oxidans* include adenosine-3-triphosphate (not adenosine-5-triphosphate which occurs in muscle, yeasts *Esch. coli*, *B. subtilis*, *Staph. aureus* and *Ps. fluorescens*), fructose-1:6-diphosphate, glucose-6-phosphate, glucose-1-phosphate and co-enzyme I (see Chapter XV). This suggests that the internal carbon metabolism of the autotrophs is similar to that of the heterotrophs. The reactions may be expressed as :—

(1) $2S + 3O_2 + 2H_2O$ + inorg. phosphate $\longrightarrow 2H_2SO_4$ + phosphate ester.

(2) $CO_2 + 2H_2$ + phosphate ester $\longrightarrow (CH_2O) + H_2O$ + inorg. phosphate.

The symbol (CH_2O) does not necessarily represent formaldehyde but may be a carbohydrate.

The anaerobic organism, *Th. denitrificans*, is an obligate autotroph which oxidises sulphur, hydrogen sulphide, thiosulphate or tetrathionate to sulphuric acid at the expense of the oxygen of nitrates. For example thiosulphate may be oxidised as follows :—

$$5Na_2S_2O_3 + 8KNO_3 + 2NaHCO_3 \longrightarrow 6Na_2SO_4 + 4K_2SO_4 + 4N_2 + 2CO_2 + H_2O$$

It occurs widely in soils, water and mud, from which it was isolated by Beijerinck.

The morphologically more complex sulphur bacteria, including the purple pigmented, photosynthetic *Thiocystis* and the colourless, chemosynthetic *Thiothrix* and *Beggiatoa*, are characterised by the presence of globules

of sulphur within their cells. They are obligate autotrophs which require hydrogen sulphide for their growth. The hydrogen sulphide is oxidised in two stages, first to sulphur and then to sulphate :—

$$H_2S + O \longrightarrow H_2O + S$$

$$2S + 3O_2 + 2H_2O \longrightarrow 2H_2SO_4.$$

As long as hydrogen sulphide is available the organisms contain globules of sulphur, but as soon as the supply fails the sulphur is oxidised, and on its complete disappearance the cell dies. The free access of oxygen and carbon dioxide is necessary for the growth of the colourless organisms.

The purple bacteria contain the pigment bacteriopurpurin, which is a mixture of two components, one a green chlorophyll-like pigment, bacteriochlorin, and the other a red carotenoid pigment, bacterioerythrin. The bacteriochlorin apparently is the active pigment in the respiration of these organisms, behaving, like the chlorophyll in plants, as a sort of transformer for radiant energy. That is, these bacteria require light as well as carbon dioxide and hydrogen sulphide, but can dispense with free oxygen. It seems possible that the light energy is needed in the reduction of carbon dioxide with formation of, probably, formaldehyde, which is used in the synthetic reactions, and of oxygen, which oxidises the hydrogen sulphide :—

$$CO_2 + 2H_2S \longrightarrow HCHO + H_2O + 2S.$$

These sulphur bacteria are found in fresh and salt water and the mud of lakes and rivers, but not in soils.

The sulphur oxidising bacteria play an important rôle in nature in rendering the sulphur of proteins available again to plants as sulphate. They may also be of value in neutralising alkaline soils by the production of sulphuric acid, and probably also convert insoluble phosphates into soluble, available salts. On the other hand, it is possible

that they are responsible, in part at least, for the decay of stonework and concrete.

The Iron Bacteria.—The iron bacteria are found associated with deposits of ferric hydroxide around mineral springs, mines and similar localities. The deposit is usually in the form of a sheath round chains of rods which thus acquire a filamentous form, or it may occur as a sheath around true filamentous forms. Some of these iron bacteria are obligate autotrophs, for example *Didymohelix ferruginea*, and some facultative autotrophs, such as *Leptothrix crassa*. The reaction by which they derive their energy is probably :—

$$4FeCO_3 + O_2 + 6H_2O \longrightarrow 4Fe(OH)_3 + 4CO_2.$$

In certain cases the iron may be replaced by manganese.

The Hydrogen Bacteria.—There are a number of hydrogen oxidising bacteria occurring in such places as canal mud or swamps and in soils where large amounts of hydrogen are produced by anaerobic processes. The hydrogen bacteria are usually facultative autotrophs which oxidise hydrogen to water in the presence of carbon dioxide, but they can also utilise organic compounds. For example a member of the photosynthetic "sulphur free" purple bacteria (*Athiorhodaceæ*) is known which can oxidise simple alcohols in presence of carbon dioxide with formation of the corresponding ketone and reduction of the carbon dioxide to give cell substances. Thus isopropanol is oxidised to acetone :—

$$2CH_3.CHOH.CH_3 + CO_2 \longrightarrow 2CH_3CO.CH_3 + (CH_2O) + H_2O.$$

The oxidation of hydrogen very probably does not proceed directly to water but through the intervention of carbon dioxide with formation of formaldehyde :—

$$H_2CO_3 + 2H_2 \longrightarrow HCHO + 2H_2O.$$

Part of this formaldehyde is used in the synthetic

reactions accompanying growth and part is oxidised to carbonic acid :—

$$HCHO + O_2 \longrightarrow H_2CO_3$$

The mechanism

$$2H_2 + CO_2 \longrightarrow HCHO + H_2O$$

has also been suggested.

Some strains will only grow in symbiosis with one another, for example when one of the pair needs the pyrimidine moiety of aneurin and the other the thiazole moiety. (See Chapter IX). Pure cultures of each may be grown if small amounts of the growth factors are provided.

The Carbon Monoxide and Methane Bacteria.—Strictly speaking these are not autotrophic bacteria since they can utilise the carbon of their substrates for their growth. They are best regarded as intermediate types between the autotrophs and the heterotrophic bacteria. An organism, *Carboxydomonas oligocarbophila*, which oxidises carbon monoxide to carbon dioxide was isolated by Beijerinck from soil. It is a facultative autotroph which exists as a filamentous actinomyces-like organism when grown in carbon monoxide, but exhibiting a coccal form when grown in the presence of organic compounds.

The methane-oxidising organism, *Methanomonas methanica*, was isolated from the mud of canals and marshes by Söhngen. It oxidises methane, but not other hydrocarbons, to carbon dioxide and water :—

$$CH_4 + 2O_2 \longrightarrow CO_2 + 2H_2O.$$

Other hydrocarbon-utilising organisms are known which, although not autotrophs, may be mentioned here as forming part of the transition group between them and the heterotrophs. They are *Methanomonas aliphatica*, *Meth. aliphatica liquefaciens* and " Paraffin Bakterien," which utilise paraffins, including methane in the case of the two former, as their source of carbon and energy. They can also grow on ordinary media. It has been

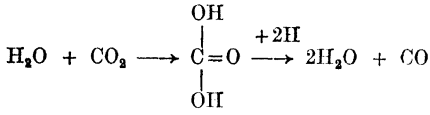
claimed that *Meth. aliphatica liquefaciens* can behave as a true autotroph and live by the oxidation of hydrogen in presence of carbon dioxide as well as on paraffins.

Sarcina methanica decomposes methanol, CH_3OH, in the presence of carbon dioxide with formation of methane. If carbon dioxide containing radioactive carbon is used, the methane is also found to contain radioactive carbon; that is the methane is produced by reduction of the carbon dioxide. *Methanobacterium omelianski* similarly oxidises primary and secondary alcohols to the corresponding fatty acids with simultaneous reduction of carbon dioxide to methane. Neither formate, methanol, nitrate, sulphate nor atmospheric oxygen can replace carbon dioxide as the oxidising agent. It is, therefore, considered that formate and methanol are not intermediate products. It has been shown by the use of radioactive carbon dioxide and carbon balance sheets that most of the carbon of the cell constituents is derived from sources other than the carbon dioxide.

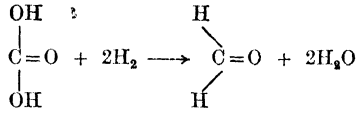
Another group of intermediate organisms comprise the strictly anaerobic purple "sulphur-free" bacteria *Athiorhodaceæ*. They are peculiar in that they are photosynthetic, but differ from the autotrophs in requiring simple fatty acids for their growth as well as carbon dioxide. In the absence of the fatty acids, carbon dioxide is not taken up. It is claimed that when subjected to infra-red radiation they can use hydrogen, that is, they are true autotrophs. The pigment of these organisms consists of two components, one carotenoid and the other chlorophyll-like, photocatalytic, and similar to that in the purple sulphur bacteria.

We know virtually nothing of the way in which the reduction of carbon dioxide, which seems to be an essential factor in the metabolism of the autotrophs, occurs. It has been suggested that it may be by one of three routes :—

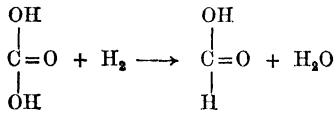
(*a*) *via* carbon monoxide,



(*b*) *via* formaldehyde,



(*c*) *via* formic acid,



By analogy with plant metabolism it would be expected that the second method, *via* formaldehyde, is the most probable. Support is lent to this view in that formaldehyde can be fixed as an insoluble complex with dimedon (see Chapter XV) in the cases of *Nitrosomonas* and an autotrophic sulphur oxidising organism.

Van Niel has suggested that all photosynthetic reactions in which carbon dioxide is reduced conform to the general equation :—

$$CO_2 + 2H_2A \xrightarrow{\text{light}} (CH_2O) + 2A + H_2O.$$

In the case of green plants H_2A is water and oxygen is set free. In photosynthesis by bacteria H_2A may be one of a variety of inorganic or organic substances characteristic of the particular organism. It is assumed that each of the necessary four quanta of light energy is associated with the activation of a hydrogen atom in the pigment and that the carbon dioxide is reduced by the activated pigment which thus becomes re-oxidised.

In order that the pigment may again become a hydrogen donor it must be reduced at the expense of the donor H_2A, with formation of A. The only essentially photosynthetic step is the activation of the reduced pigment. The reduction of carbon dioxide and of the pigment can occur in the dark. Some sulphur bacteria can reduce carbon dioxide in the dark in the presence of hydrogen suggesting that the mechanism of reduction is the same for photosynthetic and chemosynthetic organisms. In chemosynthetic bacteria the hydrogen donor is not a pigment but some other substance whose oxidation provides the necessary energy so that light activation is unnecessary. These autotrophic processes are not restricted to autotrophs since it has been shown that carbon dioxide may be reduced in the dark and fixed by heterotrophic organisms, probably by the same mechanisms as in the chemosynthetic autotrophs, the energy being provided by dissimilation reactions (see Chapter VII).

As mentioned on p. 72, the symbol (CH_2O) is used to indicate the reduction product of carbon dioxide which may or may not be formaldehyde, although the latter is a probable intermediate.

Ruben suggests that the reactions by which *Methanobacterium omelianski* reduces carbon dioxide to methane with simultaneous oxidation of an alcohol to the fatty acid (see p. 76) are as follows :—

$$RH + \text{phosphate donor} \rightleftharpoons \text{Phospho-}RH + \text{donor}$$

$$\text{Phospho-}RH + CO_2 \rightleftharpoons RCOOH + \text{phosphate}$$

$$RCOOH + \text{phosphate donor} \rightleftharpoons R.\overset{\overset{O}{\parallel}}{C}- O- PO_3H_2 + \text{donor}$$

$$R.\overset{\overset{O}{\parallel}}{C}- O- PO_3H_2 + 2H \rightleftharpoons R.CHO + \text{phosphate}$$

$$R.CHO + 6H \rightleftharpoons RH + CH_4 + H_2O$$

The energy and hydrogen yielding reactions are :—

$$2C_2H_5OH \rightleftharpoons 2CH_3CHO + 4H$$

$$2CH_3CHO + 2H_3PO_4 \rightleftharpoons 2CH_3C(=O)-O-PO_3H_2 + 4H$$

$$2CH_3C(=O)-O-PO_3H_2 + 2\ donor \rightleftharpoons 2CH_3COOH + 2\ phosphate\text{—}donor$$

Organic compounds are not only not used by the strict autotrophs but have a definite inhibitory effect on their growth, under artificial conditions at least. Thus it was not until Winogradsky grew the nitrifying organisms on media containing no carbon source other than carbon dioxide that he was able to obtain cultures of them. For solid media he employed silica gel in order to avoid organic substances. The sulphur bacteria and some of the iron bacteria are less sensitive to organic matter and can grow if only low concentrations of carbon compounds are present, especially if large inocula are used. The iron bacterium, *Leptothrix ochracea*, however, is susceptible to peptone, sucrose and asparagine.

The thermodynamic efficiency of the autotrophs is not very high, only about 5 to 10 per cent. of the energy liberated by the oxidation of the inorganic substrate being utilised in the reduction of carbon dioxide to the organic compounds used for synthesis.

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CHAPTER VII

THE NUTRITION OF THE HETEROTROPHIC BACTERIA

WHEN bacteria grow and reproduce there occurs a synthesis of all the many cell constituents, the proteins and nucleoproteins of the protoplasm, polysaccharides, fats, phosphatides and a number of other carbon compounds. The elements, mainly carbon, nitrogen, hydrogen and oxygen, but also phosphorus, sulphur and certain metals in smaller amount, required for these syntheses have to be supplied in an available form by the medium in which the organism is grown.

As would be expected from an analysis of the ash of bacteria, the inorganic constituents which must be supplied are mainly phosphorus, sulphur, sodium, potassium, magnesium, calcium, iron and chlorine. It is probable that the metals needed in only small amount form part of enzyme systems. Iron is an essential part of the cytochrome complex; phosphorus, potassium and magnesium are also intimately involved in respiratory mechanisms (see Chapter XII). *Corynebacterium diphtheriæ*, *Clostridium tetani* and *Cl. welchii* need small amounts of iron in order to produce their toxins.

We have seen that the autotrophic bacteria derive their carbon from carbon dioxide and their nitrogen from ammonia, nitrites or nitrates; accordingly, they must possess a very complete equipment of the enzymes necessary to carry on these syntheses from such simple starting materials. It seems probable that the heterotrophic bacteria, which, in general, require much more complicated sources of carbon and nitrogen, have lost some

of the synthetic power of the autotrophs and depend in more or less degree on preformed organic material for their existence. The degree of dependence varies considerably; organisms like *Esch. coli* can thrive on very simple synthetic media containing a single carbon source, like lactate or glucose, and a single nitrogen source, such as an ammonium salt, together with the appropriate mineral salts. Synthetic media are those which contain only constituents of known composition and no proteins, broth or similar components. Further along the scale are the organisms like the diphtheria bacillus which will grow on synthetic media, but which require a more or less extended number of amino-acids. Some of these amino-acids, for example, tryptophane and cystine, appear to be essential, whilst others can be replaced by alternatives. More exacting still are those organisms like the gonococcus and the influenza bacillus which demand the so-called "enriched" media, containing blood or some tissue fluid or extract, for their growth. Almost certainly these enriched media support growth because of the growth factors (see p. 98) which they contain. Finally, there are the viruses which have so far lost their synthetic powers that they can only live and grow in the presence of living tissue, on which, it seems possible, they depend for their supply of ready-made cell constituents, or at least for materials which are well on the way to being the finished product. The less exacting organisms might be compared with country people who bake their own bread, and the exacting bacteria with town dwellers who have lost the art of making bread and who must buy it ready made.

The simplest heterotrophs, from the nutritional point of view, are those which depend on an organic carbon source but which can still use inorganic nitrogen, either as gaseous nitrogen or as nitrate or as ammonia. The nitrogen-fixing organisms may be free-living, like the *Azotobacter* or symbiotic with plants like the *Rhizobium*.

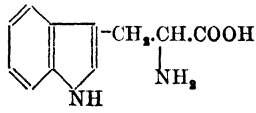
The denitrifying organisms use nitrate as a source of nitrogen. At the next stage are those organisms which cannot fix atmospheric nitrogen but can thrive on ammonium salts; of course, they also require organic compounds as energy source and to supply raw materials for synthesis. An extremely wide range of substances may serve as the sole carbon source for many micro-organisms. Of these carbohydrates and similar compounds are most readily assimilated, whilst hydroxy-acids, fatty acids and monohydric alcohols are progressively less easily utilised. Amino-acids can often serve as both carbon and nitrogen source. Amines are not very satisfactory as carbon sources. Nearly all saprophytic organisms belong to this group; as examples may be mentioned bacteria which can decompose formic acid and methyl alcohol, the genus *Chromobacterium* and the genus *Escherichia*. It is of interest to note, in this connection, that often organisms will not grow in synthetic media if they are sown in only very small numbers, but if a large inoculum is used, growth proceeds vigorously. This may be due to the introduction of essential growth factors, bacterial vitamins, which are absent from the medium but present in sufficient quantity in large inocula to allow growth to commence; once the organism has started it can synthesise sufficient of the growth factor to allow of continued growth. An alternative explanation is that in synthetic media there is not, initially, a sufficiently high concentration of carbon dioxide to permit growth. It has been shown by several workers that carbon dioxide is an essential prerequisite for the growth of many organisms, of which *Esch. coli* is one; again, large inocula carry over sufficient carbon dioxide to allow growth to start. The truth of this explanation is borne out by the fact that *Esch. coli* grows quite regularly from small inocula in synthetic media under aerobic conditions where carbon dioxide is produced by respiration; but under anaerobic conditions, where

practically no carbon dioxide is produced, large inocula are necessary to establish growth. For the satisfactory growth of *Brucella abortus* about 10 per cent. of carbon dioxide is necessary.

It has recently been shown that many heterotrophic organisms, for example yeasts, *Esch. coli* and the propionic acid bacteria, can assimilate carbon dioxide by a mechanism similar to that of autotrophic bacteria (see pp. 77, 78 and Chap. XII).

The next step in the loss of synthetic power by bacteria is probably that of the ability to utilise ammonia as nitrogen source. The nitrogen must be supplied in the form of organic compounds, usually as amino-acids. This would appear to be the step in nitrogen metabolism analogous to the loss of ability to use carbon dioxide in the change from autotrophic to heterotrophic bacteria. Just as there is an intermediate group of organisms between the autotrophs and the heterotrophs, the facultative autotrophs, so there is a group of organisms which can utilise either ammonium salts or amino-acids for their nitrogen supply. An example of this group is *Eberthella typhosa* (*B. typhosus*), which is capable of growth on either source of nitrogen but develops better on amino-acids. Usually strains of any one species in this intermediate group vary in their ability to use ammonium salts. For example, the *Salmonella*, *Proteus*, the dysentery and typhoid bacilli and the *Vibrios*, as a general rule, comprise two types of strains: (*a*) "exacting" strains which cannot utilise ammonium salts but need amino-acids, and (*b*) "non-exacting" strains which will grow on ammonium salts as well as on amino-acids. The "exacting" strains are usually pathogenic. Organisms of this group may be contrasted with those of the coli group and *Serratia marcescens* which can grow on ammonium salts or amino-acids, but of which no "exacting" strains, using only amino-acids, are known.

The amino-acid most often demanded as essential by the "exacting" strains is tryptophane,



β-indole-α-amino-propionic acid. It is one of the most complicated of the amino-acids, so that it is, perhaps, not surprising that it should be the most difficult to synthesise, and accordingly be one of the earliest to be required in a preformed condition. In many cases this lost synthetic power may be restored by "training" the organism by repeated subculture on media containing less and less tryptophane and more and more ammonium salt, until it can grow again in the entire absence of the amino-acid. This so-called "training" may not be a true change in the metabolism of the organism, but may be a concentration, by selection, of a few individual cells in the "exacting" strain which have not lost their synthetic power. Those cells which have lost the power will die out under the adverse conditions, until ultimately only non-exacting organisms are left; the acquisition of the ability to use ammonium salts is, according to this view, only apparent, the power really being present all the time in a small proportion of the bacteria.

Further along the route to complete loss of synthetic power are those organisms which, in addition to needing organic carbon and organic nitrogen in the form of one or more amino-acids, require the so-called "growth factors" or bacterial vitamins, as they are sometimes termed. The best-known organisms in this group are *Staphylococcus aureus, Clostridium sporogenes, Cl. botulinum* and *Lactobacillus casei.* It seems possible, if not probable, that the organisms of the other groups can produce their own vitamins, but that those in the present

group have lost the power of synthesis of both amino-acids and of the vitamins. Many bacterial growth factors are recognised now as being identical with the vitamins which play a large part in animal nutrition. In fact many of the fundamental metabolic reactions of bacteria are identical with, or very similar to, those of animals. They are non-specific in the sense that the *Cl. sporogenes* factor promotes the growth of *Cl. botulinum* and of *Cl. welchii*, and that they are produced by many bacteria having simpler nutritional requirements ; *Esch. coli*, for example, can synthesise the growth factor required by *Staph. aureus* (see Chapter IX).

As with the other changes of nutritional types, here, again, occurs a group of intermediately placed organisms which link those requiring growth factors with those which do not. These intermediate species either exist as two sorts of strain, one requiring the factor and the other not, or they may be trained, with more or less difficulty, to synthesise their own growth factor instead of requiring it ready made. This seems to be the case with the tubercle bacillus and such organisms as *Corynebacterium diphtheriæ*, which, when freshly isolated, require complex media containing growth factors, but can be trained to grow on synthetic media comprising only known carbon and nitrogen sources.

It is possible that the various growth stimulants which have been described for certain organisms are really essential growth factors, but which are produced so slowly by the organism concerned that their addition from an outside source causes an increased growth. If the rate of synthesis of the factor were so slow that its concentration were negligible, it would be regarded as an essential growth factor which must be supplied in the medium to enable growth to occur. If, on the other hand, the organism produced it so fast that adequate growth occurred without the necessity of adding it from outside, neither its stimulating nor essential character

would be recognised. It is probable that most, if not all, organisms require the various growth factors, and that they differ only in their ability to synthesise one or more of them.

Organisms of the *Hæmophilus* group which require two growth factors are probably the most highly evolved of the bacteria from the nutritional standpoint. The *X*-factor, derived from the hæmoglobin of the blood, may not be a generally required factor, although there is some evidence that certain bacteria (for example, *C. xerosis* and *Esch. coli*) may be able to synthesise it from iron compounds. The *V*-factor, which is also present in blood, can be derived from various bacterial and vegetable extracts and appears to be of much more general occurrence ; even bacteria which have very simple nutritional requirements produce it. The various members of the influenza group of bacteria have lost the power of synthesising one or other or both of these factors. *H. canis*, for instance, has lost the capacity to produce the *X*-factor and must be supplied with it, but it can make its own *V*-factor. The hæmolytic influenza bacilli have lost the power of synthesising the *V*-factor, but can do without added *X*-factor ; whilst *H. influenzæ* itself cannot synthesise either the *X*- or *V*-factors and must be supplied with both from an external source. The pneumococcus, meningococcus and gonococcus also belong to this highly evolved group.

Parasitic organisms, particularly pathogenic ones, have much more complex growth requirements than the saprophytic organisms. It seems reasonable to assume that this is because the parasites find in their host a source of nearly all of their needs in a preformed condition and in the course of time have lost the necessary synthetic powers to build up their own requirements. This difference of demands between saprophytic and parasitic organisms is well illustrated by the acid-fast bacteria. The purely saprophytic *Mycobacterium phlei* can grow

freely on ammonium salts and simple carbon compounds, whilst the parasitic *M. tuberculosis* and Johne's bacillus have complex requirements when freshly isolated, but on prolonged culture in the laboratory gradually become less fastidious and able to grow on ordinary or even synthetic media.

Our knowledge of the general nutritional requirements of bacteria has been summed up by Knight, who divides the organisms into four groups with increasing complexity of demands, corresponding to progressive loss of synthetic power, as follows :—

1. Carbon derived from carbon dioxide ; nitrogen from inorganic sources (elementary nitrogen, nitrites, nitrates or ammonia) ; energy from light in the case of photosynthetic autotrophs and from simple inorganic oxidations in the case of chemosynthetic autotrophs.
2. Carbon and energy from organic carbon compounds (carbon dioxide is not the main source of carbon) ; nitrogen from inorganic compounds.
3. Carbon and energy from organic carbon compounds ; nitrogen from amino-acids (some, tryptophane, for example, are in many cases essential) ; ammonium salts are not assimilated.
4. Carbon and energy from organic compounds ; nitrogen from amino-acids, of which a considerable number is usually required. One or more growth factors are also required.

A fifth group, the viruses, may be added to this list :—

5. Live and reproduce only in living tissues ; that is, exhibit an almost complete lack of synthetic powers.

It may be stated that in the last year or two the discovery of saprophytic filter-passing organisms, which can thrive independently of living tissue, has been reported.

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CHAPTER VIII

ADAPTIVE AND CONSTITUTIVE ENZYMES

IN the last chapter the " training " of *Eberthella typhosa* to assimilate ammonium salts instead of amino-acids and of *C. diphtheriæ* and *M. tuberculosis* to grow on synthetic media were cited as examples to show that organisms can be made to grow on an initially unfavourable medium. Another example is the training of certain yeasts to ferment galactose (a power which the majority of yeasts do not possess), whilst the production of lactose fermenting variants of *Esch. coli*, of rhamnose fermenting strains of *Eberth. typhosa* and of sucrose fermenting variants of *Shigella dysenteriæ* probably occur by a similar mechanism. As we have said, it has been suggested by some workers that this might not be due to a true " training " or induction of variants but to a selection of the appropriate strain from a mixed inoculum containing only very small numbers of the " non-exacting " strain, in the case of variation of nutrient requirements, or of those organisms fermenting galactose, etc., in the other cases.

There is evidence, however, that this is not always the correct explanation, although it may be true in some instances. Often there is a considerable lag before the mutant appears, much more than would be expected if organisms of the " trained " type were already present, even in very small numbers. Moreover, in the majority of cases the bacteria concerned were isolated as single colony cultures and repeatedly subcultured and would thus be expected to be pure strains, although it must

be remembered that there may be a continuous production of variants.

An alternative and more likely explanation of these variations is that the organisms produce a new enzyme or series of enzymes, under the stimulus of the changed medium, which enables them to deal with the new nutrient substances provided. Some of these enzymes may be merely capable of breaking down a new sugar, as is the case with galactose trained yeasts or the *Esch. coli* mutants; or they may be responsible for synthetic reactions which were lacking in the parent organism, as occurs when *Eberth. typhosa* is trained to grow on ammonium salts in the absence of tryptophane.

Karström, as a result of growing certain organisms on a variety of media, showed that the bacterial enzymes may be divided into two groups. To the first group belong the

1. **Constitutive Enzymes** which are always produced by a given organism, whatever the medium on which it grows. These enzymes appear to be essential members of the " battery " of enzymes carried by the cell.

The second group comprises the

2. **Adaptive Enzymes** which appear in a given organism as the result of growth on a medium containing the corresponding substrate. These enzymes only appear when their specific substrate is present, and on that account seem not to be essential enzymes.

The constitutive enzymes can be further divided into two sorts: (*a*) those which always appear in approximately the same amount even on a medium from which their particular substrates may be lacking, and (*b*) those which, although always produced to some extent, occur in increased amount when the organism is grown on the specific substrate.

According to Karström the formation of adaptive enzymes is always associated with the life processes of the cells. Dead cells can never give rise to enzymes which were not present in the living cells. If adaptive enzymes appear in a culture which exhibits no growth (for instance, where the growth has been prevented by some particular treatment, as is the case with the "resting" bacteria of Quastel and his co-workers) it must be assumed that such cells, although incapable of cell division in the given circumstances, are not dead but in a state of suspended animation.

We will describe some of Karström's experiments with *Aerobacter aerogenes* (*B. aerogenes*) which he grew on a lactose medium. He separated the cells by centrifugalisation, washed them and suspended them in solutions containing xylose and calcium carbonate (the latter to prevent development of acidity). Four solutions were used containing, besides the cells, xylose and calcium carbonate :—

1. Sodium chloride.
2. Potassium phosphate.
3. Potassium phosphate and yeast water.
4. Potassium phosphate and ammonium sulphate.

The fermentation of xylose by the cells in these solutions was measured by the amount of carbon dioxide evolved, with the results shown in Table 5.

TABLE 5

Time.	Carbon Dioxide Evolved from Solution.			
	1.	2.	3.	4.
hrs.	c.c.	c.c.	c.c.	c.c.
0	0	0	0	0
1	0	0	5	2
13	0	0	52	55
22	0	0	66	76

The enzyme fermenting xylose is adaptive, and since the organism was grown on a lactose medium it did not contain the xylose enzyme. In solutions 1 and 2 there was no growth of the cells (owing to lack of a nitrogen supply), and so xylose was not fermented. In solutions 3 and 4, however, nitrogen was present, growth (or at least synthetic activity) occurred and a delayed fermentation of xylose took place after the necessary enzyme had been elaborated.

In other experiments Karström grew the pentose fermenting lactic acid organism, *Leuconostoc mesenteroides* (*Betacoccus*), on media containing only one of a series of sugars, separated and washed the cells and tested their fermenting ability on other carbohydrates, with the results show in Table 6.

TABLE 6

Grown on	Fermentation of					
	Glucose, Fructose, Mannose.	Galactose.	Arabinose.	Sucrose.	Maltose.	Lactose.
Glucose	+	—	—	—	—	—
Galactose	+	+	—	+	—	—
Arabinose	+	—	+	+	—	—
Sucrose	+	—	—	+	—	—
Maltose	+	—	—	+	+	—
Lactose	+	+	—	+	—	+
No sugar	+	—	—	+	+	—

The glucose, fructose, mannose and sucrose fermenting enzymes are thus seen to be constitutive since they are produced when the organism is grown on any sugar medium, and even on media containing no sugar at all. The other enzymes fermenting galactose, arabinose, maltose and lactose are adaptive since they are only developed in the presence of the appropriate substrate

(except for the maltose enzyme which also appeared in the absence of any carbohydrate). That the galactose enzyme appeared when *L. mesenteroides* was grown on lactose is not surprising since lactose is built up of glucose and galactose units.

Usually the glucose splitting enzymes are constitutive, but an exception is found in the case of the pentose fermenting organism, *Lactobacillus pentoaceticus*, in which the glucose enzyme is adaptive and the xylose and arabinose enzymes constitutive, as may be seen from Table 7 (also due to Karström) :—

TABLE 7

Grown on	Ferments.		
	Arabinose.	Glucose.	Xylose.
Arabinose - -	+	—	+
Glucose - -	+	+	+

An example of the second type of constitutive enzyme, those showing increased production in presence of the substrate, is given by the formation of the sucrose splitting enzyme by *Esch. coli* in considerably greater amount when the organism is grown on sucrose then when it is grown on media containing glucose, maltose or lactose, all of which give rise to the production of some sucrase, however.

Quastel has studied the production of the enzymes catalase, urease and fumarase by the organism *Micrococcus lysodeikticus* in different media. This organism was chosen because it possesses the peculiar property of being very easily lysed by egg-white with consequent liberation of its endo-enzymes into the medium. After liberation

in this way the amount of enzyme could be easily determined. It was found that the presence of glucose in an agar-peptone medium stimulated the production of urease but depressed that of catalase. The presence of urea did not stimulate urease production nor did succinate or fumarate stimulate fumarase production, which is high in the presence of glucose. Quastel considers that the effect of the substrate on enzyme production depends on a balance of factors : (*a*) whether the substrate tends to destroy the enzyme or to protect it from destruction (perhaps by forming a reversible complex with it), and (*b*) whether the substrate has an effect on the synthesis of the enzyme by contributing some necessary molecules or configuration for that synthesis.

Another type of enzyme which is adaptive is the hydrogenlyase, produced by the coli group of organisms when grown in the presence of formate, and which breaks formic acid down to carbon dioxide and hydrogen :—

$$HCOOH \longrightarrow H_2 + CO_2.$$

It is also produced in presence of glucose or glycerol, which yield formic acid as a result of their fermentation. Some specific factor in the medium also appears to be necessary since, generally speaking, the enzyme is only produced when the organism is grown on a tryptic digest of casein. If the bacterium is grown on synthetic media, even one containing formate, no hydrogenlyase is produced in spite of good growth. It is almost certain that in this case the enzyme is not produced as a result of selection of a mutant strain, since the addition of formate to a young growing culture on tryptic casein digest caused the appearance of the enzyme in less than an hour, during which time the number of organisms had increased by only 18 per cent. A maximum production of enzyme occurred in two hours with an increase of the viable count by 34 per cent. In other words a maximum production of hydrogenlyase had occurred before the

organisms had doubled in numbers, which certainly seems to rule out selection as the mechanism in this case. In another experiment washed suspensions of *Esch. coli* were added to a tryptic digest plus formate, and the production of enzyme (as measured by evolution of hydrogen in a Barcroft apparatus) was followed. Initially there was no enzyme, but it began to appear after forty-five minutes and reached a maximum value in 150 minutes, during which time there was less than a 5 per cent. increase in the number of organisms; the hydrogen production in the same time increased by more than a thousandfold.

Other examples of adaptive enzymes are hyaluronidase (the "spreading factor" produced by *Clostridium welchii*) which is only formed when its substrate, hyaluronic acid, is present and the enzyme, formed by a soil organism, which hydrolyses the specific polysaccharide of Type III pneumococcus.

The results of experiments on adaptive and constitutive enzymes depend largely on the time for which the organism is allowed to grow or to remain in the medium. The amounts of some enzymes change considerably with time (that is, with the state of growth of the organism), whilst others remain more or less constant in amount. Thus an organism examined after twelve hours' growth may have quite a different set of enzymes from that which it possesses after, say, seventy-two hours. When grown on a particular substrate an organism usually tends to maintain a normal concentration of the corresponding enzyme for a considerably longer time than it ordinarily does, and to maintain that concentration after the activity of most of the other enzymes has fallen off. Hence the apparent increase in activity of an enzyme in presence of its substrate may not always be due to a true increase in production of the enzyme but to a contrast with the low values of the other enzymes. This, of course, will be particularly marked if old cultures are examined; if the cultures are examined during the

logarithmic phase of growth all the enzymes may be of approximately equal intensity. Undoubtedly, though, in many cases a real stimulation of enzymes does occur under the influence of the specific substrates. The production of enzymes by bacteria is also influenced by substances other than the specific substrate. Thus calcium is necessary for the formation of gelatinase by *Proteus vulgaris* and magnesium for the phosphatases of propionic acid bacteria. The presence or absence of growth substances in the medium and the *p*H of the medium are factors which must be taken into account when considering enzyme synthesis.

Some adaptive enzymes are more easily produced than others. Karström showed that *Esch. coli*, for instance, produced enzymes to deal with mannitol in seventy-five minutes, sucrose in 105 minutes and lactose in 165 minutes. The production of the necessary enzymes in training "exacting" strains is usually much slower.

The production of the galactose fermenting enzyme by yeast is a true adaptation, since it has also been shown to occur in the absence of cell division, which rules out the selection hypothesis. On the other hand, the production of lactose fermenting variants by *Esch. coli mutabile* seems to be a true selection, since it has been shown that such variants are being continuously produced even in the absence of lactose. In this case the effect of lactose in the medium is to provide the most favourable conditions for the growth and identification of the mutants.

The majority of constitutive enzymes are those which bring about the respiratory and synthetic processes of the bacteria, whilst most of the adaptive enzymes are to be found among the hydrolases which break down the more complex nutrient materials to a form suitable for the attack of the constitutive enzymes. Exceptions to this rule are the production of the enzymes which enable "exacting" organisms to become "non-exacting" and

use ammonium salts instead of amino-acids in their synthetic processes.

For further reading;—

R. J. Dubos, "The Adaptive Production of Enzymes by Bacteria." *Bact. Rev.* **4** (1940), 1.

E. F. Gale, "Factors Influencing the Enzymic Activities of Bacteria." *Bact. Rev.* **7** (1943), 139.

B. C. J. G. Knight, "Bacterial Nutrition." Medical Research Council Special Report No. 210. H.M. Stationery Office. London, 1936.

H. Karström, "Formation of Enzymes in Bacteria. I, II." *Suomen Kem.*, **2** (1929), 63; **3** (1930), 42.
"Enzymatische Adaptation bei Mikro-organismen." *Ergebnisse für Enzymforschung.* **7** (1938), 350.

CHAPTER IX

GROWTH FACTORS

PRIOR to the last two or three decades it was considered that an adequate supply of protein, fat and carbohydrates, together with some mineral salts, was all that was necessary for the normal growth and development of animals and man. Then knowledge of the more detailed composition of foodstuffs led to the recognition of the part played in nutrition by minor and formerly unsuspected constituents. The cause of several of what are now called " deficiency diseases " was shown to be a lack of certain essential growth factors in the diets of the afflicted persons. Well-known examples are scurvy, rickets and beri-beri. As the use of diets containing, as far as possible, only known constituents for experimental investigation of vitamins became common more and more such substances were discovered. The stages in the development of our knowledge have usually been first, the recognition of a condition as due to a deficiency of some "essential metabolite," secondly, the discovery of some crude preparation which would supply the lacking factor or vitamin and finally the identification of a chemically defined substance which could replace the crude preparation. Micro-organisms are similar to animals in that they, too, require essential growth factors in addition to the normal sources of carbon, nitrogen, mineral salts and other elements, necessary for the supply of energy and raw materials for growth. These growth factors were sometimes called " bacterial vitamins " by analogy with the vitamins concerned in

animal nutrition and health. It is now becoming evident that many bacterial vitamins are in fact identical with those involved in animal metabolism. Many of these substances, possibly all, are intimately connected with enzyme or co-enzyme systems, often constituting the prosthetic group of such systems, and in other cases serving as an essential intermediate step in a cycle of reactions.

Whereas, formerly, vitamins which were first discovered in connection with animal nutrition were later found to be necessary for the growth of micro-organisms, the position is now rather the reverse. For instance, *p*-aminobenzoic acid, biotin and riboflavin were first investigated in connection with the metabolism of micro-organisms and not until then was their activity in other fields suspected. As the metabolism of the micro-organisms is considerably easier to study than is that of the much more complex animals it is very probable that considerable improvement in our knowledge of general metabolism will result from investigation along such lines.

The investigation of bios affords an example of the way in which knowledge of growth factors evolves.

In 1901 Wildiers showed that yeast would not grow on synthetic media if small inocula were used, but that the introduction of large inocula was followed by satisfactory growth. He demonstrated that the addition of boiled yeast to the synthetic medium permitted the growth of small inocula. He attributed this phenomenon to the presence in yeast of an essential growth promoter which he called bios ; he suggested that small inocula did not contain enough of it to allow growth to start, but large inocula carried sufficient bios into the new medium for growth to occur. He showed that it could be extracted from yeast with water, and that it was soluble in 80 per cent. alcohol but not in absolute alcohol nor in ether. It was stable to heat and moderately stable to

acids, being destroyed by boiling with 20 per cent. sulphuric acid but not by 5 per cent. acid. Boiling with sodium hydroxide solutions stronger than 1 per cent. destroyed bios. It was dialysable through semipermeable membranes.

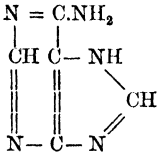
When yeast was shown to be a rich source of the vitamin-B complex it was thought that bios might be identical with it. This has been shown to be nearly, but not entirely, true, the two complexes having many factors in common. It was soon shown that bios was not a single substance but a mixture of several factors. It was first split into bios I and bios II by the action of barium hydroxide solution, which precipitates bios I but not bios II. Neither fraction alone is active, but mixing them restores the potency of the preparation. Bios I has been shown to be optically inactive *meso*-inositol.

Further fractionation has shown that bios contains the following substances :—

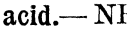
Bios I	*meso*-Inositol
Bios IIa	Pantothenic acid
Bios IIb	Biotin
Bios V	Aneurin
	β-Alanine
	l-Leucine
	Nicotinic acid
	Pyridoxine.

Several other substances of known constitution and some of unknown constitution are also involved in small amounts in metabolism. The known substances include adenine, *p*-aminobenzoic acid, folic acid, hæmatin, phosphopyridine nucleotides, pimelic acid, riboflavin and uracil. Among the substances of unknown composition are the " sporogenes factor " and a fraction from *Mycobacterium phlei* which stimulates the growth of Johne's bacillus.

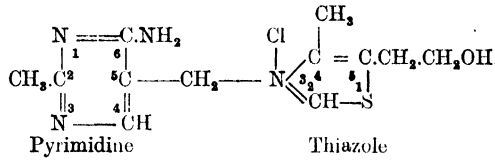
The properties of these growth factors will be considered in more detail.

Adenine.—  It has been shown that adenine is a necessary constituent of media for the growth of *Clostridium tetani*. It can be replaced by hypoxanthine. Very probably adenine is concerned in the synthesis of nucleic acids and diphosphopyridine nucleotide (see p. 202 *et seq.*). It has recently been shown that adenine can inhibit the bacteriostatic action of some of the sulphonamide drugs (see Chapter X).

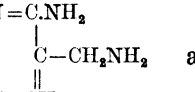
β-Alanine. — $NH_2.CH_2.CH_2.COOH$. *Saccharomyces* species and *Corynebacterium diphtheriæ* need β-alanine as a growth factor in synthetic media. Since it forms a part of the molecule of pantothenic acid it is probably required for its synthesis. It is effective in promoting the growth of *C. diphtheriæ* in concentrations of the order of 1 μg. per millilitre or less. (1 μg.=0·001 mg. Sometimes the symbol γ is used instead of 1 μg.) β-Alanine cannot be replaced by α-alanine. It may be derived by many organisms from asparagine or aspartic acid.

***p*-Aminobenzoic acid.**— NH_2  $COOH$. It was shown by Woods and by Woods and Fildes in 1940 that *p*-aminobenzoic acid was the substance in yeast extracts, peptone and other substances which inhibited the bacteriostatic action of sulphonamide drugs (see Chapter X). It has since been shown to be a growth factor for certain strains of *Neurospora crassa*, *Acetobacter suboxydans* (0·005 μg/ml.), *Clostridium acetobutylicum* (1×10^{-6} μg./ml. in presence of $1{\cdot}5\times10^{-6}$ to $1{\cdot}5\times10^{-4}$ μg./ml. of biotin) and *Cl. butyricum*.

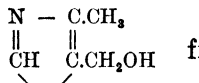
Aneurin.—



Vitamin B_1, aneurin or thiamine was shown to be a constituent of bios by Williams in 1940. It was shown by Knight that the substance required by *Staphylococcus aureus*, in addition to nicotinic acid, and supplied by a gelatin hydrolysate, meat extract or "marmite" (an autolysed yeast preparation) was aneurin. It is active in concentrations of 0·003 μg./ml. It can be replaced by a mixture of the corresponding 2-methyl-6-amino-5-amino-



methyl pyrimidine, and 4-methyl-

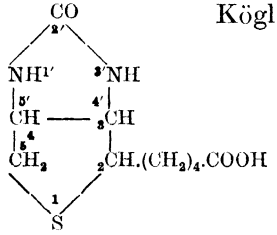


5-β-hydroxyethyl thiazole, fragments but not by differently substituted fragments nor by differently substituted aneurins. It was shown that the pyrimidine moiety attached to an inactive thiazole as in thiochrome (an oxidation product of aneurin) can act as a source of the pyrimidine and that the thiazole moiety attached to an inactive pyrimidine can serve as a source of thiazole.

Aneurin, or a mixture of the two components, has also been shown to be essential for the growth of *Phycomyces blakesleeanus*, of lactic acid bacteria and of propionic acid bacteria. Some protozoa and some parasitic fungi need intact aneurin and will not grow if the separate components are supplied instead. Some organisms need only one component and can synthesise the other. Thus *Mucor rammanianus* can synthesise the pyrimidine but not the thiazole whilst the red yeast, *Rhodotorula*

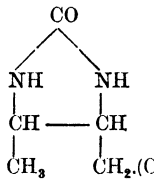
rubra, can synthesise the thiazole but not the pyrimidine part. The two organisms can be grown together in symbiosis, each producing the component needed by the other. Many organisms, of which *Escherichia coli*, *Proteus vulgaris*, *Aerobacter aerogenes*, *Alkaligenes faecalis*, *Bacillus mesentericus* and *Thiobacillus thio-oxidans* are examples, are independent of added aneurin, being capable of synthesising it themselves.

It is highly probable that the aneurin is active in the decarboxylation of pyruvic acid, since aneurin pyrophosphate is known to be co-carboxylase (see Chapter XII).

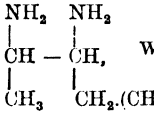
Biotin.—  Kögl isolated biotin in 1935

from egg yolk and from yeast. It occurs in these sources in very minute quantities, 360 tons of yeast or one and a half million eggs being required to yield 1g. of the crystalline substance. It has m.p. 230—231°C. and $[\alpha]_{22}^{D} = +92°$ (in 0·1 N NaOH). It is probably the most active biological substance known at present; a dilution of one part in 10^{10} is sufficient to promote half the maximum growth of yeast. It has been synthesised and the synthetic material is indistinguishable from the natural substance in biological activity. It is inactivated by treatment with nitrous acid, oxidising agents or by benzoylation or acetylation. The methyl ester, however, can be utilised in place of free biotin, but in some cases less readily. By reduction in presence of the Raney nickel catalyst the sulphur atom is eliminated and

replaced by two hydrogen atoms to give desthiobiotin,



which, very surprisingly, is as active as biotin itself. Observable growth of *Saccharomyces cerevisiae* occurs in the presence of a dilution of 1 in 4×10^{11}. Desthiobiotin will not promote the growth of *Lactobacillus casei*. Hydrolysis of desthiobiotin by acid or by alkali yields a diaminopelargonic acid,



which on oxidation gives pimelic acid, $COOH.(CH_2)_5.COOH$. Biotin has been shown to be identical with vitamin H which is protective against "egg white injury," and with "co-enzyme R" which is necessary for the respiration of the nitrogen fixing organisms, *Rhizobium*. The action of biotin is inhibited by avidin, the substance in egg white which is responsible for "egg white injury." Since the activity of desthiobiotin is also inhibited by avidin it seems probable that it is the urea grouping of biotin which combines with avidin.

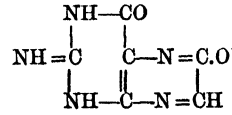
O-heterobiotin, the analogue of biotin in which the sulphur atom is replaced by an oxygen atom, has about half the activity of biotin for *L. casei*, *L. arabinosus* and *Sacch. cerevisiæ*. It, also, is inactivated by avidin.

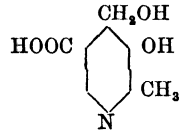
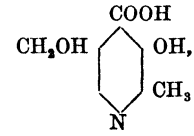
Added biotin is required for the growth of lactobacilli, the propionic acid bacteria, *Cl. acetobutylicum*, *Cl. butylicum*, *Staphylococcus* (0·005 to 0·01 μg./ml.), *Brucella* and hæmolytic streptococci. At present the mechanism of its activity is obscure.

Folic acid.—Williams isolated folic acid from the leaves of spinach and from yeast, liver and kidney. It is possibly identical with, or forms a part of liver

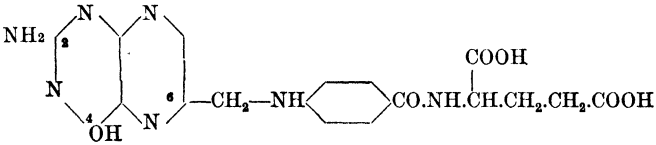
" eluate factor." It occurs in most green leaves, including grass. It contains vitamins B_{10} and B_{11} which are necessary in the growth of chicks and for the production of feathers by chicks respectively. It also increases the growth of rats.

Folic acid is required as a growth factor by many lactic acid bacteria. In a concentration of 0·00012 μg./ml. it gives half its maximum growth effect on *Str. lactis R.* It also stimulates the growth of *L. casei* and *L. delbrückii.* There appear to be at least two factors in folic acid preparations required for bacterial growth because, although an extract from spinach has equal activity in supporting the growth of *Str. lactis* and *L. casei*, extracts from liver and yeast have different values for the two organisms. The difference is due to a factor which is active for *Str. lactis* but not for *L. casei.* The factor has been isolated and it was found that 1μg. of it was equivalent in activity to 56 μg. of a folic acid concentrate towards *Str. lactis* but that 1 μg. was less active than 0·004 μg. of the concentrate for *L. casei.* It differs from an active orange yellow crystalline substance of the composition $C_9H_{10}N_3O_3$, probably identical with vitamin B_c, the chick anti-anæmia factor, which has been obtained from folic acid concentrates. The crystalline material induces half the maximum growth of *L. casei* at a concentration of 0·00005 μg./ml. but is considerably less active for *Str. lactis.* A liver extract stimulating the growth of *L. casei E* can be replaced by orotic acid, uracil-4-carboxylic acid, but not by uracil itself. Orotic acid was at one time thought to be a constituent of folic acid.

Folic acid, vitamin B_c, the *L. casei* factor and xanthopterin,  , are closely related and

probably concerned in the synthesis of thymine,  . Thymine can replace vitamin B_c in the nutrition of *L. casei* but not in that of *Str. lactis R* (identical with *Str. fœcalis*). *L. casei* factor can be partially converted into folic acid by incubation with chick liver; if α- or β-pyracin,  or  is added to the mixture even better conversion occurs.

Folic acid has been shown to have the constitution N-[4-(2-amino-4-hydroxy-6-pteridyl)methyl-aminobenzoyl]-glutamic acid, or pteroylglutamic acid,

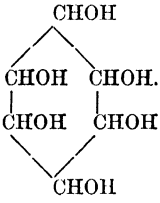


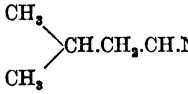
and has been synthesised as bright yellow crystals in 15 per cent. yield from glutamic acid, *p*-aminobenzoyl chloride, propionaldehyde and 2 : 4 : 5-triamino-6-hydroxy-pyrimidine. If the synthesis is carried out using *p*-aminobenzoic acid instead of *p*-aminobenzoyl-L-glutamic acid a compound, pteroic acid, is obtained which promotes the growth of *Str. fœcalis R* but not that of *L. casei* nor of the chick.

Glutamine.—$CH_2(CONH_2).CH_2.CH.NH_2.COOH$. This amino-acid has been shown to be necessary for the initiation of growth of many strains of *Streptococcus*, *Lactobacillus*, *Diplococcus pneumoniæ*, and *B. anthracis*. The organisms appear to be able to synthesise adequate

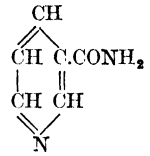
quantities of glutamine once growth has commenced. For some organisms it can be replaced by considerably larger amounts of glutamic acid.

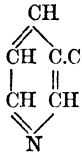
Haematin.—It has been established that hæmatin is the *X*-factor required by members of the genus *Hæmophilus*. It can be replaced by certain inorganic iron compounds which have oxidase or catalase activity. It is, apparently, only necessary for the aerobic growth of these organisms, since anaerobically they can grow in its absence. It is highly probable that hæmatin, or the other iron compounds, are necessary for the synthesis of the cytochrome system which plays an important part in bacterial respiration (see Chapter XII).

***Meso*-Inositol.**—  This substance is essential for the growth of yeasts of the genus *Saccharomyces*. It is noteworthy that it is required in amounts considerably larger than for the majority of growth factors, milligrams rather than micrograms being needed. Its function is still unknown although Eastcott, who isolated it from bios, states that it is stored unchanged in the yeast cell. In animal metabolism it can prevent the development of "fatty liver" which normally arises when there is an excess of cholesterol in the diet. Inositol occurs as the phosphoric ester in the phytin of wheat germ, which interferes with the normal metabolism of calcium in bone formation.

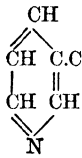
***l*-Leucine.**—  This amino-acid is required by yeast for growth in synthetic media and is a constituent of bios. It is classed as one

of the essential amino-acids for the growth of most bacteria.

Nicotinamide.—  . Knight found that nicotinamide was one of the constituents of meat or yeast extract required for the aerobic growth of *Staph. aureus* on gelatin hydrolysate with the addition of tryptophane, cystine and glucose. It was active at a dilution of 0·05 μg./ml. of medium. Nicotinic acid,



was found to be somewhat less active than the amide. That the activity is not due to an impurity is shown by the fact that pyridine-3-



nitrile, is inactive until it is hydrolysed to the acid. Nicotinamide is also required by Shiga's dysentery bacillus and by *Hæmophilus pertussis*. The ratio of the activity of the amide to that of the acid is not constant but differs for various organisms. Thus for *C. diphtheriæ* the acid is ten times as active as the amide, for *Proteus vulgaris* the two are equally active, for *Staph. aureus* the amide is five times as active as the acid, for *Shigella dysenteriæ* the ratio is ten to one, whilst some *Pasteurella* strains cannot use the acid at all.

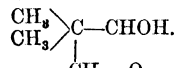
Shigella dysenteriæ requires 0·1 μg./ml. of nicotinamide for growth but higher concentrations have an inhibiting effect. This is probably analagous to the inhibition of enzyme action by an excess of one or more of the products.

Nicotinamide forms part of the molecule of Harden and Young's cozymase or Co-enzyme I, a diphosphopyridine nucleotide, and of Co-enzyme II, a triphosphopyridine nucleotide, which are concerned in carbohydrate metabolism (see Chapter XII). It seems obvious that nicotinamide is required by some organisms for the synthesis of these co-enzymes. Bacteria like *Esch. coli*. *Eberthella typhosa*, and *Vibrio comma* can synthesise the whole co-enzyme, whilst *Staphylococcus* and the dysentery bacilli, for example, cannot synthesise the pyridine moiety. *H. influenzæ* cannot synthesise any part of the co-enzyme which must be supplied intact and cannot be replaced even by adenylic acid (adenine+d-ribose+phosphate, see p. 332). Nicotinamide can be replaced by Co-enzymes I or II.

In America the name niacin has been given to nicotinamide in order to avoid the unfortunate association in the public mind with nicotine. This is important as nicotinamide is of interest in human metabolism, a deficiency of it giving rise to pellagra.

Pantothenic acid.— $\begin{matrix} CH_3 \\ CH_3 \end{matrix}\!\!>\!C(CH_2OH)-CHOH.CO.NH.CH_2.CH_2.COOH.$

Williams isolated pantothenic acid from a number of sources such as yeast, rice bran, milk, liver and egg white by extraction with 80 per cent. methanol. It was shown to be identical with the chick anti-dermatitis factor of liver and to be an important constituent of bios. It is of very widespread occurrence in bacteria, moulds and many plant and animal tissues, a fact which gave rise to its name. It is active in very low concentrations, of the order of 0·008 μg./ml., in stimulating carbohydrate fermenting organisms, but not non-carbohydrate fermenters. Pantothenic acid loses its activity towards some organisms, e.g. *Str. hæmolyticus*, on cleavage,

by acid hydrolysis, into α-hydroxy-β β-dimethyl-γ-butyrolactone,  and β-alanine, $NH_2.CH_2.CH_2.COOH$. This is similar to the failure of some organisms to utilise glutamic acid instead of glutamine, and to the readier use of nicotinamide than of the acid. It appears to be associated with the inability to form amide linkages other than in the α-position, that in pantothenic acid being β- and that in glutamine being γ-.

The effect of pantothenic acid is usually increased by relatively large amounts of *meso*-inositol and by extremely small amounts of biotin.

Active pantothenic acid has been synthesised from its inactive component parts.

Among organisms for which pantothenic acid is a growth factor are the lactic acid bacteria, hæmolytic streptococci, *C. diphtheriæ gravis* and *Proteus morganii*.

The way in which panthothenic acid enters into the metabolism of micro-organisms is not yet understood.

Phosphopyridine Nucleotides. — Diphosphopyridine nucleotide, Co-enzyme I, which is constituted as nicotinamide-ribose-phosphate-phosphate-ribose-adenine, has been shown to be identical with the *V*-factor required by *H. influenzæ* and which is supplied by extracts of many bacteria, yeasts, blood and plant and animal tissues. A mixture of nicotinamide and adenylic acid cannot replace Co-enzyme I in the metabolism of *H. influenzæ*. The co-enzyme is a co-dehydrogenase (see Chapter XII).

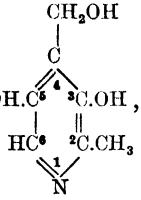
The closely related triphosphopyridine nucleotide, Co-enzyme II, which is concerned in reduction and phosphorylation reactions, is also required by *H. influenzæ* and *H. parainfluenzæ*.

Organisms of the genus *Hæmophilus* afford another example of symbiosis. *H. canis* needs hæmatin but

synthesises Co-enzyme I; *H. parainfluenzæ* needs co-enzyme I but synthesises hæmatin. If sown separately in peptone water neither grows. If they are sown together good growth results, each organism supplying the other with the lacking factor.

Pimelic acid.—$COOH.CH_2.CH_2.CH_2.CH_2.CH_2.COOH$. It was shown by Mueller that pimelic acid was one of the substances in liver extract which was required for the growth of *C. diphtheriæ* in synthetic media. The synthetic acid is equally effective, but other dibasic acids, such as azelaic acid, were not effective. Pimelic acid has an observable effect in concentrations of 0·005 μg./ml. and optimum effect at 0·01 μg./ml.

Although nothing is known of the way in which pimelic acid acts it is, perhaps, significant that the acid arises as a result of the hydrolysis and oxidation of biotin (see p. 104). It is possible that pimelic acid may be required in the synthesis of the carbon chain of biotin, the other two carbon atoms of the chain being derived by condensation with acetaldehyde.

Pyridoxine.— 

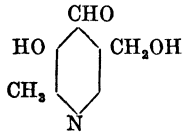
,2-methyl-3-hydroxy-4:5-di-(hydroxymethyl) pyridine. This substance has been shown to be a rat anti-dermatitis factor, vitamin B_6, which occurs in the vitamin B complex. It may also be concerned as an anti-pernicious anæmia factor in liver extracts. It has been isolated from rice bran, liver, molasses and similar sources. It was shown by Williams to be necessary for the growth of *Saccharomyces cerevisiæ* and *Streptobacterium plantarum*. It is required by *Lactobacillus casei, L. delbrückii* and *L. lactis* but not by *L. arabinosus* or *L. pentosus*. *Leuconostoc mesenteroides* can

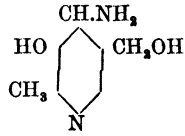
grow without it but is stimulated by its presence. The last three organisms are able to synthesise pyridoxine but *L. mesenteroides* only to the extent of about one-fourth of the production by the other two. The amount required by *L. casei* depends on the oxygen tension of the medium, the lower the oxygen tension the lower is the amount of pyridoxine required. It stimulates the growth of staphylococci.

The 4:5-diacetyl derivative is nearly as active as pyridoxine itself, but the triacetyl derivative is inactive. Substitution usually reduces or destroys the activity.

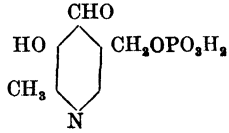
It has been shown that pseudopyridoxine, which is formed by the action of hydrogen peroxide on pyridoxine or by autoclaving solutions of the latter in presence of cystine, is considerably more active than pyridoxine in stimulating the growth of *Str. lactis* and *L. casei*, although it had no greater effect on the growth of several moulds, *Neurospora sitophila*, yeasts such as *Saccharomyces carlsbergensis* and *Sacch. oviformis* or rats.

Pseudopyridoxine is now known to be a mixture of

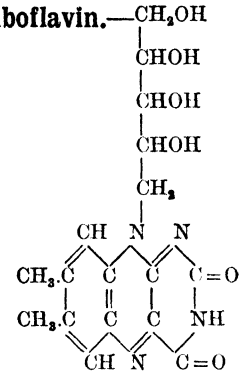
pyridoxal 

and
pyridoxamine, 

These two compounds are reversibly interconvertible and act as the co-enzyme of transamination (see p. 341.)

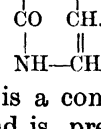
Pyridoxal is converted, in presence of adenosine triphosphate, to the phosphate 

which acts as the co-decarboxylase for the amino acid decarboxylases for tyrosine, lysine, asparagine, arginine and glutamic acid (see p. 228).

Riboflavin.— 

Warburg and Christian, in 1932, showed that riboflavin was an essential part of the "yellow" respiratory enzyme, which, together with Co-enzymes I and II, is concerned in the carbohydrate metabolism of yeasts and bacteria (see Chapter XII). It occurs in yeasts and those bacteria which do not require it as a growth factor and in many animal tissues. Since it was originally isolated from milk it is sometimes known as lactoflavin. It is a growth factor for most lactic acid bacteria, propionic acid bacteria, streptococci, *Thermobacterium* and *Clostridium tetani*.

Its function seems, obviously, to be built into the enzyme by those organisms which cannot synthesise it for themselves.

Uracil.— 

Richardson showed that for the anaerobic growth of *Staphylococcus aureus* on synthetic media it was necessary to add uracil. It is a component of nucleic acids (see Chapter XVIII), and is, presumably, required for their synthesis under anaerobic conditions. *Staph. aureus* appears to be able to synthesise uracil under aerobic conditions only. It has been shown to be necessary for *Lactobacillus arabinosus*, *Leuconostoc mesenteroides* and Group C hæmolytic streptococci. It can be replaced by orotic acid (uracil 4-carboxylic acid) for the latter. Uracil is not necessary as a growth factor for Group A hæmolytic streptococci if carbon dioxide is present at a partial pressure above 40 mm. of mercury.

***Mycobacterium phlei* Extract.**—In 1912 Twort and Ingram showed that Johne's bacillus, on first isolation, would only grow in the presence of some substance occurring in an extract of *M. tuberculosis hominis* or, better, in *M. phlei*. The organism could be "trained," with some difficulty, to grow in the absence of the extract, or, in other words, to produce its own growth factor. The substance, which is acidic in nature, is very stable and can be extracted by glycerol, hot water, hot alcohol or hot acetone. It can be replaced by alcoholic extracts of a number of vegetable tissues and fungi. It can be partially replaced by 0·1 μg./ml. of phthiocol, 3-hydroxy-2-methyl-1 : 4-naphthoquinone, a constituent of tubercle bacilli (see p. 392), or of 2-methyl-1 : 4-naphthoquinone, the anti-hæmorrhagic vitamin K.

The Sporogenes Factor.—*Cl. sporogenes*, when inoculated as a spore suspension into synthetic media shows no growth. When active preparations from yeast or from urine are added in very small quantities (0·4 μg./ml.) good growth ensues. The factor is widely distributed in animal and vegetable tissues from which it can be extracted by 75 per cent. alcohol. It can be purified by conversion into an alcohol soluble barium salt. The regenerated acid is active in concentrations of 0·02 μg./ml. It can be further purified by distillation of its methyl ester in a high vacuum (boiling point, 80 to 100°C. at 0·001 mm. of mercury). The ester is inactive but the activity is restored on hydrolysis. The sporogenes factor is an unsaturated hydroxy fatty acid, $C_{11}H_{14}O_4$ or $C_{11}H_{12}O_4$, of molecular weight about 200. Its presence appears to be essential for the growth of *Cl. botulinum* and *Cl. welchii* as well as of *Cl. sporogenes*. It is produced by many, probably all, aerobic bacteria, for example, *Salmonella typhimurium*, *Eberthella typhosa*, and *M. tuberculosis* and by the mould *Aspergillus versicolor*. It is probable that it is required by all micro-organisms

but that the *Clostridia* have lost the power of synthesising it for themselves.

It will have been noticed that many of the substances listed as growth factors are the prosthetic groups or the parent substances of prosthetic groups of enzymes or co-enzymes which are essential for the metabolism of micro-organisms. It is this which, probably, accounts for the fact that most growth factors are needed only in very small amounts, since they remain in circulation, as it were, and are used over and over again. If they are not available the particular metabolic process in which the corresponding enzyme or co-enzyme is involved is brought to a standstill and the organism fails to grow. It is almost certainly true that all organisms make use of these substances in their metabolism. Some organisms, however, appear to be able to make them all for themselves, whilst others need one or more of them to be provided from outside sources. It is only when a substance has to be supplied ready made to a micro-organism that it is regarded as being a growth factor for that organism. An organism, although capable of synthesising a particular growth factor, may do so relatively slowly so that it is in a chronic state of deficiency and accordingly develops only poor growth. The addition of the metabolite then has a stimulating effect on growth. Fildes has suggested that, in reality, most, if not all, of these compounds can be regarded as " essential metabolites."

That bacteria really can synthesise " essential metabolites " themselves is shown by the fact that *Thiobacillus thio-oxidans*, for instance, when grown autotrophically on sulphur containing media with no organic material initially present contains aneurin, biotin, nicotinic acid, pantothenic acid, pyridoxine and riboflavin. The same is true of *Esch. coli*, *Proteus vulgaris*, *Aerobacter aerogenes*, *Alkaligenes fæcalis*, *B.*

anthracis, *B. mesentericus*, *B. vulgatus*, *Vibrio comma* and *Serratia marcescens* in which aneurin, biotin, nicotinic acid and other substances have been detected after their growth on synthetic media containing none of these compounds originally. It is possible that intestinal bacteria may serve as a source of vitamins for human and animal nutrition, since it has been observed that vitamin deficiency symptoms often develop when the growth of intestinal bacteria is suppressed by the use of such drugs as sulphaguanidine or sulphasuxidine.

The *Lactobacilli* require a considerable proportion of the known growth factors for their adequate growth on synthetic media. This fact is used for the detection and estimation of the amounts of such substances present in various extracts or foods. Thus Landy and Dickens have shown that *L. casei* will grow well on a synthetic medium containing the appropriate amino-acids and mineral salts together with aneurin, biotin, nicotinamide, pantothenic acid, pyridoxine and riboflavin. The amount of growth can be estimated by titrating the lactic acid formed. If any one of the growth factors is omitted from the medium growth does not occur. If the missing factor is added in amounts less than that required for maximum growth, the degree of growth is proportional to the quantity of the factor added. The growth of *L. casei* on the medium lacking one of the factors can, therefore, be used as a test for the presence of that factor in anything added to the medium ; by comparing the amount of growth in presence of the addendum with that occurring on the full medium the test can be made quantitative. By omitting each growth factor in turn from the medium an analysis of the factors in an extract can be made.

Another valuable method of assaying growth factors and essential amino-acids has recently developed from the use of mutants of the mould *Neurospora crassa*

obtained by the action of X-rays on the asexual spores and crossing them with the heterothallic strain of opposite character. A series of mutants has been obtained which can grow on a complete medium but which fail to grow on a medium lacking a single constituent specific to each mutant. This is due to a lack of synthetic ability to form the particular compound, occasioned by destruction of the controlling gene. Mutants failing to synthesise *p*-amino benzoic acid, aneurin, choline, inositol, nicotinic acid, pantothenic acid, pyridoxine and the essential amino-acids arginine, leucine, lysine, methionine, proline, threonine, tryptophan and valine are known. The growth of such mutants is proportional to the amount of the specific substance (up to the amount necessary for optimum growth) which is added to an otherwise adequate medium.

These and other mutants are also extremely valuable for elucidating the mechanism of certain metabolic reactions (see p. 343).

In contrast with the term "Antibiotics" which has recently come into use for those substances produced by micro-organisms which inhibit the growth of other organisms, it has been suggested that those substances capable of stimulating metabolism which are produced by micro-organisms, and which are usually highly specific in their action, should be called "Biotics" as an alternative to growth factors.

A list of some micro-organisms and their requirements of growth factors is given in Table 8. It must be realised that different strains of a given species may vary in their ability to synthesise one or more of the biotics mentioned so that the lists given cannot apply rigidly to all strains. Some of the strains may also require growth factors of still unknown composition in addition to those listed. Considerable variation of this sort is found amongst strains of *C. diphtheriæ*, *Lactobacillus*, *Streptococcus* and the yeasts.

TABLE 8

Organism.	Growth Factors.	Amount required per ml. of medium.
		μg.
A. suboxydans	*p*-Aminobenzoic acid	0·005
Brucella	Biotin (or methyl ester)	0·000003 to 0·0001
	Nicotinamide	0·02
	Pantothenic acid	0·02
	Pyrimidine (or aneurin)	0·02
Cl. acetobutylicum	*p*-Aminobenzoic acid	0·000001
	Biotin	0·00015 to 0·000001
Cl. botulinum	"Sporogenes Factor"	...
Cl. butylicum	Biotin	...
	"Sporogenes Factor"	...
Cl. butyricum	*p*-Aminobenzoic acid	...
Cl. sporogenes	"Sporogenes Factor"	0·02
Cl. tetani	Adenine	5·0
	Aneurin	0·1
	Biotin	0·001
	Folic acid	0·001
	Nicotinic acid	1·0
	Oleic acid	2·5
	Pantothenic acid	0·05 to 0·25
	Pyridoxine	1·0
	Riboflavin	0·1
	Tryptophane	50·0
	Uracil	2·5
Cl. welchii	Oleic acid	...
	Pyridoxine	...
	"Sporogenes factor"	...
	Uracil	...
C. diphtheriae	β-Alanine	0·1
	Nicotinic acid or	1·0
	Nicotinamide	10·0
	Oleic acid	...
	Pantothenic acid	...
	Pimelic acid	0·005 to 0·01

Continued on next page

Table 8 (*Continued*)

Organism.	Growth Factors.	Amount required per ml. of medium.
		μg.
H. canis	Hæmatin	...
H. ducreyii	Hæmatin	...
H. influenzæ	Hæmatin	...
	Co-enzyme I or II	...
H. parainfluenzæ	Co-enzyme I or II	...
H. pertussis	Nicotinamide	...
Needed by all following strains of *Lactobacillus*	Aneurin	0·2
	Biotin	0·0001 to 0·0004
	Folic acid	0·00005 to 0·3
	Nicotinamide	0·1 to 0·2
	Pantothenic acid	0·03 to 0·2
	Riboflavin	0·04 to 0·2
	Tryptophane	100·0
L. arabinosus	Adenine	20·0
	p-Amino benzoic acid	0·0002 to 0·6
	Inositol	20·0
	Uracil	5·0
L. casei	Adenine	20·0
	p-Amino benzoic acid	0·6
	Guanine	20·0
	Inositol	5·0
	Orotic acid	0·01
	Pyridoxine	0·06 to 0·6
	Uracil	20·0
L. delbrückii	Pyridoxine	...
L. lactis	Pyridoxine	...
L. pentoaceticus	Pyridoxine	...
L. pentosus	Adenine	...
L. plantarum	Adenine	...
	Guanine	...
	Pyridoxine	...
Leuc. mesenteroides	Pyridoxine	...

Continued on next page

Table 8 (*Continued*)

Organism.	Growth Factors.	Amount required per ml. of medium.
		μg.
Pneumococcus	Biotin	...
	Pantothenic acid	1·0
Propionic acid bacteria	Aneurin	0·005 to 0·05
	Biotin	...
	Riboflavin	...
Proteus morganii	Pantothenic acid	0·2
	Nicotinamide	...
Proteus vulgaris	Nicotinamide	...
Rhizobium	Biotin	...
Rhodotorula rubra	Aneurin	0·016
Rhodotorula flava	Aneurin	0·016
Shigella dysenteriæ	Nicotinamide or Co-enzyme I or II	0·1
Staph. aureus	Aneurin	0·003
	Biotin	0·005 to 0·01
	Nicotinamide	0·05 to 0·2
	Pyridoxine	0·3 to 1·2
	Uracil (anaerobic only)	0·005
Streptobacterium plantarum	Biotin	0·001
	Nicotinic acid	0·2
	Pantothenic acid	0·2
	Pyridoxine	1·0
Streptococcus hæmolyticus	Aneurin	0·001
	Biotin	...
	Glutamine*	...
	Nicotinamide	0·1
	Pantothenic acid	0·008 to 1·25
	Pyridoxine	2·0
	Riboflavin	0·004 to 0·1
	Uracil (for Group C)	10 to 20
	*Synthesised once growth has commenced.	

Continued on next page

Table 8 (*Concluded*)

Organism.	Growth Factors.	Amount required per ml. of medium.
		μg.
Str. lactis R.	Aneurin	0·2
	Adenine	10·0
	Biotin	0·0004
	Folic acid	0·0005
	Guanine	10·0
	Nicotinamide	0·6
	Pantothenic acid	0·4
	Pyridoxine	1·2
	Riboflavin	0·2
	Thymine	0·2
Neurospora crassa	*p*-Aminobenzoic acid	0·0025
N. sitophila	Pyridoxine	0·1
Phycomyces blakesleeanus	Aneurin	0·02
Saccharomyces cerevisiæ	β-Alanine	0·08
	p-Aminobenzoic acid	...
	Aneurin	...
	Biotin	0·0001
	Inositol	20·0
	l-Leucine	...
	Pantothenic acid	...
	Pyridoxine	0·004

It has been claimed that ascorbic acid is required as a growth factor by the trypanosomes *Schizotrypanum cruzi*, *Leishmania tropica* and *L. donovani* and some *Trichomonas* species. Cholesterol is also said to be required by some species of *Trichomonas*.

For further reading :—

K. Hofmann, "The Chemistry and Biochemistry of Biotin." *Advances in Enzymology.* **3** (1943), 289.

B. C. J. G. Knight, "Growth Factors in Microbiology." *Vitamins and Hormones.* **3** (1945), 108.

S. A. Koser and F. Saunders, "Accessory Growth Factors for Bacteria and Related Micro-organisms." *Bact. Rev.* **2** (1938), 99.

A. Lwoff, "Les Facteurs de Croissance pour les Micro-organismes." *Ann. Inst. Pasteur.* **61** (1938), 580.

W. H. Peterson and M. S. Peterson, "The Relation of Bacteria to Vitamins and other Growth Factors." *Bact. Rev.* **9** (1945), 49.

R. J. Williams, "The Chemistry and Biochemistry of Pantothenic Acid." *Advances in Enzymology.* **3** (1943), 253.

CHAPTER X

CHEMOTHERAPY

THE term chemotherapy introduced by Ehrlich is used to describe the treatment of diseases due to micro-organisms by means of chemicals of known composition. It is analogous to serotherapy which is used for the treatment of such diseases by the use of antibacterial or antitoxic sera. Rather curiously the treatment of other conditions, such as the " deficiency " diseases due to lack of vitamins, or of endocrinological diseases, with drugs, even when their constitution is known, is not included in the term chemotherapy.

In recent years great advances have been made in chemotherapy, particularly in that part of it dealing with bacterial infections and it is now possible to account for the mechanism of the processes on a fairly certain basis.

Chemotherapeutic substances cure by the destruction of the organism causing the disease. The action may be directly on the organism or by stimulation of the defence mechanism of the host or, frequently, by a combination of both means. The organism may be weakened, or otherwise rendered susceptible, so that the tissue defences may be strong enough to overcome the infection.

The application of chemotherapy is obviously more difficult in the case of a generalised infection, or in an infection of a deep tissue, such as the central nervous system, than when the infection is localised or readily accessible to the action of the drug. In such conditions

a substance which is more harmful to the parasite than to the surrounding cells and tissues can be applied directly, as did Lister when he used phenol to combat sepsis in surgery, or as is done when flavines are used for surface staphylococcal infections, or sulphonamides are dredged into wounds. When the infection is deep seated or general the chemotherapeutic agent must circulate in the blood or lymph in order to reach the organisms and then the body as a whole is subject to the actions of the drug which may be toxic. Moreover, the substance is liable to be excreted or destroyed or inactivated by fixation in the tissues with lowering of the effective concentration. The difficulty is even greater if the organisms are situated in avascular tissue or necrotic areas or inside cells where direct access of the drug carried in the blood is not possible.

Although a substance may be highly lethal to bacteria *in vitro* it does not follow that it will be a good chemotherapeutic agent. Thus Koch, as long ago as 1881, showed that amounts of mercuric chloride many times the *in vitro* lethal dose when injected into guinea-pigs had no effect on anthrax bacilli, subsequently injected. Similarly Hata showed that an amount of methylene blue, five hundred times that required to kill *Borrelia recurrentis* (the causal organism of relapsing fever) *in vitro* had no influence on the course of the infection in mice. The converse may also be true; prontosil is without effect on *Streptococcus pyogenes* when tested on cultures of the organism but is a most useful drug in the treatment of streptococcal infections.

In all cases its toxicity to the host is the limiting factor which determines whether or not a given substance can be used as a therapeutic agent. Almost always a substance which is harmful to micro-organisms is also harmful to the cells of the host so that the choice of a suitable drug depends on the difference in intensity between the two actions. The ordinary disinfectants

like phenol, mercuric chloride, or chlorine compounds are as toxic to animal cells as to micro-organisms and obviously cannot be used internally. The more damaging the drug to the parasite compared with its toxicity to the host the more useful is it likely to be. The ratio $\frac{\text{toxicity to micro-organism}}{\text{toxicity to host}}$, often called the chemotherapeutic index, is frequently used as a measure of the value of a drug, the higher the ratio the more useful is the substance likely to be, other things being equal. The chemotherapeutic index is sometimes expressed as the ratio between the smallest amount of drug which, when injected in one dose, will effect cure and the largest amount tolerated by the host.

The route of injection of a drug may influence its apparent efficacy, as a result of differences in absorption or excretion. Thus intramuscular injection of a relatively insoluble drug will produce a depot of the drug which will maintain a more or less uniform concentration of the drug in the circulation for a considerable time, whereas intravenous injection is followed by fairly rapid excretion. As an example salvarsan, when injected intramuscularly into fowls, protects them against infection by spirochætes for several weeks, although they become susceptible again within six days of an intravenous injection. Penicillin is of little value when taken orally because it is destroyed by the acid conditions prevailing in the stomach, and is administered intravenously. On the other hand, sulphaguanidine is effective against intestinal organisms because it is only slowly absorbed from the gut, whilst the rapidly absorbed sulphanilamide is almost useless for such infections, but very effective against the bacteræmia type of infection.

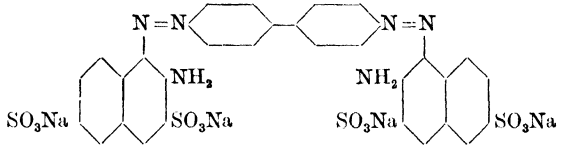
A micro-organism may be killed in one host by a drug but may be resistant to the same drug in another host.

This is possibly due to differing reactions of the host to the absorption or excretion of the drug. A further possibility may be the possession by different hosts of different amounts of substances inhibiting the drug, as illustrated by the fact that rats can be protected against streptococcal infections by pantoyltaurine, whilst mice, which normally have a higher concentration of pantothenic acid in their blood, are not (see p. 149).

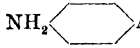
The development of chemotherapy can be regarded as commencing in 1867 with Lister's use of phenol as an antiseptic in surgery, although knowledge existed much earlier of such traditional remedies as mercury and iodides for syphilis, cinchona bark for malaria and ipecacuanha for amœbic dysentery and although, many years before the causes of the diseases themselves had been elucidated, the active principle of cinchona bark was shown to be quinine, and ipecacuanha was known to act in virtue of the alkaloid, emetine, which it contained.

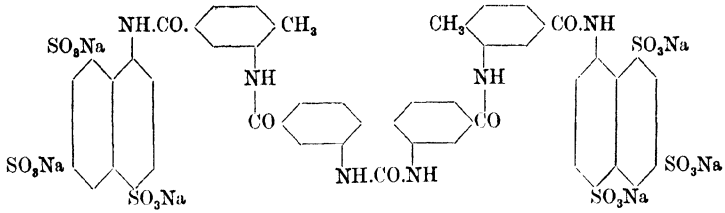
Progress in chemotherapy was greatly hampered in the early days by lack of *in vitro* methods of testing drugs. Such methods could not be developed until methods of culture of the test organisms were available. Trypanosome infections in rats and mice were originally used for testing drugs against such diseases as sleeping sickness. In untreated animals the parasites progressively multiply in the blood stream and death results in a few days. An adequate dose of an effective compound leads to permanent elimination of the parasite from the blood stream, more or less rapidly depending on the drug. In 1930 a method was devised for maintaining trypanosomes alive *in vitro* for about twenty-four hours and has been of great use in investigating trypanocidal drugs. The study of amœbecides was greatly facilitated by Dobell and Laidlaw's *in vitro* method of culturing amœba such as *Entamœba histolytica* which causes amœbic dysentery.

Protozoal Infections.—Very little progress was achieved until Ehrlich's researches gave a stimulus to the study of the subject. Ehrlich had observed that some tissues were selectively stained when certain dyes were injected into animals, whilst other tissues were almost unaffected. In 1891 he recorded that the malaria parasite was stained by methylene blue and could be differentiated from the tissues of the host in this way. This suggested that dyestuffs might be found which would be so easily adsorbed by pathogenic micro-organisms as to kill them without harming the host. As a result of these investigations Ehrlich and Shiga, in 1904, showed that trypanosomes were readily stained by the dye trypan-red,

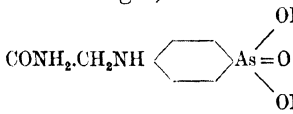


and that the substance cured the ordinarily fatal infection of mice with *Trypanosoma equinum*. Unfortunately the dye was effective only against acute laboratory infections and not against the natural disease.

In 1905 atoxyl, *p*-amino-phenyl arsonic acid,  , was shown by Thomas to be lethal to *Trypanosoma gambiense* in infected mice. This discovery led to the production of a number of arsenical drugs, many of which were dangerously near the toxic limits for therapeutic use. Their action was rarely apparent if administered in the later stages of the disease, and frequently arsenic resistant strains of trypanosomes were developed when cure was not effected. These drugs were not lethal to *T. rhodesiense*. Further research, however, led to the discovery in 1920 of Baeyer 205 or germanin (among other names),

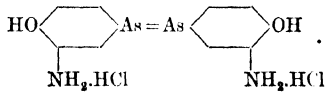


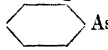
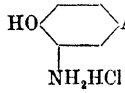
which is effective against natural trypanosome infections by *T. gambiense* and *T. rhodesiense*, but still ineffective in the later stages of the disease. This, but not the other disadvantages, was overcome by the use of tryparsamide,



, and similar drugs such as arsanine and neocryl containing pentavalent arsenic.

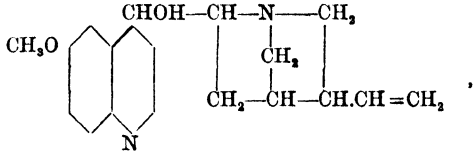
Atoxyl was shown to be effective also against the spirochæte causing syphilis. Even more effective is salvarsan or arsphenamine,



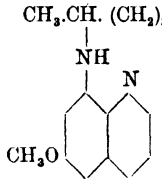
. It was later shown that these substances were not themselves active *in vitro* but that they were converted in the body, by reduction and partial oxidation respectively, to derivatives of phenyl-arsenoxide,  , which were highly lethal *in vitro* to spirochætes and trypanosomes. The therapeutic use of phenylarsenoxide derivatives in the ordinary way is not possible since they are excreted too rapidly to be effective unless given in doses which would be too toxic to the host. The arsenoxide derivative mapharsen, or mapharside,  , is used, however, by the slow intravenous drip method to give a short but intensive treatment for syphilis.

Many compounds, based on the structure of the effective drug, emetine, have been synthesised and tested for treatment of amœbic dysentery, but none, so far, has been found to exceed emetine in therapeutic value, although some are more toxic to *Entamœba histolytica* when tested *in vitro*.

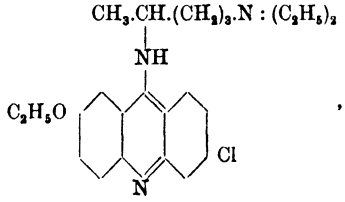
Similar search for antimalarial drugs primarily based on the structure of quinine,



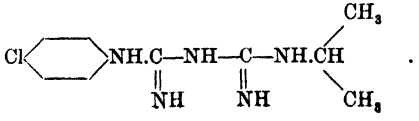
has led to the production of plasmoquin,



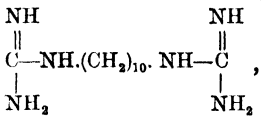
and atebrine, or mepacrine,



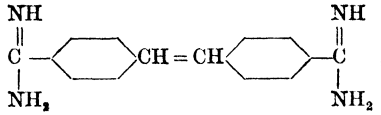
which are of considerable value. A further extension of this search in recent years has led to the discovery of paludrine, N_1-*p*-chlorophenyl-N_5-*iso*propyl-biguanidine:—



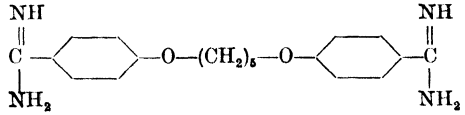
A theory of drug action different from Ehrlich's receptor theory led to the discovery of a group of drugs which have proved of great value in several trypanosome diseases. The drug synthalin, decamethylene diguanidine,



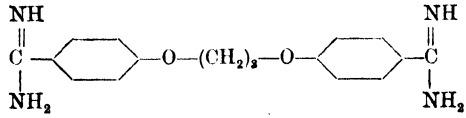
, had been used in diabetes because it had a similar effect to insulin in lowering the blood sugar content. Janscó thought that it might be effective in trypanosomiasis by lowering the blood sugar to such an extent as to starve out the trypanosomes, in the same way as he had succeeded in preventing trypanosome infections in mice by inhibiting their carbohydrate metabolism with iodoacetic acid. It so happened that the drug was active against trypanosomes, although not for the reasons which led to its trial. Investigation of drugs of similar constitution brought to light stilbamidine, diamidino-stilbene,



which is the most effective drug yet known for the treatment of kala-azar, and pentamidine,



for babesia in cattle. Propamidine,

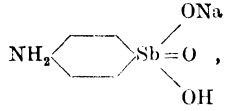


has also proved of value in trypansomiasis, kala-azar and babesiasis.

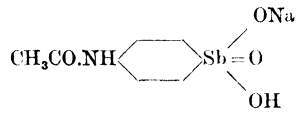
To Summarise:—Trypanosomes are susceptible to the

metallo-organic compounds of arsenic, antimony and bismuth, to derivatives of quinine, to the triphenyl methane series of dyes, to acid bis-azo dyes, such as trypan-red and trypan-blue, to acriflavine and to the amidines.

Leishmania are susceptible to pentavalent antimony compounds such as stibenyl, *p*-aminophenyl sodium stibonate,

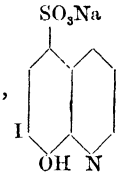


or stibacetin,

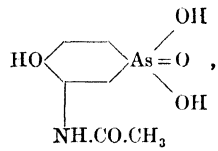


and to the amidines.

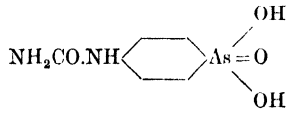
Amœbæ are susceptible to chinoform, iodohydroxyquinoline sulphonate,



and to the pentavalent arsenicals acetarsol,



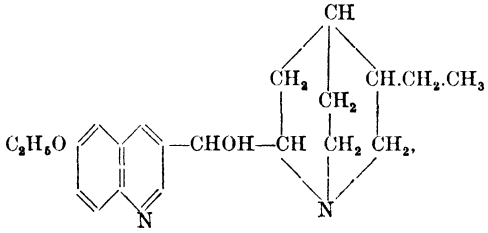
and carbasone,



Plasmodia are susceptible to quinine, plasmoquin, atebrine, and paludrine.

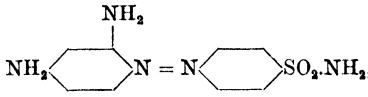
Spirochætes are susceptible to the metallo-organic compounds of arsenic, antimony and bismuth, but are relatively little affected by purely organic drugs.

Bacterial Infections.—Until 1935, apart from the local application of acridine dyes, such as acriflavine and proflavine, to wounds and surface lesions, developed during the 1914-1918 war, the only bacterial disease which had shown itself susceptible to chemotherapy was a pneumococcal infection of mice which responded to treatment with optoquin, ethyl hydrocupreine,

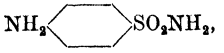


which is also active against trypanosomes.

In 1935 Domagk subjected to clinical trial the drug prontosil, sulphonamido-chrysoidin,

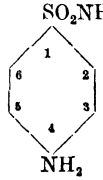


which had been discovered by Klarer and Mietzsch in 1932, and found that it was an effective agent against streptococcal infections, in spite of the fact that it was inactive *in vitro*. This phenomenon was explained by Tréfouel, Tréfouel, Nitti and Bovet in 1935, who showed that prontosil was broken down in the body to *p*-aminobenzene sulphonamide, now known as the drug, sulphanilamide,



which was active both *in vitro* and *in vivo*. This discovery led to a tremendous amount of research for similar and improved drugs. Some 2,500 derivatives of sulphanilamide have been synthesised, most of them by substitution on the nitrogen atoms. In nomenclature the sulphonamide group—

SO_2NH_2, is regarded as being at position 1, whilst the amino group is at position 4 :—



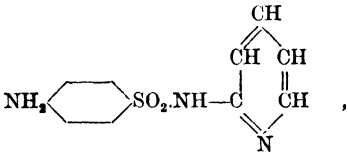
The following is a summary of the findings :—

N^1 derivatives.

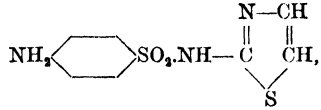
(*a*) Alkyl. The introduction of the methyl or ethyl group causes little change in effectiveness. Longer chains cause lowered activity.

(*b*) Isocyclic. About 180 have been prepared, none of which have been of much value.

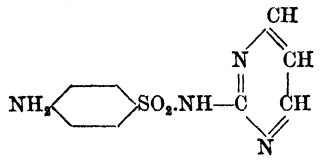
(*c*) Heterocyclic. About 250 have been synthesised. They include the most useful members known. Among them are sulphapyridine,



effective against pneumococcal, streptococcal, meningococcal, gonococcal and coli infections, sulphathiazole,

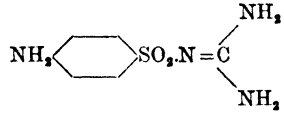


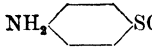
effective against staphylococci, and sulphadiazine,



which is

less toxic than sulphathiazole and is active against streptococci, pneumococci, staphylococci and gonococci.

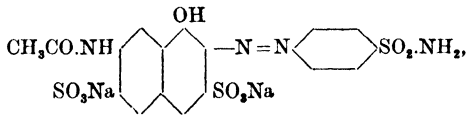
(*d*) Acyl. About 35 are known, of which sulphaguanidine,  , is very useful for intestinal diseases, including bacillary dysentery, since it is only slowly absorbed from the gut, and sulphacetamide,

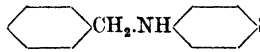


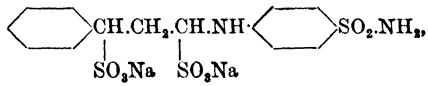
(albucid, sulamyd) which is of value in gonorrhœa and urinary infections.

N^4 derivatives.

About 550 have been made. It appears that only those which can break down in the body to give sulphanilamide or an active derivative of it are of chemotherapeutic use. Prontosil, prontosil soluble,



proseptasine, 

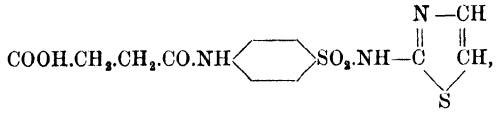
and soluseptasine 

are examples. Long chain alkyl or sulphonyl derivatives are not broken down in this way and are, therefore, inactive.

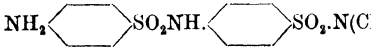
$N^1.N^4$ derivatives.

The activities of substances of this type are what would be expected from considerations of the effect of

substituents on the two nitrogen atoms mentioned above. Succinyl sulphathiazole, sulphasuxidine,

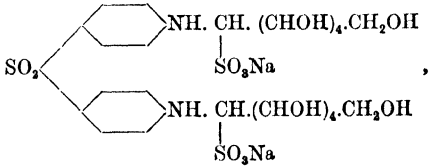


is one of the most useful, especially for intestinal infections such as dysentery because it is poorly absorbed from the gut and breaks down slowly with liberation of sulphathiazole. Uleron,

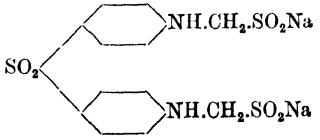


is active against staphylococci.

Tuberculous infections in guinea-pigs have been successfully treated with promin, di-aminodiphenylsulphone diglucose sulphonate,



which is also active against tubercle bacilli *in vitro*. Unfortunately it is fairly toxic to man, producing hæmolytic anæmia. The related drug diasone, diaminodiphenylsulphone disodium formaldehyde sulphoxylate

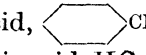


$\cdot 4H_2O$, is less toxic and has similar curative effect on guinea-pig tuberculosis. It will also cure hæmolytic streptococcal infections and pneumococcal infections in mice.

The Mode of Action of Chemotherapeutic Substances.—The earliest theory of chemotherapeutic action is that

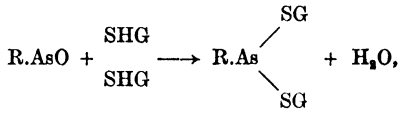
due to Ehrlich who suggested that the drugs were taken up by specific chemoreceptors attached to susceptible organisms, but lacking in cells not affected by the drug. The subsequent action of the drugs was not described except that they were considered not to kill the micro-organism but to prevent multiplication, enabling the host to deal effectively by its normal processes with the consequently milder infection.

Later, when the importance of enzymatic processes in metabolism began to be realised, suggestions were put forward that drugs in general might act by inhibiting enzyme systems and so upsetting the normal course of events in the animal body. A number of such effects had been observed *in vitro*; cyanides inhibit oxidases, atoxyl and quinine inhibit lipases, cocaine, atropine and pilocarpine inhibit yeast invertase, eserine inhibits the break down of acetylcholine by esterase, acriflavine inhibits a hydrogen transportase system in trypanosomes, which is not affected by cyanide.

Another type of enzyme inhibition is that due to the presence of excess of the products of the reaction or of substances having a constitution similar to the substrate or to the products of breakdown. For example, the breakdown of lactic acid, $CH_3CHOH.COOH$, to pyruvic acid, $CH_3CO.COOH$ is partially inhibited by α-hydroxybutyric acid, $C_2H_5CHOH.COOH$, glyceric acid, $CH_2OH.CHOH.COOH$, mandelic acid,  $CHOH.COOH$, glyoxylic acid, $HCO.COOH$, or oxalic acid, $HO.CO.COOH$. The action of succinic dehydrogenase in converting succinic acid, $COOH.CH_2.CH_2.COOH$, to fumaric acid, $COOH.CH{=}CH.COOH$, is inhibited by the presence of malonic acid, $COOH.CH_2.COOH$ or glutaric acid, $COOH.CH_2.CH_2.CH_2.COOH$, containing the $-CH_2COOH$ group (see p. 196). The heavy metals such as mercury and barium also have an inhibitory effect on many

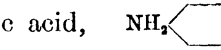
enzymes. The effect of mercuric chloride as an antiseptic has been regarded for a considerable time as being due to the combination of the mercury with the –SH groups of proteins. Its inhibition of some enzymes such as papain or invertase is held to be due to a similar reaction.

In 1923 Voegtlin suggested that the phenyl-arsenoxides were lethal to trypanosomes and spirochætes because they reacted with the sulphydryl groups of glutathone :—



and so interfered with the respiratory mechanism of the organisms. The addition, in sufficient amount, of compounds containing SH groups was capable of reversing the inhibition of enzymes or respiration by the arsenoxides or mercury by themselves combining with the inhibitors.

Accordingly when it was discovered that sulphanilamide was effective against micro-organisms *in vitro* only when small inocula were used or when the medium did not contain peptone, the suggestion was soon forthcoming that some substance or substances contained in peptone or large inocula were inhibiting the action of the drug. Confirmation of this view was afforded by Stamp in 1939 who showed that addition of killed streptococci to a medium containing sulphanilamide permitted even small inocula of living organisms to survive and flourish. He succeeded in extracting the inhibitor from streptococci with dilute ammonia solution and obtained it as an alcohol soluble substance stable to acid and heat; it contained an amino group. He regarded it as possibly a complex amino acid required for growth or as an essential part of an enzyme system. Similar results have been reported for *Brucella abortus* and other bacteria.

Woods found a similar effect with yeast extracts and brought forward strong evidence that the responsible substance was *p*-aminobenzoic acid,  , which he showed to have a powerful inhibitory effect on sulphanilamide. He suggested that *p*-aminobenzoic acid is essential for the growth of the organism, and is normally synthesised in adequate amounts. Sulphanilamide, which has a structure very similar to that of *p*-aminobenzoic acid, competes with the enzyme involved in its further utilisation and so prevents growth. Addition of *p*-aminobenzoic acid overcomes the competition for the enzymes and inhibits the action of sulphanilamide. Very small amounts of *p*-aminobenzoic acid, about one five-thousandth of the inhibitory concentration of sulphanilamide, may suffice to reverse the effect of the latter. He suggested that the varying sensitivity to sulphanilamide of different organisms was due to differences in their power of synthesising *p*-aminobenzoic acid.

The explanation that sulphanilamide intervenes at the stage of synthesis of *p*-aminobenzoic acid by combining with the synthesising enzyme seems to be wrong, because if it were true it would be expected that the amount of *p*-aminobenzoic necessary to reverse the effect would be independent of the amount of sulphanilamide present. This is not so, the ratio of *p*-aminobenzoic acid to sulphanilamide being constant.

Other theories of the action of sulphanilamide have been put forward but have been displaced by the "essential metabolite" theory. It was, for instance, suggested that in presence of sulphanilamide, streptococci lost their power to produce a capsule and that, as a result, they were much more susceptible to phagocytosis. This hypothesis does not account for the fact that many normally non-capsulated organisms are susceptible to sulphanilamide, nor for the *in vitro* bacteriostatic effect

of the drug. Another explanation was that sulphanilamide was oxidised to the hydroxyl-amino compound, NHOH.C$_6$H$_4$.SO$_2$.NH$_2$, which was known to inhibit the power of catalase to destroy hydrogen peroxide; in presence of the drug, therefore, organisms such as streptococci and pneumococci, which are sensitive to hydrogen peroxide, are killed by its accumulation.

p-Aminobenzoic acid inhibits the bacteriostatic effects not only of sulphanilamide, but also of the other sulphonamide drugs, such as sulphapyridine and sulphathiazole, which have the common grouping —NH.C$_6$H$_4$.SO$_2$.NH$_2$. Neither *ortho-* and *meta*-amino benzoic acids, *p*-aminophenyl acetic acid, NH$_2$.C$_6$H$_4$.CH$_2$.COOH, nor *p*-aminophenylglycine dihydrochloride, HCl.NH$_2$.C$_6$H$_4$.CH(NH$_2$.HCl).COOH, can replace *p*-aminobenzoic acid in inhibiting the sulphonamides On the other hand *p*-aminobenzoic acid can reverse the effect of other drugs which have groupings similar to sulphanilamide, such as *p*-aminobenzamide, NH$_2$.C$_6$H$_4$.CO.NH$_2$, or atoxyl, NH$_2$.C$_6$H$_4$.AsO$_3$H, which slows down the respiration of *Esch. coli*. However, it has no effect on the drug marfanil, NH$_2$CH$_2$.C$_6$H$_4$.SO$_2$.NH$_2$, *in vitro* although it reverses its effect *in vivo*.

It has been found that over a wide range of concentrations the molar concentration of *p*-aminobenzoic acid necessary to reverse the bacteriostatic action of sulphanilamide is proportional to the molar concentration of the latter. The ratio $\frac{\text{concentration of drug}}{\text{concentration of } p\text{-AB}}$ which just

results in bacteriostasis is known as the antibacterial index, or bacteriostatic constant. It varies, with any particular drug, from organism to organism, and with a particular organism from drug to drug. These variations in value are proportional to the potency of a drug against the organism, as measured by the minimum bacteriostatic concentration *in vitro*. These findings are illustrated in the following tables, from the work of Wyss, Grubaugh and Schmelkes (*Proc. Soc. Exp. Biol. Med.* **49** (1942) 618).

TABLE 9

Concentration of sulphonamides permitting 50 per cent. of the maximum growth in 16 hours.

	MEDIUM 1		MEDIUM 2
	Staph. aureus	*E. coli*	*E. coli*
	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.
Sulphanilamide	14·0	15·0	5·8
Sulphaguanidine	12·0	16·2	5·8
Sulphapyridine	3·0	2·9	0·61
Sulphacetamide	2·0	2·8	0·57
Sulphadiazine	0·6	0·65	0·06
Sulphathiazole	0·3	0·65	0·06

TABLE 10

Effectiveness of drugs in overcoming *p*-aminobenzoic acid.

	Amount of drug	*Staph. aureus*			*Esch. coli*		
		p-Amino-benzoic acid	Mol. ratio Drug/ *p*-AB	Efficiency	*p*-Amino-benzoic acid	Mol. ratio Drug/ *p*-AB	Efficiency
	Mg. per 100 ml.	Mg. per 100 ml.			Mg. per 100 ml.		
Sulphanilamide	50	0·0086	4660	1	0·012	3330	1
Sulphaguanidine	50	0·0070	4510	1	0·0081	3960	0·8
Sulphapyridine	5	0·0066	1416	11	0·0061	450	7
Sulphacetamide	5	0·0060	934	9	0·0060	534	6
Sulphadiazine	5	0·030	92	51	0·064	43	78
Sulphathiazole	5	0·050	53	88	0·065	41	81

TABLE 11

Neutralisation of *p*-aminobenzoic acid in presence of various bacteria.

	Molecular Ratio		Efficiency
	Sulphanilamide/ *p*-aminobenzoic acid	Sulphathiazole/ *p*-aminobenzoic acid	Sulphathiazole/ Sulphanilamide
E. coli	2000	27	74
A. aerogenes	3220	45	72
Staph. aureus	4660	53	88
Ps. æruginosa	13350	184	73
Sal. typhimurium	6650	92	72
L. acidophilus	8000	133	60
Prot. vulgaris	4000	55	73

Table 11 shows that the relative efficiency of the two drugs in inhibiting the use of *p*-aminobenzoic acid by various organisms is constant, which means that the drugs act in the same way in preventing the growth of all the organisms. This confirms the suggestion by Woods that different sulphonamides are more effective against some bacteria than against others, because the microbes have different abilities to synthesise *p*-aminobenzoic acid.

It is clear from Table 10 that the sulphonamide drugs are widely different in their ability to compete with *p*-aminobenzoic acid in the enzyme system involving the latter, sulphathiazole being some 80 times as effective as sulphanilamide. This has been explained by Bell and Roblin as due to the closeness of resemblance of the drug to the *p*-aminobenzoic acid ion in molecular structure and distribution of electric charges. That the effectiveness of the various sulphonamide drugs is closely related to their degree of ionisation is shown by the following values taken from C. L. Fox and H. M. Rose (*Proc. Soc. Exp. Biol. Med.* **50** (1942) 142) :—

TABLE 12

	Min. effective concentration of drug	Acid dissociation constant *p*Ka	% ionised at *p*H 7·0	Concn. of ionised drug at *p*H 7·0	Min. amt. of *p*-aminobenzoic acid required to inhibit	Ratio drug/*p*-AB	Ratio ionised drug/*p*-AB
	M × 10^{-6}			M × 10^{-6}	M × 10^{-6}		
Sulphanilamide -	2500	10·5	0·03	0·71	0·5	5000	1·4
Sulphapyridine -	20	8·5	3·4	0·68	0·5	40	1·4
Sulphathiazole -	4	6·8	61·6	2·46	0·5	8	4·9
Sulphadiazine -	4	6·4	80·0	3·2	0·5	8	6·4

It will be seen that the effective drug concentration is inversely proportional to the degree of ionisation and that the amount of *p*-aminobenzoic acid required for inhibition of the drug is very nearly proportional to the amount of drug in the ionised state. *p*-Aminobenzoic acid is completely ionised at *p*H 7. Its ion may be represented as at A in Fig. 5.

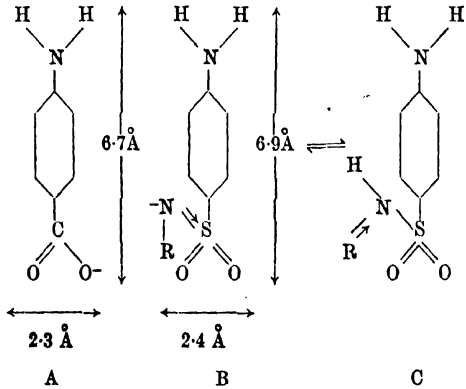


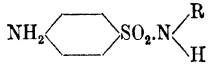
FIG. 5

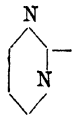
The electrons belonging to the sulphur atom of a sulphonamide (C in Fig. 5) are attracted by the oxygen

atoms and the pull is transmitted to the electrons on the amide nitrogen atom, which, consequently, exerts less attraction for the hydrogen atom which, accordingly, becomes capable of ionisation. It behaves as a very weak acid with a dissociation constant of $Ka = 3{\cdot}7 \times 10^{-11}$ in the case of sulphanilamide (B in Fig. 5). In the ionised state the electron pair which formed the covalent bond with the hydrogen atom is available to increase the electronegative character of the $-SO_2$ group, although the effect is not very great because the degree of ionisation is low. The combined effect is approximately equivalent to the ionised $-CO.O^-$ of the carboxyl group. Substitution of a group R on the amide group has two opposing effects : (1) a competition with the $-SO_2$ group for the electron pair, which decreases the resemblance to the charge distribution on the carboxyl ion and so reduces the activity of the drug ; this effect is considerable when both hydrogen atoms in the amide group are substituted so that ionisation is impossible ; the second effect (2) is observed when the hydrogen atom is present, since then ionisation is increased because the extra competition for the ions by the substituent group reduces still more the attraction of the nitrogen atom for the hydrogen atom. As a result the more electronegative the substituent group, the greater is the acid strength of the derivative, with corresponding increase in activity. There is however, an optimum degree of electronegativity since increase beyond a certain value involves too great competition for electrons, to the extent that they are withdrawn from the $-SO_2$ group to the R group with loss of similarity to the carboxyl ion ; that is, the activity of the drug is lowered. It is possible, therefore, to predict the activity of a new derivative from a knowledge of the electronegative character of the substituent group R. These effects are illustrated by Table 13 and Fig. 6 which are taken from Bell and Roblin's paper (*J.A.C.S.* **64**, (1942) 2905).

TABLE 13

Relation between Acidity and Activity of Sulphonamides :—



COMPOUND	R	Ka	*p*Ka	Percentage Ionisation at *p*H 7	Minimum molecular concentration for bacteriostasis $C_R \times 10^5$
p-Aminobenzoic acid	——	$2{\cdot}1 \times 10^{-5}$	4·68	99·0	——
Sulphanilamide	H—	$3{\cdot}7 \times 10^{-11}$	10·43	0·03	20·0
N^1-Methylsulphanilamide	CH_3—	$1{\cdot}7 \times 10^{-11}$	10·77	0·01	30·0
N^1-Phenylsulphanilamide	C_6H_5—	$2{\cdot}5 \times 10^{-10}$	9·6	0·25	3·0
Sulphapyridine		$3{\cdot}7 \times 10^{-9}$	8·43	3·5	0·6
Sulphathiazole		$7{\cdot}6 \times 10^{-8}$	7·12	43·0	0·08
Sulphadiazine		$3{\cdot}3 \times 10^{-7}$	6·48	77·0	0·08
Sulphathiadiazole		$1{\cdot}7 \times 10^{-5}$	4·77	99·0	0·6
Sulphacetamide	CH_3CO—	$4{\cdot}2 \times 10^{-6}$	5·38	98·0	0·7
N^1-Chloracetylsulphanilamide	$CH_2Cl.CO$—	$1{\cdot}6 \times 10^{-4}$	3·79	100·0	10·0
N^1-Ethylsulphonylsulphanilamide	$CH_3CH_2.SO_2$—	$7{\cdot}9 \times 10^{-4}$	3·10	100·0	1000·0

Since the curve shows a maximum, which corresponds very nearly with the compounds sulphadiazine and

sulphathiazole (see pp. 132, 133) it is probable that the most active of the sulphonamide drugs are already known. Possibilities of more therapeutically useful sulphonamides lie in eliminating the objectionable side effects, toxicity, nausea and so on, and in appropriate modification of solubility and rates of absorption. Thus, although

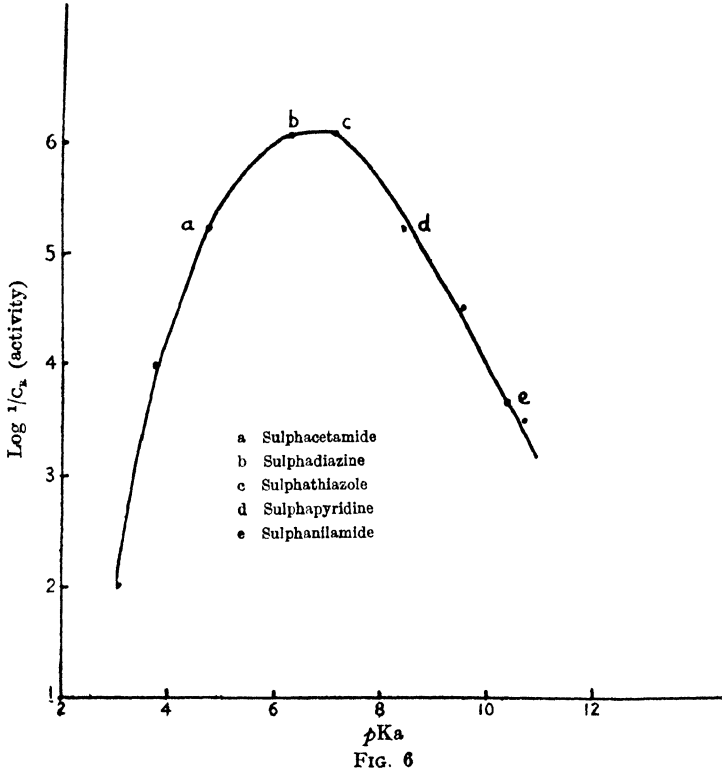
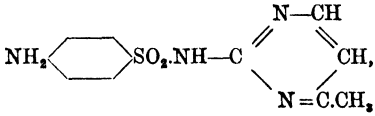


FIG. 6

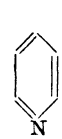
sulphadiazine is at the peak of activity, it has a low solubility, especially in acid solution, and tends to crystallize out in the urinary tract when the urine is acid or of small volume, as in hot countries. Sulphamethazine, 2-sulphanilamido-4:6-dimethylpyrimidine, is

about ten times as soluble as sulphadiazine at *p*H 7 and 37°C. and, although it has about twice the toxicity, it would probably be of greater use in the tropics. To take another example sulphamerazine, sulphamethyldiazine,



is as active as sulphadiazine, but is less readily eliminated from the body so that an adequate concentration in the blood could be obtained by less frequent administration.

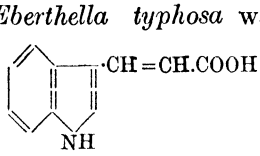
It has been claimed that the bacteriostatic power of sulphonamides can be reversed by adenine, and by methionine. Adenine sulphate, when administered to mice infected with *Str. pyogenes* in a dose of 0·8 mg. per gram prevents the chemotherapeutic effect of 2 mg. per gram of sulphanilamide or of 4 mg. per gram of sulphadiazine, sulphapyridine or sulphathiazole, being more effective than the same amount of *p*-aminobenzoic acid. Guanine and uracil had no such anti-sulphonamide action. Adenine is an essential metabolite for streptococci, forming part of the codehydrogenase and cophosphorylase systems, and it is considered that the sulphonamides may interfere with these enzyme systems. Methionine, $CH_3.S.CH_2.CH_2.CH.NH_2.COOH$, inhibits the effect of sulphadiazine on *E. coli* in synthetic medium. This property of methionine is eliminated by urea which also reverses the effect of *p*-aminobenzoic acid on sulphanilamide, and increases the potency of sulphadiazine and sulphanilamide, possibly by increasing the penetration of the drugs into the tissues. Guanidine, $\begin{matrix}NH_2\\ \diagdown\\ \quad C=NH,\\ \diagup\\ NH_2\end{matrix}$ and thiourea, $\begin{matrix}NH_2\\ \diagdown\\ \quad C=S,\\ \diagup\\ NH_2\end{matrix}$ are even more active than urea.

Following the lead given by the discovery of the *p*-aminobenzoic acid-sulphonamide inhibition mechanism of drug action a number of other systems have been investigated with analogous results. In some cases it has been possible to devise a substance which should have antibacterial properties in virtue of its close chemical relationship with a compound participating in the metabolism of bacteria. As examples may be quoted the effect of pyridine-3-sulphonic acid,  , and its amide,  , on the growth of *Staph. aureus* and *Proteus vulgaris* which require nicotinic acid for their metabolism. Pyridine-3-sulphonamide inhibits the growth of these organisms in presence of ordinarily adequate amounts of nicotinamide, and the effect is lost on increasing the amount of nicotinamide present. *E. coli* does not require nicotinamide as a growth factor and is only little affected by pyridine-3-sulphonamide at a concentration of 10^{-2} molar, but the inhibition is completely reversed by nicotinic acid or the amide. Pyridine-3-sulphonic acid, however, at a concentration 10^{-2} molar completely inhibits the growth of *E. coli* and the effect is not reversed by addition of nicotinic acid, nicotinamide or co-enzyme.

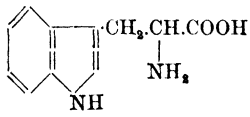
It is possible that the greater potency of sulphapyridine compared with that of sulphanilamide is due to its effect on nicotinic acid metabolism in addition to that on *p*-aminobenzoic acid. A similar inhibition by sulphapyridine is observed on the respiration of the dysentery bacillus stimulated by co-enzyme I (diphosphopyridine

nucleotide) or by nicotinamide. Sulphanilamide, sulphathiazole, and sulphapyridine all inhibit the growth of Sonne's bacillus, and this effect, but not the respiratory inhibition, is reversed by *p*-aminobenzoic acid. Sulphathiazole is said to have a similar inhibitory effect on co-enzyme or nicotinamide stimulated metabolism. The explanation may be that sulphathiazole and sulphapyridine are isosteric and accordingly could replace one another in adsorption on the co-enzyme or that sulphathiazole may act on a different part of the metabolism chain, involving decarboxylation (see p. 43). The primary action of the sulphonamides, which is reversed by *p*-aminobenzoic acid, does not affect the respiration of the cells. The secondary effects due to the pyridine or thiazole groups, for instance, which are not reversed by *p*-aminobenzoic acid, are usually concerned with respiratory processes.

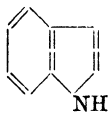
Fildes showed that the growth of *Esch. coli* and of *Eberthella typhosa* was inhibited by indole-acrylic acid,



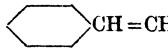
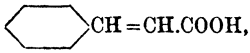
, but not by other indole derivatives and that the inhibition was removed by the addition of traces of tryptophane,

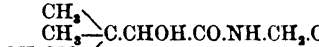
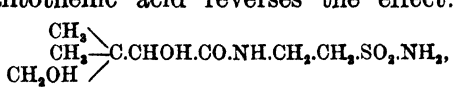
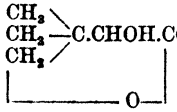


but not by indole,



. It is, therefore, assumed that the indole-acrylic acid interferes with the synthesis of tryptophane from indole. By analogy of the equivalence of α-naphthyl-acetic acid and indole-acetic acid

as plant hormones it was expected that β-naphthyl-acrylic acid,  , would inhibit the growth of *E. coli* and that the effect would be reversed by tryptophane. Inhibition is brought about by a concentration of 0·0002 M β-naphthyl-1-acrylic acid and is reversed by 0·0002 to 0·00004 M tryptophane. Styryl-acetic acid,  , and cinnamic acid,  , have similar but much weaker effects, whilst dihydro-cinnamic acid, benzoic acid and fumaric acid, COOH.CH=CH.COOH, have no such effect.

Pantothenic acid is an essential metabolite for many organisms and it has been shown that the growth of such organisms is inhibited by the addition of pantoyltaurine,  , the sulphonic acid analogue of pantothenic acid. For *Str. pyogenes* about 500 times as much pantoyltaurine as there is pantothenic acid present is required to cause inhibition. The addition of pantothenic acid reverses the effect. Pantoyltauramide,  , has a similar effect. Pantoyltaurine can also inhibit the growth of yeast. Mixtures of taurine, $NH_2.CH_2.CH_2.SO_3H$, and αγ-dihydroxy-ββ-dimethyl-butyrolactone,  , or the compounds alone had no inhibitory effect on *L. arabinosus* which is inhibited by pantoyltaurine. N-pantoyl-β-aminoethyl thiol,



bis (pantoyl-β-aminoethyl) disulphide,



are about equal in their activity against *L. arabinosus* and *Str. pyogenes*, *in vitro* and *in vivo* respectively.

It has been found possible to protect rats against many thousand lethal doses of *Str. pyogenes* by frequent subcutaneous injections of pantoyltaurine in amount

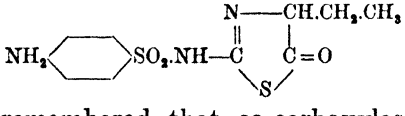
TABLE 14

Organism	Addenda		Growth
	Inhibitor	Metabolite	
	0	0	+
Str. pyogenes -	Sulphanilamide	0	0
E. coli - -	3×10^{-4} M	*p*-Aminobenzoate, 10^{-7}M	+
	,,	Pantothenate, 10^{-7} to 10^{-4}M	0
	,,	Nicotinamide, 10^{-7} to 10^{-4}M	0
	0	0	+
Staph. aureus -	Pyridine-3-sulphonamide 10^{-3}M	0	0
	,,	*p*-Aminobenzoate, 10^{-7}M	0
	,,	Pantothenate, 10^{-7} to 10^{-4}M	0
	,,	Nicotinamide, 10^{-5}M	+
Str. pyogenes -	0	0	+
Dip. pneumoniæ -	Pantoyltaurine	0	0
C. diphtheriæ -	,,	*p*-Aminobenzoate, 10^{-7}M	0
	,,	Pantothenate, 10^{-6}M	+
	,,	Nicotinamide, 10^{-7} to 10^{-4}M	0

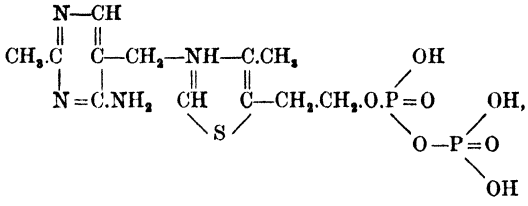
sufficient to counteract the pantothenic acid present in the blood. Mice, which normally have a considerably higher content of pantothenic acid in the blood, are not protected by such treatment because enough pantoyltaurine cannot be administered. Since human blood has somewhat less pantothenic acid than rat blood it should be possible to protect man against streptococci and *C. diphtheriæ*, which is also sensitive to pantoyltaurine.

The specific effects of metabolities on certain inhibitors is illustrated in Table 14, due to McIlwain, which shows that inhibition is reversed only by the corresponding metabolite.

By testing the effect of a number of drugs, such as sulphathiazole, containing the thiazole ring, it has been shown that they can interfere with the decarboxylation of pyruvic acid (see p. 277), by *Staph. aureus*, *E. coli*, yeast and by a carboxylase preparation from yeast. Sulphathiazole is most effective against *Staph. aureus* and *E. coli*, whilst sulphanilamide and sulphapyridine have very little effect. The most active compound was sulphanilamido-5-ethyl-4-thiazolone,

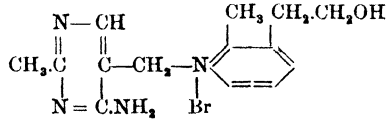


It will be remembered that co-carboxylase is aneurin diphosphate (p. 43),



which contains a thiazole ring. This may, in part, explain the greater potency of sulphathiazole, as compared with sulphanilamide, against many organisms.

Those species of bacteria, yeasts and fungi which require aneurin for their maximum growth are inhibited by pyrithiamine, the pyridine analogue of aneurin,



whilst other organisms are not inhibited. The inhibition is overcome by the addition of aneurin. The more exacting a species is in its requirement for aneurin the more readily is it inhibited by pyrithiamine. The ratio of pyrithiamine to aneurin is about 700 for *Staph. aureus* and 20,000 for *Esch. coli*. Species requiring intact aneurin are much more sensitive than those requiring only the pyrimidine moiety or those requiring both the pyrimidine and thiazole portions of the aneurin molecule. The organisms which are not affected by pyrithiamine do not synthesise increased amounts of aneurin in its presence in the same way that sulphonamide resistant organisms synthesise greater quantities of *p*-aminobenzoic acid. By growing the yeast, *Endomyces vernalis*, in the presence of small amounts of pyrithiamine a strain resistant to 25 times the normally inhibitory concentration has been developed. It still required aneurin, or its pyrimidine moiety, as a growth factor but, in their absence, was capable of converting pyrithiamine into the pyrimidine part of the aneurin molecule.

The respiration of *Plasmodia* species causing malaria is stopped by the inhibitory action of quinine, plasmoquin, or atebrine on the hydrogenase and cytochrome oxidase systems involved.

Drug Resistant Strains.—During the investigation of chemotherapy it very soon became apparent that micro-organisms developed resistance to drugs. In fact most organisms which have survived treatment by a drug became resistant to its action. Thus Ehrlich showed

that trypanosomes became resistant to dyes and to arsenic compounds. He showed that such organisms no longer took up the arsenical drug, or were not stained by the dye as were susceptible organisms. He explained this as being due to loss of affinity of the specific receptor in the organism for the drug. Trypanosomes which were resistant to atoxyl were also resistant to (and unstained by) dyes of the acridine, oxazine, and thiazine series but not to those of the trypan-blue type nor to those of the triphenylmethane series. Ehrlich noted that although trypanosomes might be resistant to atoxyl or tryparsamide they were not resistant to arsenophenyl glycine. It has since been shown that tryparsamide resistant organisms are not resistant to phenylarsenoxide or derivatives of it containing carboxyl groups and that they take up the compounds in the same way as non-resistant strains. It is considered that the active compounds are either readily water soluble or lipoid soluble and therefore easily penetrate the parasite. Trypanosomes may contain up to 60 per cent. of lipoid substances. The lethal arsenic atom can then come into contact with the susceptible groups in the organism and cause its death. The arsenicals or dyes to which the organism is resistant fail to act because they are not taken into the organism or get held up on some non-vital structure. Arsenoxides react very readily with sulphydryl groups and may kill the organism by inhibiting essential enzymes which contain SH groups, in the same way as mercury does.

Strains of pneumococcus become resistant to sulphapyridine if the dosage of the drug used for treatment has been inadequate. They can also be produced *in vitro* by growing the organism in media containing gradually increasing amounts of sulphapyridine. Such resistant organisms have the same morphology, virulence and immunological properties as the parent strains. Usually a bacterium which has become resistant to one of the sulphonamide drugs is also resistant to other

sulphonamide drugs, but not to drugs of different types such as the dyestuffs of the acridine series or the propamidines. It has been found that sulphonamide resistant organisms have acquired the property of increased production of *p*-aminobenzoic acid so that their growth is no longer inhibited by the drug.

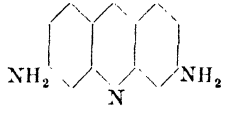
Strains of hæmolytic streptococci and of *C. diphtheriæ* have been produced which are resistant to the action of pantoyltaurine. Some such strains also occur naturally. These strains are sensitive to the sulphonamides, and sulphonamide resistant strains are sensitive to pantoyltaurine. The varying resistance of naturally resistant strains of *C. diphtheriæ* to pantoyltaurine is associated with their ability to convert β-alanine into pantothenic acid, instead of having to be supplied with the latter, which reverses the effect of pantoyltaurine. McIlwain has shown that this mechanism cannot apply to *Str. pyogenes* since many naturally resistant strains and all experimentally produced resistant strains still need to be supplied with pantothenic acid and cannot utilise β-alanine instead. Resistant strains of *Str. pyogenes* and strains of *Proteus morganii*, *Leuconostoc mesenteroides*, *Lactobacillus* and propionic acid bacteria become susceptible to pantoyltaurine when salicylate is added. This is explained on the assumption that salicylate acts on the same groups as does pantoyltaurine, that is on enzymes involved in pantothenic acid metabolism. It should be pointed out that pantothenic acid antagonises the action of salicylate. If this is true, pantoyltaurine resistant strains of *Str. pyogenes* differ from susceptible strains in having alternative processes for utilising pantothenic acid which are not blocked by pantoyltaurine, although they may still be inhibited by salicylate. Resistant strains of a normally susceptible organism are found occurring naturally and must have arisen by a means other than "training" in the presence of the drug. A clue to the mechanism by which this can happen

is found in the fact that pantoyltaurine resistant strains of *C. diphtheriæ* can utilise β-alanine instead of pantothenic acid. If susceptible strains were grown in the presence of large amounts of β-alanine, or were gradually trained to do without pantothenic acid, it would be expected that they would become resistant to pantoyltaurine and in fact this has been shown to take place. The converse of this can also happen; if *C. diphtheriæ* is repeatedly subcultured in media rich in pantothenic acid it becomes progressively more exacting in its need for pantothenic acid and at the same time more susceptible to pantoyltaurine. This behaviour is very similar to that postulated by Knight to account for the more complex demands of the parasitic organisms and viruses as compared with those of saprophytic and autotrophic bacteria (see Chapters VI and VII). In general drug resistance, once acquired, is stable through many generations of subculture on ordinary media. The development of drug resistance by "training" appears to take place in two stages. In the early stages the resistance is easily reversed, does not survive continued sub-culture on ordinary media, and is specific to the drug used, related drugs being active. The resistance is probably due to the stimulation of a reserve, less efficient, growth mechanism which is present in the organism but normally plays only a minor rôle in metabolism. In the early resistant phase it serves to tide over the organism, until it has elaborated the final alternative mechanism which confers permanent resistance on the organism. This permanent mechanism may be a new way of by-passing the mechanism normally inhibited by the drug, or it may involve the development of an enzyme system which can synthesise enough of an antagonist to the drug to overcome its effects.

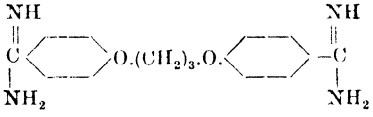
The ease with which it is possible to produce resistance to different drugs by "training" varies considerably. Streptococci and staphylococci become readily resistant

to sulphonamides and to penicillin, but resistance to the acridine dyes and propamidine is harder to induce.

Staphylococci resistant to proflavine,



are also resistant to propamidine,



and *vice versa*.

CHAPTER XI

ANTIBIOTICS

THE term antibiotics is used of those substances produced by micro-organisms which have an antagonistic effect, usually specific, on other organisms. Antibiosis results, therefore, from the growth of an organism evolving an antibiotic, in presence of another, susceptible, organism, in contrast to symbiosis which occurs when two micro-organisms grow together with mutual benefit (see pp. 102, 109).

The antagonistic effect of some micro-organisms on others has been known for many years. The anthrax bacillus, for example, was shown by Pasteur to be inhibited by aerial contaminants ; lactic acid bacilli will overgrow *Cl. butyricum* in the butyl alcohol/acetone fermentation, because the large amount of lactic acid which they form produces conditions under which *Cl. butyricum* cannot survive (see p. 315). Substances like lactic acid which act in a non-specific way by altering the physical condition of the environment are not, as a rule, called antibiotics ; the expression antibiotic is reserved for substances which act specifically on a few species of organisms and which are usually active in very small amounts. That is they have an action which is almost the reverse of that of growth factors, probably by interfering with enzyme systems involved in metabolism. Antagonism also results from other causes such as the " swamping " of a slow growing organism by a fast growing one which competes successfully for the available nutrients, or by the production by one organism of conditions of oxidation-reduction potential unfavour-

able to another which then dies out. We are concerned here only with bacteriostatic or bactericidal substances produced by micro-organisms. Soil is a rich source of micro-organisms which have antibiotic properties.

A considerable number of antibiotic substances is now known. The more important of them will be dealt with in turn.

Actinomycetin.—Many species of *Actinomycetes* produce substances which are lytic to living and dead bacteria. Thus *Actinomyces albus* yields the water soluble, thermolabile protein-like material actinomycetin which will lyse living or dead Gram-positive organisms and dead Gram-negative organisms. It can be precipitated by alcohol and appears to have the properties of a proteolytic enzyme. A similar substance has been isolated from *A. violaceus*, which, although heat stable, otherwise resembles the enzyme lysozyme which occurs in egg white and tears and lyses most non-pathogenic bacteria and also streptococci and staphylococci. Its substrate is a mucopolysaccharide which it breaks down to an acetylated amino-hexose and a ketohexose.

Actinomycin.—The brown pigmented soil organism *A. antibioticus* is very active against almost all bacteria, and fungi, especially Gram-positive bacteria. An active substance, actinomycin, was isolated from it by extraction with ether and fractionated into actinomycin A, soluble in alcohol and in petrol and giving a clear aqueous solution, and actinomycin B which is soluble in petrol, difficultly soluble in alcohol and gives a turbid suspensino in water.

Actinomycin A is a bright red crystalline polycyclic nitrogen compound, C, 59·0 per cent.; H, 6·68 per cent.; N, 13·35 per cent.; m.p. 250°C. (with decomposition), $[\alpha]_D^{25} - 320°$ It has a molecular weight about 800. It has the properties of a reversible oxidation-reduction indicator and is probably of quinonoid structure. It is

thermostable. It is very strongly bacteriostatic to many Gram-positive organisms even at a dilution of 1 in 10^8. Gram-negative bacteria are usually less sensitive (dilutions of 1 in 5000 to 1 in 10^5 being necessary for bacteriostasis) but there is no clear dividing line. It is only slowly bactericidal. Actinomycin A inhibits the fibrinolytic activity of cultures or filtrates of hæmolytic streptococci and the coagulase activity of staphylococci. It is highly toxic to animals, when injected intraperitoneally, intramuscularly or intravenously.

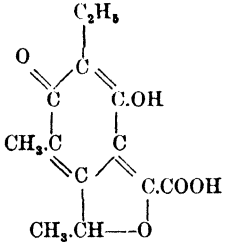
Actinomycin B is a colourless compound which is only slightly bacteriostatic but highly bactericidal to Gram-positive organisms at concentrations of 1 mg. in 100 ml. Gram-negative bacteria are more resistant. It is also highly toxic to animals.

Actinomycin B predominates in young cultures of *A. antibioticus* and Actinomycin A in old cultures.

Aspergillic Acid.— *Aspergillus flavus*, when grown as a surface culture on a peptone medium gives yields of 250 to 400 mg. of crystalline aspergillic acid per litre of medium. Aspergillic acid is a monobasic, amphoteric acid, $C_{12}H_{20}N_2O_2$, m.p. 93°C., $[\alpha]_D + 14°$. It can be distilled in steam or *in vacuo* without loss of activity and is stable to acid and to alkali. When grown on a peptone medium containing 2 per cent. of brown sugar *A. flavus* gives a closely related substance, $C_{12}H_{20}O_3N_2$, containing one oxygen atom more than aspergillic acid, and having m.p. 149°C, $[\alpha]_D + 42°$ and about one tenth the activity of aspergillic acid. The substance isolated by Glister from a species of *Aspergillus* related to, but not identical with *A. flavus*, active against *Esch. coli*, *Eberthella typhosa*, *Salmonella paratyphi*, *Sal. schottmülleri*, *Shigella dysenteriæ* and *Vibrio comma*, as well as against Gram-positive organisms, at a dilution of one in 200,000 is aspergillic acid. It is bacteriostatic to Gram-positive and Gram-negative bacteria, *e.g.*, streptococci, staphylococci, pneumococci, *Esch. coli*, *Aerobacter aerogenes*, in

concentrations of 1 in 100,000 to 1 in 400,000. It is bactericidal at dilutions of 1 in 25,000 to 1 in 50,000. It is relatively highly toxic to animals. It will not protect mice against infection with hæmolytic streptococci or pneumococci but prevents the lethal action on mice of gonococci suspended in mucin solution and saves guinea-pigs from the action of gas gangrene organisms. Its antibacterial activity can be measured by its inhibition of the luminescence of *Photobacterium fischeri*. *A. flavus* when grown as a submerged culture in agitated Czapek-Dox medium does not produce aspergillic acid but a substance which is very similar to or identical with penicillin in chemical and biological properties (see p. 171 *et seq.*).

Citrinin.—*Penicillium citrinum* when grown on Czapek-Dox or Raulin medium produces citrinin,



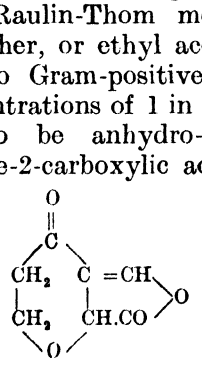
which can be precipitated from the medium, in yields of about 2 g. per litre, as a yellow microcrystalline substance by the addition of hydrochloric acid. It is also formed by *Aspergillus candidus*. It has m.p. 168° (decomp.). Its sodium salt is soluble in water. The culture filtrate (containing about 2 g./litre of citrinin) is inhibitory to *Staph. aureus* in dilutions of 1 in 160 to 1 in 320. Citrinin itself is bacteriostatic to Gram-positive and Gram-negative organisms in concentrations of 1 in 9000 to 1 in 30,000, the Gram-positive organisms, in general, being the more sensitive.

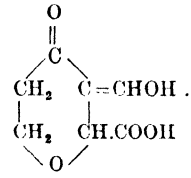
Clavacin.— *Aspergillus clavatus* when grown on Czapek-Dox medium gives antibacterial filtrates from which clavacin can be isolated by extraction with ether or chloroform or by adsorption on charcoal followed by elution with ether. It is a relatively stable substance even in strongly acid solution. It is bactericidal in concentrations of 1 in 10^5, to Gram-positive and Gram-negative organisms. It has also been called clavatin.

At about the same time *Penicillium claviforme* was shown to yield an optically inactive, colourless, crystalline substance, m.p. 110°C. which could be extracted from culture filtrates with chloroform. It was stable to boiling dilute acid, but not to alkali, nor to boiling in neutral solution. It was bactericidal to pathogenic Gram-positive and Gram-negative bacteria and killed leucocytes at a dilution of 1 in 800,000. It is lethal to mice (0·25 mg. intravenously, 2 mg. subcutaneously or 2·5 mg. *per os*). It was given the name claviformin.

Later *Aspergillus giganteus*, when grown on a medium containing 4 per cent. of glucose, 0·1 per cent. of sodium nitrate and 0·1 per cent. of potassium dihydrogen phosphate, was also shown to yield claviformin. The substance is also a product of the growth of a species of *Gymnoascus*.

A fifth mould, *Penicillium patulum*, produced a colourless, crystalline substance, given the name patulin, when grown on Raulin-Thom medium, which could be extracted by ether, or ethyl acetate. It was found to be inhibitory to Gram-positive and Gram-negative organisms at concentrations of 1 in 30,000 to 1 in 80,000. It was shown to be anhydro-3-hydroxymethylene-tetrahydro-γ-pyrone-2-carboxylic acid,



which on treatment with dilute alkali or on boiling in water gives the acid,  . Patulin is toxic

to mice and rabbits in doses of the order of 0·25 to 0·5 mg. per 20 g. body weight. In concentrations of 1 in 2000 it is inhibitory to phagocytosis. Conflicting reports have been published about its efficacy in curing the common cold. The result probably depends on the particular organisms concerned in the secondary stages of the cold, some being susceptible to patulin and some not. The primary virus stage of the cold appears not to be affected by patulin.

Since clavacin is inactivated by excess of SH compounds it is possible that it exerts its action by inhibiting SH-containing essential metabolites or bacterial enzymes.

It has been shown by chemical, biological and X-ray evidence that clavacin, claviformin and patulin are identical compounds.

Clavatin.—This substance is identical with clavacin.

Claviformin.—This substance is identical with clavacin.

Flavacidin.—This substance, produced by *A. flavus* in deep, agitated, aerated cultures in a modified Czapek-Dox medium, is very probably identical with penicillin.

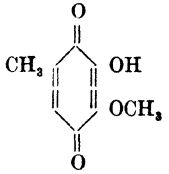
Flavicin.— *Aspergillus flavus* when grown on a modified Czapek-Dox medium containing corn steep liquor gives rise to a bacteriostatic substance which can be extracted with *iso*propyl ether. The purified material is active against the Gram-positive organisms, staphylococci, streptococci, *C. diphtheriæ*, and *B. anthracis* at concentrations of 0·006 to 0·008 mg./ml. Gram-negative organisms are much less sensitive, *E. typhosa*, *Shigella*

dysenteriæ and *Vibrio comma* being inhibited by 0·8 mg./ml. Flavicin is bactericidal as well as bacteriostatic. When injected in small doses, 50 mg./kg. of body weight, it protects mice against infection by Type I pneumococci.

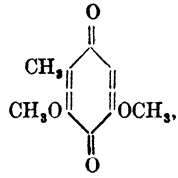
Flavicin resembles penicillin in being a soluble acid which is unstable in acid solution. It differs from penicillin in being more active against *C. diphtheriæ*, *B. anthracis* and *Brucella abortus*.

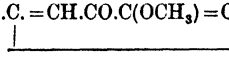
Fumigacin.— *Aspergillus fumigatus* produces, in synthetic media, in the first few days of growth the antibiotic fumigacin, which is readily soluble in chloroform or ethanol and to a limited extent in ether or water. It is precipitated from alcoholic solution on cooling as colourless long, slender, needle-shaped crystals. Fumigacin has m.p.220°C. and $[\alpha]_D = -132°$ (c = 0·4 in chloroform), contains neither nitrogen nor sulphur, is weakly acidic, and gives a methyl ester corresponding to the formula $C_{29}H_{38-40}O_7$ for the original substance. The silver salt, however, corresponds to $C_{32}H_{44}O_3Ag$. Fumigacin contains a lactone group in addition to the carboxyl group and on treatment with alkali gives a crystalline, inactive sodium salt. Its general properties agree with those of helvolic acid (see p. 167) which has a formula $C_{32}H_{44}O_8$, corresponding to that of the silver salt mentioned above. The specific rotation of helvolic acid is given as $[\alpha]_D = -49·4°$ in chloroform, a difference which may, possibly, be due to complete or partial conversion of the lactone to the free acid. It is active against Gram-positive organisms at dilutions of 1 in 200,000 to 1 in 600,000 and only slightly active against Gram-negative bacteria (dilution of 1 in 1000). In concentrations of 200 μg. per ml. it is bactericidal. It is thermostable, and not very toxic to animals but doses near the toxic limit are necessary to protect mice against infection with *Streptococcus pyogenes*.

Fumigatin.— *Aspergillus fumigatus*, in addition to fumigacin, synthesises the pigment fumigatin which has been shown to be 3-hydroxy-4-methoxy-2 : 5-toluquinone,



which inhibits the growth of Gram-positive bacteria at dilutions of 1 in 33,000 to 1 in 50,000, and Gram-negative organisms at 1 in 12,000. It is particularly active against *V. comma* which is inhibited at a concentration of 1 in 100,000. A number of mono-, di- and tri-methoxy derivatives of toluquinone and of benzoquinone have been tested for activity against *Staph. aureus*. 4-Methoxy-, 4 : 6-dimethoxy-, 3 : 4 : 6-trimethoxy- (spinulosin dimethyl ether), and 6-hydroxy-4-methoxy- toluquinones and 2 : 6-dimethoxybenzoquinone have considerably greater activity than fumigatin. Introduction of the OCH_3 group increases the activity whilst an OH group reduces it. The most active is 4:6-dimethoxy-toluquinone,

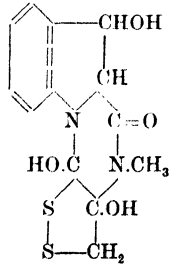


, of which 10μg. per ml. is inhibitory. The active structure seems to be $=C=C.CO.C(OCH_3)=C.CO$ which is also present in penicillic acid (see p. 170). Replacement of the terminal — CO group by oxygen (as in the methyl derivative of kojic acid ) (see p. 294) causes a great loss in activity.

Gigantic Acid.—The growth of *A. giganteus* on a 2 per cent. malt extract containing 1 per cent. of peptone

and 5 per cent. of tri-ethanolamine buffer at *p*H 8·2 yields a product which is very similar to penicillin in its properties.

Gliotoxin.—*Gliocladium fimbriatum*, when grown in an agitated culture medium containing sucrose and peptone at *p*H 3 to 3·5, yields gliotoxin which can be extracted with chloroform. It is also formed along with fumigacin by *A. fumigatus* in surface or submerged cultures and from an unidentified strain of *Penicillium*. The material can be recrystallised from methanol and has m.p. 221°C. (decomp). It is optically active, having $[\alpha]_D^{25} -290°$ in ethanol, $-270°$ in pyridine, and $-255°$ in chloroform. In ethanol containing sodium hydroxide it undergoes mutarotation from $[\alpha]_D^{25} + 111°$ to $+ 80°$ after 48 hours and to 0° after 5 days. Gliotoxin has an ultra-violet light absorption curve similar to those of tryptophane and indole. It is a neutral substance with the composition $C_{13}H_{14}N_2O_4S_2$. It appears to be an indole derivative having a third 6-membered ring at positions 1 and 2. The third ring contains the second nitrogen atom, which carries a methyl group, and is bridged by a disulphide group. The oxygen is present in the form of hydroxyl groups. On heating with hydriodic acid or with dilute alkali, gliotoxin loses its sulphur to give an α-pyrazindole derivative, $C_{13}H_{12}O_2N_2$, m.p. 122°C., which is biologically inactive. Gliotoxin very probably has the structure :



Alteration of the S linkages, even by the mildest alkali,

causes loss of bacteriostatic activity. Gliotoxin is inactivated by cysteine and by thioglycollic acid which change the disulphide grouping to the disulphydryl grouping. The activity is restored by mild oxidation by air or iodine. It is suggested that gliotoxin is active by the oxidation of SH groups of enzymes to the inactive disulphide, -S-S-, form.

In a concentration of 10μg./ml. gliotoxin prevents the growth of all pathogenic organisms tested; 0·2 to 0·3 μg. per ml. was adequate to inhibit hæmolytic streptococci and Type III pneumococci. It is also inhibitory to *A. niger*, *P. italicum* and *Rhizopus*. It is lethal to mice and rabbits in doses of 45 to 65 mg. per kg. of body weight. Less than the lethal dose causes kidney lesions and hæmaturia.

Gramicidin.—In 1939 Dubos isolated from soil a spore bearing bacillus, which has since been named *B. brevis*. Autolysates of the organism contained a soluble substance, tyrothricin, capable of lysing living Gram-positive cocci. The substance is non-volatile, does not dialyse through collodion and is heat stable. It is very stable to alkali but not to acid. 0·02 μg. per ml. inhibits the growth of pneumococci, whilst staphylococci and streptococci are not quite so sensitive. Gram-negative organisms are not affected even by large amounts of the substance. The intraperitoneal injection of 2 mg. of the extract protected mice against infection with pneumococci. Tyrothricin is not to be confused with the enzyme, produced adaptively by some soil bacilli, which destroys the capsule of Type III pneumococci only, rendering them susceptible to phagocytosis. Tyrothricin inhibits the fibrinolytic action of β-hæmolytic streptococcal filtrates and the coagulase activity of staphylococcal filtrates. Tyrothricin has been shown to be a mixture. By extraction of the crude material with equal parts of acetone and ether, evaporation to dryness and extraction with warm acetone a crystalline substance, gramicidin, is obtained on cooling

the acetone solution. The residue insoluble in the acetone-ether mixture is taken up in boiling ethanol and acidified with hydrochloric acid when a crystalline material, tyrocidine hydrochloride, separates out (see p. 182). Tyrothricin contains about 10 to 20 per cent. of gramicidin and 40 to 60 per cent. of tyrocidine. Gramicidin is a neutral substance which is specifically bacteriostatic to Gram-positive bacteria. Gramicidin has the property of depressing the surface tension of aqueous solutions; the property is not lost on heating, although the bacteriostatic and hæmolytic effects are destroyed by heat. Gramicidin does not lose its bacteriostatic properties in the presence of serum. It will protect mice from infection by Gram-positive organisms when it is injected in contact with the organisms but not otherwise. Gramicidin has been shown to detoxify tetanus and diphtheria toxins. It is too toxic to be of use for internal chemotherapy but has been used for local application. Gramicidin has been shown to be a closed ring polypeptide containing 24 amino acid residues, having a molecular weight of 2790, m.p. 230 — 231°C., $[\alpha]_D^{25}$ + 2·5°. It contains no free amino or carboxyl groups. The amino acid residues comprise 6 of leucine, 6 of tryptophane, 5 of valine, 3 of alanine, 2 of glycine and 2 of a hydroxy-amino compound, possibly *iso*-serine. The leucine and two or three of the valine units are in the *d*-form. It is resistant to the action of trypsin, pepsin and papain. It is possible that the *d*-amino acids, very rarely found in nature, may be responsible for its bacteriostatic properties.

The action of formaldehyde on gramicidin greatly reduces its toxicity and hæmolytic activity without harming its bacteriostatic properties. The modified gramicidin may possibly be used for internal therapy.

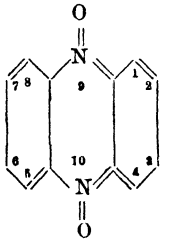
Gramicidin-S.—A substance of a similar character to gramicidin has been obtained by Russian workers from a soil bacillus. Gramicidin-S is very stable to heat,

has m.p. 268-270°C. and molecular weight about 1250. It is of peptide nature but contains free NH_2 and COOH groups. It also differs from gramicidin in containing proline and ornithine but no tryptophane. It is different in that it is bacteriostatic to some Gram-negative organisms, e.g., *E. typhosa* at 50μg./ml., *Sh. dysenteriæ* at 12μg./ml., *V. comma* at 25 μg./ml. and *P. vulgaris* at 100 μg./ml., as well as to Gram-positive organisms.

Helvolic Acid.—A mutant of *A. fumigatus* mut. *Helvola* when grown on a glucose-salts medium gives a substance which can be adsorbed on charcoal at *p*H 4 and eluted with 80 per cent. acetone. It can be purified by chromatography of a chloroform solution and recrystallised from acetone. The yield is 0·4 g. from 100 litres of medium. It is a monobasic acid, $C_{32}H_{44}O_8$, containing three active hydrogen atoms per molecule, m.p.205°C, $[\alpha]_D^{20} -49{\cdot}4°$ in chloroform. It is almost insoluble in water, but gives a soluble sodium salt; salts with other metals are very sparingly soluble. Its activity is not affected by heating to 100°C. with 2 N acid for fifteen minutes, or in neutral solution or with alkali at *p*H 10. It is bacteriostatic to Gram-positive but not to Gram-negative organisms, but has no effect on the respiration of suspensions of staphylococci. Human tubercle bacilli are inhibited by a 1 in 10,000 dilution of helvolic acid. Mice will tolerate 5 mg. of sodium helvolate intravenously and 20 mg. orally. Leucocytes are not injured by a dilution of 1 in 1600 nor are tissue cultures by 1 in 2500 sodium helvolate. It is absorbed from subcutaneous tissue and from the gut and is excreted in the urine and bile. Although antibacterial concentrations can be maintained in the blood stream, repeated injection leads to liver damage. This probably accounts for the fact that the lives of mice infected with staphylococci or streptococci can be prolonged but not saved by sodium helvolate.

Helvolic acid is probably the acid corresponding to the lactone, fumigacin (see p. 162).

Iodinin.—*Chromobacterium iodinum* gives the pigment odinin which is the di-N-oxide of a dihydroxy phenazine,



. The position of the two hydroxyl groups is unknown, but they are probably not in the 2 : 3 or 2 : 5 positions. The growth of streptococci is partially inhibited by 3×10^{-7} molar concentrations and completely inhibited by 1 to 2×10^{-6} molar iodinin. Phenazine di-N-oxide has a similar but weaker effect. When grown in the presence of sub-inhibitory quantities of iodinin or phenazine di-N-oxide, organisms multiply and destroy the compounds, probably by reduction. Extracts of a wide variety of yeasts, bacteria and plant and animal tissues have no effect on the inhibition, but those which are active contain anthraquinones or naphthoquinones. Pure hydroxyanthraquinones and 2-methyl-1 : 4-naphthoquinone (vitamin K) antagonise 2×10^{-6} molar iodinin at concentrations between 5×10^{-7} and 5×10^{-6} molar, the iodinin being destroyed. The destruction does not take place in the absence of the organisms. It is suggested that the quinones are probably concerned in hydrogen transportase systems which can be interfered with by iodinin or phenazine di-N-oxide.

Notatin.—*Penicillium notatum*, during growth on a modified Czapek-Dox medium, produces an antibiotic which is different from penicillin (see p. 171). It has been called notatin, penicillin-A, penicillin-B, or penatin. The formation of notatin is repressed by the addition of

corn-steep liquor, yeast extract, brown sugar or malt extract to the Czapek-Dox medium. It can be separated by concentration of the culture filtrate and precipitation with acetone, or by tannic acid. It is a buff coloured powder, soluble in water but not in organic solvents. It is decomposed by 70 per cent. aqueous methanol at 30°C. or by aqueous solutions of trichloracetic acid to give a protein and a prosthetic group, neither of which is active alone. Its activity is lost below *p*H 2 and above *p*H 8 or by heating at 60°C. It is not destroyed by pepsin at *p*H 3·8, trypsin at *p*H 5·7, takadiastase or emulsin. It is almost completely inactived by activated papain at *p*H 3·8. Blocking of the amino groups by formaldehyde, nitrous acid, or phenyl-isocyanate does not cause loss of activity. It has the properties of a "yellow enzyme," that is a flavoprotein enzyme (see p. 42). In the presence of glucose and oxygen it has a very powerful bactericidal effect on all bacteria which are sensitive to hydrogen peroxide, e.g., *Staph. aureus*, *Str. pyogenes*, *Dipl. pneumoniæ*, *E. typhosa*, *Sal. paratyphi*, *Sal. schottmülleri*, *Sal. typhi-murium*, *V. comma*, *B. anthracis* and *Proteus vulgaris*. The effect is reversed by the presence of catalase. Notatin is a glucose aerodehydrogenase which, under aerobic conditions, oxidises glucose to gluconic acid with production of hydrogen peroxide which is the directly lethal agent. Glucose can be replaced by galactose or xylose but not by other sugars.

The xanthine-oxidase in milk is also a flavoprotein enzyme which catalyses the oxidation of xanthine or hypoxanthine to uric acid and hydrogen peroxide. In the presence of its substrate (present in ordinary meat-broth media but not in peptone water or hydrolysates of purified proteins) the growth of organisms sensitive to hydrogen peroxide is inhibited. The effect is eliminated by the presence of catalase, which destroys the hydrogen peroxide formed, or by the absence of the appropriate

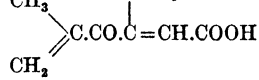
substrate. The growth of *E. coli*, *Bact. lactis aerogenes*, *Shigella flexneri* or *C. diphtheriæ*, which are insensitive to hydrogen peroxide, is not affected by either of these enzymes.

Parasiticin.— *A. parasiticus*, when grown on a medium containing peptone or 7 to 8 per cent. of corn-steep liquor and small amounts of glucose at *p*H 7, gives culture filtrates which are active at dilutions of one in 200 to 600 against Gram-positive organisms but not against Gram-negative organisms. The substance responsible for the activity can be adsorbed on charcoal and eluted by aqueous acetone. It has properties very similar to those of penicillin and to the substances obtained from *A. flavus* (see p. 161) and *A. giganteus* (see p. 163) and may be identical with them.

Patulin.—This substance is identical with clavacin (see p. 160).

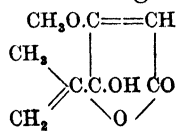
Penatin.—This substance is identical with notatin (see p. 168).

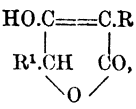
Penicillic Acid.—The growth of *P. cyclopium* on Raulin's medium (but not on Czapek-Dox medium) leads to the formation of about 2g. per litre of penicillic acid

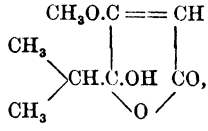


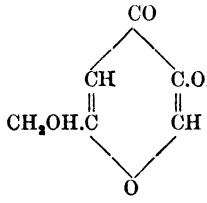
It is inhibitory to staphylococci, *E. coli* and Gram-positive and Gram-negative pathogens at dilutions of 1 in 30,000 to 1 in 100,000 but is only poorly bactericidal.

Penicillic acid, which is a colourless, crystalline substance with m.p. 86°C, soluble in water and chloroform, can also exist in a closed ring form,



which is β-methoxy-γ-hydroxy-γ-*iso*propylidene tetronic acid. Other tetronic acids produced by fungi, 

such as carlic, carolic, carolinic and carlosic acids (see p. 296) and dihydropenicillic acid, 

are not antibacterial. The somewhat similarly constituted kojic acid,  (see p. 294) is much less active than penicillic acid.

It is possible that penicillic acid is active because it combines with such amino-acids as glycine, alanine and *p*-aminobenzoic acid, all of which reverse its effect.

Penicillin.—In 1929 Fleming noted that the growth of *Staph. aureus* on a plate contaminated by *Penicillium notatum* was inhibited in the neighbourhood of the mould. He showed that the growth of *P. notatum* on a fluid medium gave a culture filtrate which was strongly bacteriostatic to certain Gram-positive bacteria. The active substance, penicillin, which is also formed by *P. chrysogenum* and *A. flavus*, can be extracted from acidified culture filtrates with ether or other organic solvents in the cold and can be taken back into aqueous

solution by washing the extract with dilute alkali, which converts penicillin into the readily soluble sodium salt; concentration of the penicillin can be effected at the same time by use of a small volume of alkali. Further purification can be achieved by chromatography on alumina or silica gel carrying an alkaline earth carbonate, and conversion to the barium salt. The barium salt is fairly stable between *p*H 5·5 and 7·5, but is easily inactivated by heating or by acid or alkali. The free acid, penicillin, is hygroscopic and loses its activity readily, although in ether or amyl acetate solution it is stable for some days. It is inactivated by copper or mercury, primary alcohols, ammonia, amines, hydrazine, hydroxylamine and oxidising agents.

Culture filtrates have been shown to contain one or more of several closely related compounds first recognised by somewhat different degrees of activity against various bacteria. They have the general empirical formula, $C_9H_{11}O_4SN_2.R$, and differ in the nature of the radical R, as is shown :—

British Name	American Name	R
Penicillin I - -	Penicillin F	Δ^2-pentenoyl, $-CH_2.CH=CH.CH_2CH_3$
Dihydropenicillin I	—	*n*-Amyl
Penicillin II - -	Penicillin G	Benzoyl
Penicillin III -	Penicillin X	*p*-Hydroxybenzyl
Penicillin K - -	Penicillin K	*n*-Heptyl

That the penicillins probably have either the β-lactam structure, I, or the incipient azlactam structure, II, is in harmony with the following findings :—

II (Penicillin, incipient azlactam)
V (Penicillamine)
VI (Penaldic acid)
VII (Penillo-aldehyde)
Hot, dilute acid
Alkali or Penicillinase
VIII (Penicilloic acid)
cold, dilute acid
I (Penicillin, β-lactam)
Methanol
III (Penillic acid)
HgCl2
IV (Penillamine)
+CO2
X (Isopenillic acid)
Ba(OH)2
IX (Methyl ester, inactive)

They are strong monobasic acids with $pK=2{\cdot}8$. The probable presence of a masked basic group is shown by a slow titration with perchloric acid in acetic acid. Hydrolysis of penicillin with dilute acid at 30°C. gives the penillic acids, III, which are dicarboxylic acids containing a basic group but no SH group. Treatment of the penillic acids with cold aqueous mercuric chloride causes loss of carbon dioxide and gives rise to penillamines, IV, which contain an SH group, whilst treatment with baryta converts penillic acid to *iso*-penillic acid, X. Hydrolysis of penicillin with hot dilute mineral acids gives carbon dioxide, penicillamine, V (*d*-$\beta\beta$-dimethyl cysteine) and penilloaldehydes, VII; that from penicillin I is Δ^2-hexenoylamino acetaldehyde, whilst penilloaldehyde II is phenylacetylamino acetaldehyde. The carbon dioxide and the aldehydes are derived from the corresponding penaldic acids, VI. Treatment of penicillin with alkali or with penicillinase causes opening of the lactone ring with formation of dibasic penicilloic acids, VIII. Treatment of penicillin with methanol gives rise to the inactive methyl ester, IX.

The potency of penicillin preparations is defined in terms of an arbitrary international unit, the "Oxford unit," which is the amount of activity contained in 0·6 μg. of a particular standard crystalline preparation. The measurement is carried out by comparing the effect of the unknown sample with that of a sample of known potency on cultures of a suitable organism *in vitro*. Staphylococci are inhibited by a concentration of penicillin equivalent to 0·01 to 0·02 units per ml. The purest solid preparations of penicillin so far described contain about 1650 units per milligram, so that dilutions of 1 in 3×10^7 are sufficient to inhibit staphylococci; culture filtrates from surface growth contain about 20 to 80 units per millilitre, whilst those from submerged growth may contain up to 250 units per millilitre, depending on the strain and conditions.

The majority of organisms which are sensitive to penicillin are Gram-positive, namely, *Staphylococcus*, *Streptococcus*, pneumococcus, *C. diphtheriæ*, *B. anthracis*, *Actinomyces*, *Cl. welchii*, *Cl. œdematiens* and other clostridia. The sensitive Gram-negative organisms are the gonococcus, meningococcus and *Micrococcus catarrhalis*. The other Gram-negative pathogens are not affected by penicillin nor are Gram-negative saprophytes, yeasts or moulds. The tubercle bacillus is not sensitive to penicillin. The activity of penicillin is not affected by the presence of serum, blood or pus nor by the number of organisms present.

Penicillin is almost completely non-toxic to man or animals and has no deleterious effect on leucocytes or tissue cells. It is, therefore, superior to the sulphonamides in these respects. From the chemotherapeutic point of view it has the defects that it cannot be given by mouth as it is destroyed by the acid of the stomach, and that it is very rapidly excreted in the urine after intravenous or intramuscular injection. In order to maintain an adequate concentration in the blood it must be administered by the continuous intravenous drip method or by intramuscular injections repeated at least every three hours for as long as the infection persists. A daily dosage of about 120,000 Oxford units is necessary. Penicillin is of particular value in treating staphylococcal septicæmia, which is often resistant to treatment with sulphonamides, osteomyelitis, gas gangrene, gonorrhœa and infections by sulphonamide resistant strains of pneumococci. Penicillin can be used effectively in the local treatment of burns, wounds and skin infections by application in a cream or as a powder (usually mixed with sulphanilamide). The methyl and ethyl esters of penicillin are more stable than penicillin to such an extent that they can be given by mouth. They have only about one hundredth of the activity of penicillin

in vitro, probably due to slow hydrolysis. In doses of about 2 mg. they will protect mice against many thousand lethal doses of hæmolytic streptococci. The crystalline sodium or calcium salts of penicillin, which are more stable than the free acid, are normally used in practice.

As with other drugs, bacteria develop resistance to penicillin when subjected to concentrations which are inadequate for bacteriostasis. Organisms which have acquired resistance to penicillin are still susceptible to sulphonamides and *vice versa*. The mode of action of penicillin is unknown but it does not interfere with respiration; susceptible organisms subjected to less than the bacteriostatic dose continue to grow but lose their power of subdivision so that giant forms are produced.

Penicillin is destroyed by enzymes (penicillinase) secreted by several species of bacteria including *E. coli*, *Micrococcus lysodeikticus*, *Proteus* and some Gram-positive bacilli such as *B. subtilis*.

Penicillin B.—This substance is identical with notatin.

Proactinomycin.—A species of *Proactinomyces*, when grown on a glucose agar medium, forms an alkaloid like base, soluble in organic solvents and in water at *p*H 4, which has bacteriostatic properties similar to, but weaker than, those of penicillin. It is more stable, undergoing only small loss of activity on boiling at *p*H 2 or 7 for ten minutes; it is inactivated by boiling at *p*H 10. Proactinomycin is toxic to mice in doses of 2 to 5 milligrams.

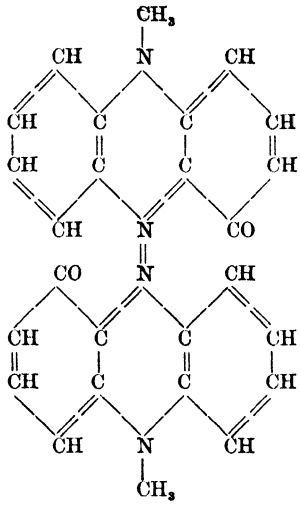
Puberulic Acid.—Several species of *Penicillium*, *P. puberulum*, *P. aurantio-virens*, *P. johannioli* and *Cyclopium viridicatum* yield puberulic acid, $C_8H_6O_6$,

and puberulonic acid, $C_8H_4O_6$. The former is a colourless, crystalline, dibasic acid of m.p. 316 to 318°C., whilst puberulonic acid is a bright yellow, crystalline substance having m.p. 298°C., which is thought to be the quinonoid form of the quinol, puberulic acid. Puberulic acid inhibits Gram-positive organisms at dilutions of 1 in 6000 to 1 in 33,000, whilst puberulonic acid is less effective, inhibiting at 1 in 6000 only. They have little effect on Gram-negative organisms.

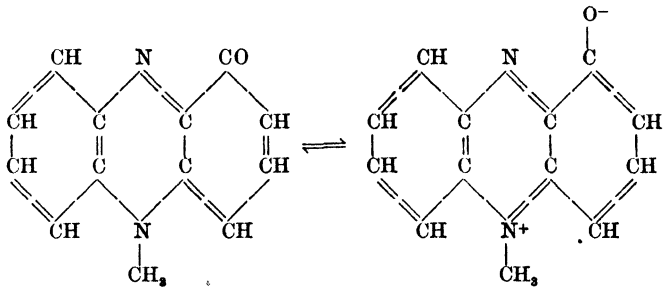
Pyocyanase.—*Pseudomonas æruginosa* (B. pyocyaneus), which was among the earliest of organisms shown to produce antibiotic substances, forms pyocyanase, pyocyanin and α-hydroxy-phenazine (hemipyocyanin). Pyocyanase is lytic to many bacteria, such as *E. coli*, *Eberthella typhosa*, *C. diphtheriæ*, *V. comma*, streptococci and staphylococci, and also detoxifies the toxins of *Cl. tetani* and other bacteria very rapidly, a property also possessed by sodium lauryl sulphate and zephiran (a sulphonated mixture of the fatty acids, C_8 to C_{17}, contained in coconut oil).

Pyocyanase, in spite of its heat stability, was at one time thought to be an enzyme attacking nucleic acids but it is now regarded as being of lipoidal nature, the activity depending largely on the presence of unsaturated fatty acids; it is said to contain a phosphatide and free fat in addition. The facts that it is soluble in ether, chloroform and benzene and that its activity is not much affected by changes in temperature between 0 and 37°C. are not in accord with the view that pyocyanase is an enzyme. It has been obtained as a colourless oil forming an ether soluble lead salt, so that it is probably an unsaturated fatty acid.

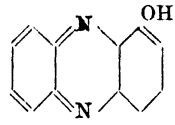
Pyocyanin.—The chloroform soluble blue pigment, pyocyanin, produced by *Ps. æruginosa*, has been shown to be a phenazonium compound :—



which breaks down to



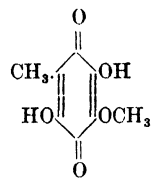
with a structure not unlike that of iodinin (see p. 168) which is also a phenazine derivative. It is formed in the first two or three days of growth of the organism; pyocyanase occurs in cultures two to three months old. Cultures of intermediate age contain a second pigment, yellow in colour, which is a degradation product of pyocyanin, known as hemipyocyanin, and having the structure, α-hydroxy-phenazine,



Pyocyanin, which is thermostable, has strong bacteriostatic power against Gram-positive and Gram-negative bacteria and comparatively little effect against moulds or yeasts. Hemipyocyanin is less active against bacteria but considerably more active against yeasts and fungi. The similar pigment, chlororaphin, formed by *Ps. chlororaphis* (see p. 391), is also somewhat inhibitory to bacteria.

In virtue of its oxonium structure, pyocyanin is known to act as an oxygen carrier in the oxidation of α-hydroxyglutaric acid to α-ketoglutaric acid in presence of a dehydrogenase occurring in animal tissues. It is possible that the antibiotic effect of pyocyanin is due to interference in some such process in bacterial metabolism.

Pyocyanin is highly toxic to animals as well as being bactericidal; hemipyocyanin is much less toxic and has about the same bactericidal power as the flavines. It has no staining properties.

Spinulosin.—*P. spinulosum* gives rise to the red pigment spinulosin, 3 : 6-dihydroxy-4-methoxy-2 : 5-toluquinone,  , which is hydroxy-fumigatin (see p. 163). It is a weaker bacteriostatic agent than fumigatin, being active against Gram-positive organisms at concentrations of 1 in 6000 to 1 in 10,000. The loss in activity as compared with fumigatin is attributed to the additional hydroxyl group since, in a series of tolquinone derivatives, those members containing hydroxyl groups are less active than those without.

Streptomycin.—*Streptomyces griseus*, one of the soil organisms, the Actinomycetes, forms the substance streptomycin which resembles streptothricin in inhibiting the growth of Gram-negative as well as Gram-positive organisms, but is more active against *Pr. vulgaris* and *Ps. æruginosa* among the former and against *B. mycoides* and *M. tuberculosis* among the latter. It is also bacteriostatic to *E. typhosa*, *Sal. schottmülleri*, *Br. abortus*, *H. influenzæ*, *H. pertussis*, *Serratia marcescens*, *B. subtilis*, *Staph. aureus* and *Cl. butylicum*. Streptomycin is poorly absorbed from the intestine but is not destroyed and so may be useful for controlling intestinal infections. In man it is rapidly absorbed and excreted in the urine after parenteral administration, but therapeutic levels can be maintained in the blood and urine more easily than with penicillin. It has low toxicity for animals. Preliminary trials indicate that it may be of therepeutic use against typhoid fever and human tuberculosis. If these claims are substantiated streptomycin is likely to be as valuable as penicillin.

Streptomycin can be adsorbed on charcoal from the culture fluid resulting from the growth of *S. griseus* on a corn steep liquor medium. After elution from the charcoal with acid ethanol it can be purified and recrystallised as the hydrochloride, $[\alpha]_D$ −84°, the sulphate, the helianthate or reineckate, m.p. 162-164°C. It is an organic base soluble in water but not in most organic solvents. It is fairly stable to heat, losing about half its activity in ten minutes at 100°C. It is inactivated by cysteine, 2-aminoethanethiol and, to a less extent, by thioglycollic acid. The inactivation is reversed by iodine. Its activity increases with the alkalinity of the medium up to *p*H 9. Streptomycin is also inactivated by reagents such as hydroxylamine and semicarbazide which react with carbonyl groups.

Streptomycin hydrochloride is completely inactivated by standing for 24 hours with anhydrous methanol

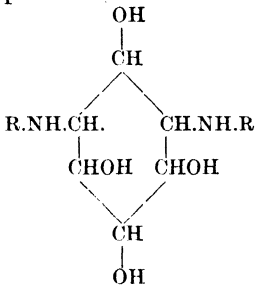
containing N HCl. Addition of ether to the solution precipitates a diguanidine base, streptidine, leaving an optically active substance, methyl streptobiosaminide dimethylacetal hydrochloride, in solution :—

$$\underset{\text{(Streptomycin hydrochloride)}}{C_{21}H_{37-39}N_7O_{12}.3HCl} + 3\,CH_3OH \longrightarrow \underset{\text{(Streptidine)}}{C_8H_{18}N_6O_4.2HCl} + H_2O + \underset{\text{(Streptobiosaminide dimethyl acetal)}}{C_{13}H_{20-22}NO_7(OCH_3)_3.HCl}$$

On hydrolysis streptidine loses ammonia and carbon dioxide and forms a new base, streptamine :—

$$C_8H_{18}N_6O_4 + 4\,H_2O \longrightarrow C_6H_{12}N_2O_4 + 4NH_3 + 2CO_2$$

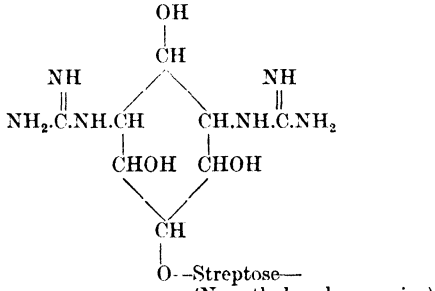
The six nitrogen atoms of streptidine are present as two mono-substituted guanidine groups which are replaced by two amino groups in streptamine. The oxygen atoms are present as hydroxyl groups since acetyl and benzoyl derivatives can be obtained. Streptamine is a diamino tetrahydroxy cyclohexane with the amino groups at positions 1 : 3 :—



I

Streptamine, R = H

Streptidine, R = —C(=NH)—NH$_2$



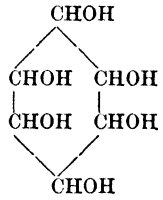
II

Streptomycin

Methyl stretobiosaminide dimethyl acetal is most probably derived by the action of methanol on a nitrogen containing disaccharide, streptobiosamine. Streptobiosamine is composed of N-methyl-L-glucosamine and streptose, which is a hexose of unknown constitution.

The first carbon atom of streptose is involved in the linkage of streptobiosamine with streptidine as shown in formula II, above.

The structure of the streptidine moiety of streptomycin makes it tempting to speculate that the antibiotic is active in virtue of its resemblance to the growth factor inositol,



Streptothricin.—A species of *Actinomyces*, occurring in the soil, *A. lavendulæ*, gives rise to the antibiotic streptothricin, which has a selective action on Gram-negative organisms, for example *E. coli* and *Shigella dysenteriæ*, but also acts on some Gram-positive bacteria, for example *B. subtilis* and *Staph. aureus*, moulds and yeasts. Streptothricin is a base and appears to be built up of amino-acid residues, resembling tyrocidine. It is only inhibitory in the undissociated state ; factors such as the presence of salts and *p*H values which cause dissociation lower the activity of streptothricin. Bacteria subjected to its action increase in size and tend to form chains. Resistant strains of bacteria can be developed. It has a low toxicity for animals and could probably be used for internal chemotherapy.

Tyrocidine.—Tyrothricin, isolated from autolysates of *B. brevis*, is a mixture of gramicidin and tyrocidine (see p. 165). Tyrocidine is the fraction insoluble in acetone-ether mixtures, but soluble in hot ethanol and crystallised out as the hydrochloride. Tyrocidine hydrochloride has m.p. 237-239°C. (decomposition), and $[\alpha]_D^{20} - 102°$ (c = 1 in 95 per cent. ethanol). Tyrocidine is a polypeptide having a molecular weight about 2500

and containing about twenty amino-acid residues including tryptophane, tyrosine, alanine, phenylalanine and aspartic acid, combined in such a way as to leave free two basic amino groups, three amide groups and one carboxyl group or a phenolic OH group. About twenty per cent. of the amino-acid residues have the *d*-configuration. It is of interest to note that the capsules of *B. anthracis*, *B. mesentericus* and *B. subtilis* are made up of a polypeptide composed of *d*-glutamic acid (see p. 338). The presence of such a large proportion of *d*-amino-acids in gramicidin and tyrocidine probably accounts for their resistance to pepsin, trypsin and papain.

Tyrocidine has marked bactericidal and lytic action *in vitro* against Gram-negative as well as Gram-positive organisms. Fifty to 100 μg. affords definite protection to mice infected intraperitoneally with pneumococci. Tyrocidine blocks the oxidative processes of metabolism. It is antagonistic to certain lower fungi such as *Achorion schœnlandii*, *Microsporium gypseum*, *Trichophyton gypseum* and *Candida albicans*.

Tyrocidine has a strong hæmolytic effect on human and rabbit red blood corpuscles, hæmolysis occurring in the presence of 0·005 μg. of the substance, which is also highly toxic to animals. Tyrocidine loses much of its bactericidal power in the presence of blood, serum or pus. It appears to prevent the loss of activity which tetanus toxin undergoes on heating at 55°C.

Both tyrocidine and gramicidin can be used chemotherapeutically by local application, to wounds for example.

Un-named Antibiotics.—When *B. mesentericus*, in the smooth phase, is grown in nutrient broth it gives rise to a substance which has a specific bactericidal effect on *C. diphtheriæ* at a dilution of 1 in 1250. The toxic effects of *C. diphtheriæ* are eliminated when the organism is injected along with the *B. mesentericus* filtrate into guinea-pigs.

Aspergillus candidus produces a thermostable substance, similar to citrinin, but which is more powerful against *Staphylococcus* and *B. mycoides*, being bacteriostatic at concentrations of about 1 in 100,000 and bactericidal at 1 in 8000.

A. flavipes, when grown on tryptone medium or corn-steep liquor medium, gives an alkaline bacteriostatic filtrate active at dilutions of 1 in 320 to 1 in 1300 against *Str. pyogenes*, pneumococci, and *Cl. welchii*, very slightly active at 1 in 5 to 1 in 10 against staphylococci and inactive towards *Str. fœcalis*, *E. coli* and *Aerobacter œrogenes*. It can be obtained as a gummy precipitate by concentration of the medium *in vacuo* and addition of ten volumes of acetone.

Penicillium resticulosum, grown on Czapek-Dox medium, gives a metabolism solution which inhibits the growth of *Staph. aureus* at dilutions of 1 in 320 to 1 in 2500. The antibacterial substance can be precipitated from solution by the addition of acetone after concentration, the yield being about 1 g. per litre from a filtrate inhibiting *Staph. aureus* at a dilution of 1 in 80. It is readily soluble in water, contains 3·8 per cent. of nitrogen, and causes complete inhibition at a concentration of 1 in 160,000. It is very sensitive to acid, being destroyed by contact with 0·1 *N* hydrochloric acid at room temperature.

Of thirty-nine moulds which were tested seventeen, all of the genus *Penicillium*, had antibacterial activity. They could be divided into two groups ; I, active against *Staph. aureus*, *Str. viridans* and *C. diphtheriæ*, were contaminants of laboratory media, and II, which were active against *E. coli* and *Eberthella typhosa* in addition to the above organisms, were mainly fruit contaminants.

Among a large number of species of Fungi imperfecti, Wilkins and Harris showed that about 40 per cent. of *Aspergillus* species and 25 per cent. of *Penicillium* species gave substances antagonistic to one or more of

the test bacteria, *E. coli*, *Staph. aureus* and *Ps. æruginosa*. Very few representatives of other genera were active, the exceptions being *Botrytis cinerea* and *Helminthosporium avenæ* against *Staph. aureus* and *Fusarium javanicum* against *E. coli*.

Among the number of antibiotics recently isolated and about which little is known at present the following may be mentioned. Allicin, from garlic, *Allium sativum*, probably having the structure

$$CH_2 : CH.CH_2.S.S.CH_2.CH : CH_2$$
$$\underset{O}{\|}$$

which is active against Gram-positive and Gram-negative organisms; bacitracin, from a *B. subtilis* like organism, which is non-toxic to animals but is very active against Group A hæmolytic streptococci, staphylococci and the gas gangrene organisms; mycophenolic acid (see p. 296), formed by *Penicillium brevi-compactum*, which inhibits staphylococci, streptococci, *C. diphtheriæ*, *B. subtilis* and many pathogenic fungi but not Gram-negative organisms; viridin, from *Trichoderma viride*, which is inhibitory to the growth of *Botrytis*, *Fusarium*, *Trichothecium* and *Cephalosporium* strains and to a less extent to that of *Penicillium* and *Aspergillus* strains; a substance formed by Group N streptococci which inhibits many Gram-positive organisms, including streptococci, *Bacillus*, *Clostridium* and *Lactobacillus* species; subtilin, from *B. subtilis*, active against Gram-positive but not against Gram-negative organisms; violacein, the pigment from *Chr. violaceum* (see p. 389), which is very inhibitory to Gram-positive organisms (meningococcus, the only Gram-negative organism affected, is inhibited by 0·0005 per cent. of violacein), *Saccharomyces cerevisiæ* and some moulds.

It is interesting that the basic proteins, protamine and histone, which are of comparatively low molecular weight, are bacteriostatic or bactericidal to *E. typhosa*,

Shigella paradysenteriæ, *E. coli*, *B. subtilis*, *Staph. albus*, *Staph. aureus*, *Cl. welchii*, *Cl. tetani*, *Cl. histolyticum*, streptococci and pneumococci in dilutions varying from 1 in 40,000 to 1 in 3000. It is seen that they have properties similar to the polypeptide antibiotics gramicidin and tyrocidine, the resemblance being especially close to the latter. The effects are eliminated by the presence of phosphatides, with which all the substances combine. It is possible that they may be active by interference with the phosphatide metabolism of the organisms.

Antibiosis appears to be a very widespread phenomenon, existing among micro-organisms of all sorts. It is probably the chief mechanism by which the majority of species manage to survive in natural surroundings, particularly in the soil where they must be subjected to intense competition. Obviously the antibiotic substances produced by micro-organisms influence profoundly the ecology of their surroundings.

Antibiotic substances have found several applications in the control of plant and animal diseases, the outstanding example in human treatment being the use of penicillin. It is possible that the closed plaster-cast method of treating wounds may depend for its efficacy on the antagonistic action of saprophytic organisms towards any pathogens which might be present originally.

Another use of antibiotics has been in the preparation of selective media for the isolation of bacteria from mixed cultures. For instance, the inclusion of penicillin in Bordet-Gengou medium suppresses the growth of most organisms occurring in the throat and enables *Hœmophilus pertussis* to be isolated with greater ease from cough plates or swabs.

For further reading :—

S. A. Waksman, "Antagonistic Relations of Micro-organisms." *Bact. Reviews*, **5**, (1941), 231.

S. A. Waksman, "Microbial Antagonisms and Antibiotic Substances." The Commonwealth Fund. New York, 1945.

CHAPTER XII

BACTERIAL RESPIRATION

THE term respiration has undergone a continuous expansion in its meaning. Originally "respiration" in animals signified the exchange of oxygen and carbon dioxide through the lungs, then it was used to describe the transfer of oxygen to and the removal of carbon dioxide away from tissues. Later still the term connoted the general oxidation processes of cells, and, finally, now that it is recognised that these processes are almost always concerned with the energy requirements of the cells, the expression has come to mean any energy producing biological reaction, even when the reaction takes place under anaerobic conditions. It is in this sense of a chemical reaction producing energy in the cell, whether aerobically or anaerobically, that we shall employ it.

Chemists tend to regard bacteria, yeasts and moulds, merely as useful reagents which can bring about many reactions, such as the synthesis of acetone, butyric acid, butyl alcohol and so on, some of which he cannot yet carry out in the laboratory. The tendency of the biologist, on the other hand, is to regard these reactions or products as accidents more or less incidental to the life of the cell, useful accidents it may be, in that they sometimes provide a means of identifying or helping to identify the organism (as is the case with sugar fermentation reactions), or in that they provide some product like alcohol which he values.

These reactions should be regarded as the life processes of the cell, providing both the energy required and the raw materials for building up new cells.

As far as we know only two sources of energy are available for living cells, light and chemical energy. Of these, light can only be utilised by chlorophyll-containing plants, by the blue-green algæ and by a few autotrophic bacteria ; for all other forms of life the requisite energy must be derived from chemical reactions. Heat, electricity and mechanical energy cannot be utilised by organisms, probably because they lack appropriate " transformers " ; the only transformers we know are chlorophyll and similar pigments for light. Heat, or in other words a rise in temperature, may cause increased growth and metabolic activity of a cell, but it is only in so far as the chemical changes (which supply the essential energy) are speeded up by a rise in temperature. A cell cannot economise on food by using the heat energy of the medium; a starving cell, for instance, derives no benefit from a rise in temperature.

It follows that the energy liberated in one cell is of no use to any other cells ; neighbouring cells, even those closely linked as in tissues, have no direct energy exchange system. Moreover, the chemical energy necessary for growth must be liberated within the cell, since if it were produced outside the cell it would have to take the form of heat or electricity, which cannot be utilised by the cell. As a result of this the only foods of value to the organism are those which can diffuse into the cell. Thus complex proteins, fats and carbohydrates like starch and cellulose, are not directly available to the organism, but first have to be broken down or hydrolysed to appropriate smaller, soluble, diffusible compounds. This is the work of the class of exo-cellular enzymes or hydrolases which are secreted into the medium by the organism.

As we have already seen in Chapter IV the reactions catalysed by these enzymes involve only relatively small energy changes, whilst, on the other hand, those reactions brought about by the endo-enzymes, inside the cell, where the energy liberated is of real value to the organism, involve large energy changes. This is illustrated in Table 15, which shows the energy liberated from 1 gram of the appropriate substrate by the action of different enzymes.

TABLE 15

Exo-enzymes.		Endo-enzymes.	
	cals.		cals.
Pepsin, trypsin, rennet	0	Lactacidase	82
Lipase	4	Alcoholase	149·5
Invertase	9·3	Urease	239
Maltase	10	Vinegar oxidase	2,530
Lactase	23		

An exception to the general rule that hydrolytic reactions involve little energy change is the case of the breakdown of urea, which is used as energy source by the urea bacteria. This is only an apparent exception because the reaction takes place in two stages, first the hydrolysis of urea to ammonia and carbon dioxide :—

$$\begin{matrix} NH_2 \\ CO \\ NH_2 \end{matrix} + H_2O \longrightarrow 2NH_3 + CO_2 + \text{about 0 Cals.}$$

which involves practically no liberation of energy. The second step is the formation of ammonium carbonate from the ammonia and carbon dioxide :—

$$2NH_3 + CO_2 + H_2O \longrightarrow (NH_4)_2CO_3 + 12 \text{ Cals.}$$

and it is this part of the reaction which supplies the energy.

The products formed by the action of the endo-enzymes in the cell are usually essentially different from the substance fermented, and are in the main useless, often even harmful, to the organism. It is this accumulation of end products which is often responsible for the cessation of growth after a time ; for instance, in yeast fermentation when about 8·5 per cent. of alcohol has been produced, further growth of the yeast cells is inhibited.

The majority of organisms can utilise several kinds of food and therefore bring about various fermentations in the course of their metabolism. They may even be able to utilise the same food in different ways, according to the conditions. For instance, certain typical sugar fermenting bacteria and the yeasts can be grown in the absence of sugars ; lactic acid bacteria will grow on peptone. Yeast normally ferments sugar to alcohol, but it can also oxidise it completely to carbon dioxide and water if a sufficient supply of oxygen is available. Most moulds appear to be omnivorous, attacking almost any substrate with which they may be supplied. At the other extreme are the autotrophic bacteria, most of which can utilise only one substrate as a source of energy (see Chapter VI).

The chemical activity of the micro-organisms is, generally speaking, vastly greater than that of animals or plants. For example, it has been calculated that 1 gram of *Micrococcus ureæ* can decompose 180 to 1,200 grams of urea per hour ; and that 1 gram of certain lactose fermenting bacteria can hydrolyse 180 to 15,000 grams of lactose per hour. If man were capable of metabolism on the same scale he would consume several thousand tons of food per hour. These figures are probably subject to a certain margin of error, but they are sufficiently accurate to indicate the enormous difference between the metabolic activities of the bacteria and animals. A major cause for this difference in activity is the much greater area in bacteria available for the absorption of nutrients. The area to weight ratio in

bacteria is of the order of 200,000 times that for man. Moreover, the whole of the bacterial surface allows the passage into the cell of foodstuffs, whilst in man and animals the absorption of nutrients takes place through only a limited part of the total surface.

If an organism can use a given compound as a nutrient, its value will depend on its calorific value. For instance, it has been shown that when *Aspergillus niger* is grown on various compounds as the source of energy and carbon, the weight of mycelium grown runs parallel with the heats of combustion (that is, the calorific values) of the compounds, as may be seen from Table 16.

TABLE 16

Nutrient.	Heat of Combustion.	Weight of Mycelium.
Tartaric acid - - -	2,618 cals./1·5 g.	0·155 g.
Citric acid - - -	3,711 ,,	0·240
Glucose - - - -	5,614 ,,	0·278
Glycerol - - -	6,461 ,,	0·475
Olive oil - - -	13,972 ,,	0·810

The value of a compound as a food or energy source also depends on the degree of oxidation which it undergoes; the more complete the oxidation the higher the energy available. Glucose may be taken as an example, and the energy liberated with varying degrees of oxidation compared :—

(*a*) Complete aerobic oxidation

$$C_6H_{12}O_6 + 6O_2 \longrightarrow 6CO_2 + 6H_2O + 674 \text{ Cals.}$$

(*b*) Partial aerobic oxidation

$$2C_6H_{12}O_6 + 9O_2 \longrightarrow \underset{\text{(oxalic acid)}}{6C_2H_2O_4} + 6H_2O + 493 \text{ Cals.}$$

(*c*) Anaerobic oxidation

(i) $C_6H_{12}O_6 \longrightarrow \underset{\text{(lactic acid)}}{2C_3H_6O_3} + 22{\cdot}5 \text{ Cals.}$

(ii) $C_6H_{12}O_6 \longrightarrow \underset{\text{(ethyl alcohol)}}{2C_2H_5OH} + 2CO_2 + 22 \text{ Cals.}$

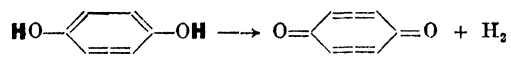
(iii) $C_6H_{12}O_6 \longrightarrow \underset{\text{(acetic acid)}}{3CH_3COOH} + 15 \text{ Cals.}$

It follows from this consideration that the less complete the oxidation the more of a given substance must be broken down to supply the needs of the organism. The incomplete oxidation of non-nitrogenous compounds is what we usually call fermentation, and the incomplete oxidation of nitrogenous compounds is usually referred to as putrefaction.

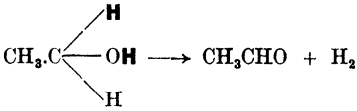
Pasteur considered that the main factor controlling fermentation was the oxygen supply. He showed that under anaerobic conditions the growth of yeast cells is much restricted but that fermentation is very active, whilst under aerobic conditions with a good oxygen supply the growth of the cells is rapid but fermentation is repressed. The aerobic breakdown of 7 grams of sugar is associated with the formation of 1 gram of yeast cells, whilst anaerobically the production of 1 gram of cells needs 70 grams of sugar, a striking illustration that complete oxidation is much more economical than incomplete oxidation.

Various theories have been propounded to explain the fact that such compounds as carbohydrates, amino-acids and fatty acids are readily oxidised in part or completely by cells at ordinary temperatures, whilst in the laboratory they are only oxidised by much more drastic means. Usually these theories involve the activation of either the substrate or the hydrogen acceptor (whether it be oxygen or some other compound) or sometimes both of them.

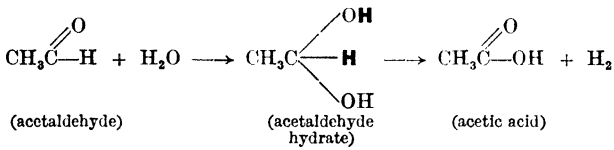
Wieland has put forward the idea that all oxidations are due to a hydrogen transfer. He came to this conclusion as a result of experiments in which he found that many substances could be anaerobically oxidised, in presence of such metallic catalysts as spongy palladium or platinum, by loss of hydrogen which is taken up by the catalyst. Examples are the oxidation of hydroquinone to quinone :—



and the oxidation of ethyl alcohol to acetaldehyde :—



The hydrogen atoms shown in bold type are those transferred. In cases where there is an actual increase of oxygen in the oxidised molecule, he regards the first step as being the formation of a hydrate, the second step being the loss of hydrogen from the hydrate. Thus the oxidation of acetaldehyde to acetic acid is considered to follow the equation :—



Lactic acid and glucose were found to be oxidised in a similar way by these metallic catalysts, especially if a hydrogen acceptor such as oxygen or some easily reduced compound was added. Methylene blue proved a very useful hydrogen acceptor.

Wieland regards biological oxidations as being similar in character, the difference being that the metallic catalyst is replaced by an enzyme. The enzymes are variously known as a **reductase** when the hydrogen acceptor is a dye or a nitrate or some similar compound, as a **mutase** when the acceptor is a second molecule of the substance being oxidised (as, for example, when acetaldehyde is converted into acetic acid and alcohol by the acetic acid bacteria), or as an **oxidase** when atmospheric oxygen is activated as the hydrogen acceptor.

That bacteria are able to activate a large variety of compounds in this way has been shown by the use of the " methylene blue technique." In this procedure a washed suspension of the organism under investigation

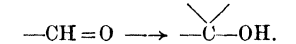
is incubated with a solution of the compound to be examined and a standard quantity of methylene blue solution, all buffered at an appropriate *p*H value in an evacuated system. If the bacterium is capable of activating the substrate as a donator of hydrogen (that is, if it is capable of oxidising it) the methylene blue accepts the hydrogen and becomes reduced to the colourless leuco-compound, the loss of colour serving as an indicator that the reaction has occurred. By this means numerous fatty acids, hydroxy- and amino-acids, polyhydric alcohols and sugars have been shown to be activated as hydrogen donators. Some of these substrates are much more readily activated than others, the most active, glucose, being some ten thousand times more effective than the least active of the lower fatty acids. Formic, lactic and succinic acids are all fairly active. All bacteria do not activate the same compounds, and there are marked differences between the activation by plant and animal tissues and by bacteria.

Thunberg, who originated the methylene blue technique, considered these activations as being due to a series of specific enzymes. This is rather hard to believe, however, since *Esch. coli*, for instance, would need to contain over fifty such enzymes, including some for substances like chlorates which the organism would be extremely unlikely to meet in the ordinary course of its existence. Quastel has suggested that one general mechanism is responsible for all these activations, which, after all, are alike in that they are all hydrogen transfers. He considers that the enzyme action depends on two factors ; first that the substrate is adsorbed on an active surface in the cell, and secondly that the adsorbed molecule is rendered unstable in such a way that it is liable to lose hydrogen if it is a donator or to gain hydrogen if it is an acceptor (like methylene blue). The cell is pictured as having a network of internal interfaces, probably composed of protein and lipoid constituents.

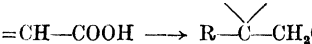
Certain areas of these interfaces are endowed with activity as a result of the arrangement of the molecules composing them; certain arrangements of polar groups in the molecules set up local electric fields of varying intensity depending on the particular molecular arrangement. Any molecule, especially one containing polar groups, like —COOH, =CO, or double bonds, coming into close contact with such an active surface by adsorption becomes distorted with a resulting shift of the hydrogen atoms rendering the molecule unstable or activated. For instance, a double bond is supposed to be activated according to the scheme :—



or an aldehyde group in this way :—



The presence of a polar group like carboxyl in a molecule favours a concentration of hydrogen in its direction :—



whilst a non-polar group like methyl favours a concentration of hydrogen away from it :—



The mechanism, as described so far, suggests how a compound can be activated to act as a hydrogen donator or acceptor, but it does not account for specificity. For instance, it affords no explanation of the fact that *Esch. coli* activates glucose whilst *Alcaligenes fœcalis* (*B. fœcalis alcaligenes*) does not, although both organisms strongly activate lactates. Nor does it explain why succinic acid is oxidised by both bacteria and muscle tissue, but that formic acid is a very active hydrogen donator in presence of bacteria but quite inactive with muscle. This activity is held to be due to the presence of definite groupings of molecules in the active centres of the cells which

selectively adsorb different types of compound which have a corresponding arrangement of their own polar groups (see p. 31). Thus sugars are adsorbed by one grouping, succinic and similar acids by another sort of grouping, lactic and other hydroxy-acids by a third grouping, and so on. Once this specific adsorption has occurred the general mechanism of the activation is the same in each case. As would be expected, substances having a configuration similar to those activated, but which are themselves not activated (possibly because the field of force is not strong enough), will partially inhibit the activation of the latter, since they can be adsorbed on to the active centres to the partial exclusion of the normal substrate. For example, α-hydroxybutyric acid, $CH_3CH_2CHOH.COOH$, or tartaric acid, $COOH.CHOH.CHOH.COOH$, will inhibit the activation of lactic acid, $CH_3CHOH.COOH$, in virtue of the common structure —CHOH.COOH which enables them to compete for places at the active centres; but they will not inhibit the activation of succinic acid, $COOH.CH_2.CH_2.COOH$, because it is adsorbed at different active centres not affected by the hydroxy-acids. Conversely malonic acid, $COOH.CH_2.COOH$, or glutaric acid, $COOH.CH_2.CH_2.CH_2.COOH$, will inhibit the activation of succinic acid but not that of lactic acid, because they are adsorbed on the same centres as succinic acid which has the common group, $—CH_2.COOH$. This suggestion of competitive adsorption at enzyme centres has also been used in the explanation of the mode of action of chemotherapeutic substances (see Chapter X).

Warburg suggested that all aerobic oxidations, that is, those in which atmospheric oxygen is involved, are brought about through the intervention of iron compounds, such as hæmoglobin, whereby molecular oxygen is transferred to the substrate in an activated form. He showed that amino-acids could be oxidised by molecular oxygen when at the surface of charcoal prepared by heating blood, and

that the action could be inhibited by low concentrations of hydrocyanic acid, the concentration necessary being proportional to the iron content of the catalyst. Narcotics can also inhibit the reaction, and this he attributed to their adsorption on the active surface preventing the access of oxygen.

Kluyver has combined the above ideas into a general scheme, applicable to all fermentations, which involves the transfer of hydrogen to oxygen in the case of aerobic oxidations or to some other acceptor, suitably activated by an enzyme, in other cases. Where oxygen is concerned, an iron compound such as Keilin's cytochrome is usually also involved. The enzymes involved in such hydrogen transfers are called **dehydrases, dehydrogenases** or **hydrogen transportases.**

It is seen that all these processes are coupled oxidation-reduction reactions, hydrogen being given up by the substrate, the donator, and transferred to a second substance, the acceptor. For the purposes of our study these reactions can be divided into three types :—

Type I.—The hydrogen acceptor is atmospheric oxygen, that is, direct oxidation occurs, as is the case with the production of acetic acid in the vinegar fermentations, or the action of many moulds and of the Mycobacteria on sugars.

Type II.—The hydrogen donator and acceptor are the same molecule, giving rise to an intra-molecular fermentation. As an example one may take the conversion of glucose, $C_6H_{12}O_6$, into two molecules of lactic acid, $2C_3H_6O_3$. Apparently no hydrogen or oxygen is required from outside, the new compound resulting from a rearrangement of the distribution of the hydrogen and oxygen within the molecule. Actually the process is not so simple as this, since the final effect is brought about by a whole series of intermediate reactions. What it really amounts to is that a single substance is sufficient for the growth of the organism.

Type III.—The hydrogen donator and acceptor are different compounds, resulting in inter-molecular fermentation. The Type I oxidation is a special case of Type III in which the acceptor is oxygen. Examples of this type are the anaerobic fermentations at the expense of the oxygen of fumarates, nitrates, sulphates, or similar highly oxidised substances. *Rhizobium*, for instance, can be made to grow as much as 1 cm. below the surface of agar containing a small amount of permanganate, whereas normally it grows only on the surface of solid media. Obviously for Type III reactions to occur the energy liberated by the oxidation (dehydrogenation) of the donator must be greater than that required to cause the reduction (hydrogenation) of the acceptor (see also Oxidation-Reduction Potentials, Chapter II). Besides the factor of thermodynamic possibility the enzymic activation of the substrates comes into play. This is particularly well illustrated in the case of the streptococci which cannot use oxygen, the best of all hydrogen acceptors from the energy point of view. This is not due to an actual sensitivity to oxygen since, for example, *Str. cremoris* can grow in milk exposed to oxygen, but no oxygen uptake can be measured, whilst *Esch. coli* under the same conditions takes up oxygen freely. Again, *Str. lactis* suspended in aerated buffer solution takes up no oxygen, although the majority of aerobes and facultative anaerobes take up from 5 to 25 ml. of oxygen per hour under the same conditions. *Clostridium sporogenes* under these conditions behaves like the streptococci, and cannot utilise oxygen since it, too, lacks the appropriate enzyme system. Oxygen uptake is usually stimulated by the presence of methylene blue and inhibited by cyanide.

The growth of organisms in oxygen usually involves the production of hydrogen peroxide which is toxic to most micro-organisms. Normally this hydrogen peroxide is destroyed by the enzyme **catalase** with formation of

water and oxygen, even in the absence of any oxidisable compound to take up the liberated oxygen. On the basis of the presence of catalase McLeod has divided the bacteria into four groups :—

(*a*) **Strict Anaerobes.**—These organisms have no catalase and are very sensitive to the presence of hydrogen peroxide. Since they produce the latter in presence of oxygen they are incapable of growth aerobically. They are not sensitive to cyanide.

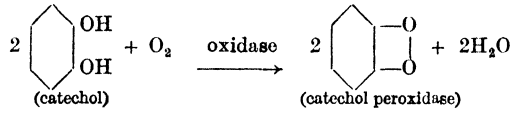
(*b*) **Micro-aerophilic Organisms.**—Members of this group have no catalase but produce hydrogen peroxide ; however, they are only moderately sensitive to it, and can therefore survive if the oxygen tension is not too great. As examples may be quoted the pneumococcus, most streptococci and the lactic acid bacteria.

(*c*) **Non-peroxide Producers.**—These produce neither catalase nor hydrogen peroxide and can therefore grow aerobically in spite of the absence of catalase. Examples are *Shigella dysenteriæ* and *Str. fæcalis.*

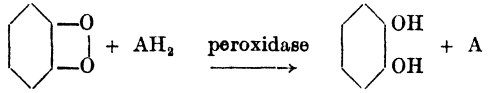
(*d*) **Catalase Producers.**—Bacteria of this group produce hydrogen peroxide, which is immediately broken down by catalase. The aerobes and most facultative anaerobes belong to this class. They are sensitive to cyanide, which inhibits catalase action.

Hydrogen peroxide and organic peroxides can also be decomposed by the enzyme **peroxidase**, which differs from catalase in that an oxidisable substance must be present to take up the liberated oxygen or to donate hydrogen, as the case may be. If such a second substance is absent no decomposition of the peroxide takes place. The organic peroxides usually arise from the oxidation by atmospheric oxygen of di- or trihydric phenols, such as catechol

under the influence of yet another enzyme, **oxidase** :—



It is to this action that the spontaneous browning of apples or potatoes in air is due. The peroxides so formed are then activated by peroxidase to regenerate the original catechol, with oxidation of the oxidisable substance :—



If the oxidisable substance, AH_2, is a compound which is coloured in the oxidised form it can be used as a test for the presence of peroxidase. The most common of these substances are α-naphthol (giving a lavender colour), guaiacol (red), benzidine (blue), *p*-phenylenediamine (greenish), dimethyl *p*-phenylenediamine (purple to black) and indophenol (purple).

The oxidation enzymes, catalase, oxidase and peroxidase, are all inhibited by the action of cyanide, sulphide and carbon monoxide. They all contain iron in the form of hæm (an iron compound of protoporphyrin) which is the prosthetic group carried by different specific proteins to give the complete enzyme. The breakdown of hydrogen peroxide by catalase is accompanied by a reduction and re-oxidation of the ferric iron in the enzyme :—

$$4Fe^{+++} + 2H_2O_2 \longrightarrow 4Fe^{++} + 4H^+ + 2O_2$$
$$4Fe^{++} + 4H^+ + O_2 \longrightarrow 4Fe^{+++} + 2H_2O$$

The oxidation enzymes play a further important part in the respiration of bacteria in that they are involved in the action of the respiratory pigments. Perhaps the best known of these is the **cytochrome** complex which consists of three iron-containing components, *a*, *b* and *c*,

related to hæmatin and distinguished by characteristic bands in their absorption spectra. Cytochrome appears to be present in all cells exposed to oxygen (with the exception of some streptococci, e.g. *Str. lactis*). Cytochrome can exist in the oxidised and in the reduced forms containing iron in the ferric and ferrous forms respectively :—

$$\text{Oxidised cytochrome} \rightleftharpoons \text{Reduced cytochrome.}$$

The oxidised form can act as a hydrogen acceptor in presence of dehydrase (or dehydrogenase) enzymes giving reduced cytochrome at the expense of the hydrogen of the donator, DH_2 :—

$$\begin{matrix} DH_2 \\ + \\ \text{Oxidised cytochrome} \end{matrix} \;\Big|\; \xrightarrow[\text{Dehydrogenase}]{} \text{Reduced cytochrome} + D.$$

Reduced cytochrome can be re-oxidised by atmospheric oxygen under the influence of cytochrome-oxidase with production of water :—

$$H_2O + \text{Oxidised cytochrome} \xleftarrow[\text{Oxidase}]{} \begin{matrix} \text{Reduced cytochrome} \\ + \\ O_2 \end{matrix}$$

so that in effect the whole system acts as a catalyst, bringing about the oxidation of the substrate, DH_2, to D and water :—

$$\begin{matrix} DH_2 \\ + \\ H_2O + \text{Oxidised cytochrome} \end{matrix} \;\Big|\; \underset{\text{Oxidase}}{\overset{\text{Dehydrogenase}}{\rightleftharpoons}} \;\Big|\; \begin{matrix} \text{Reduced cytochrome} + D \\ + \\ O_2 \end{matrix}$$

The first step, the reduction of oxidised cytochrome by dehydrogenase, can be inhibited by narcotics like chloroform or the urethanes, so that in their presence there is an accumulation of oxidised cytochrome. The second stage can be inhibited by the action of cyanide or sulphide, under whose influence reduced cytochrome accumulates.

The reduction of oxidised cytochrome to reduced cytochrome can be catalysed by a number of dehydrogenases specific for the substrate which is the hydrogen donator. Thus the dehydrogenases for the oxidation of α-glycerophosphate to glyceraldehyde phosphate, of succinic acid to fumaric acid, of lactic acid to pyruvic acid and of formic acid to carbon dioxide, all transfer hydrogen to cytochrome. Some of them can make use of acceptors other than cytochrome ; for instance methylene blue or pyocyanine are acceptors for glycerophosphate dehydrogenase but riboflavin, the flavo-proteins, glutathione or ascorbic acid cannot serve this purpose ; methylene blue can also accept hydrogen from succinic, lactic and formic dehydrogenases.

Aerobic bacteria contain all the cytochrome components, the facultative anaerobes one or two of them, whilst the strict anaerobes contain no cytochrome at all. The respiratory activity of aerobic organisms is proportional to the amounts of cytochrome and cytochrome-oxidase which they contain. Almost all aerobic respiration takes place through the cytochrome system.

In addition to the cytochrome system there are other systems which have a similar function in acting as intermediaries in hydrogen transfer reactions. Thus in alcoholic fermentation acetaldehyde is reduced to ethyl alcohol and phosphoglyceraldehyde is oxidised to phosphoglycerate by a pair of coupled reactions in which co-enzyme I, diphosphopyridine nucleotide, acts as hydrogen carrier :—

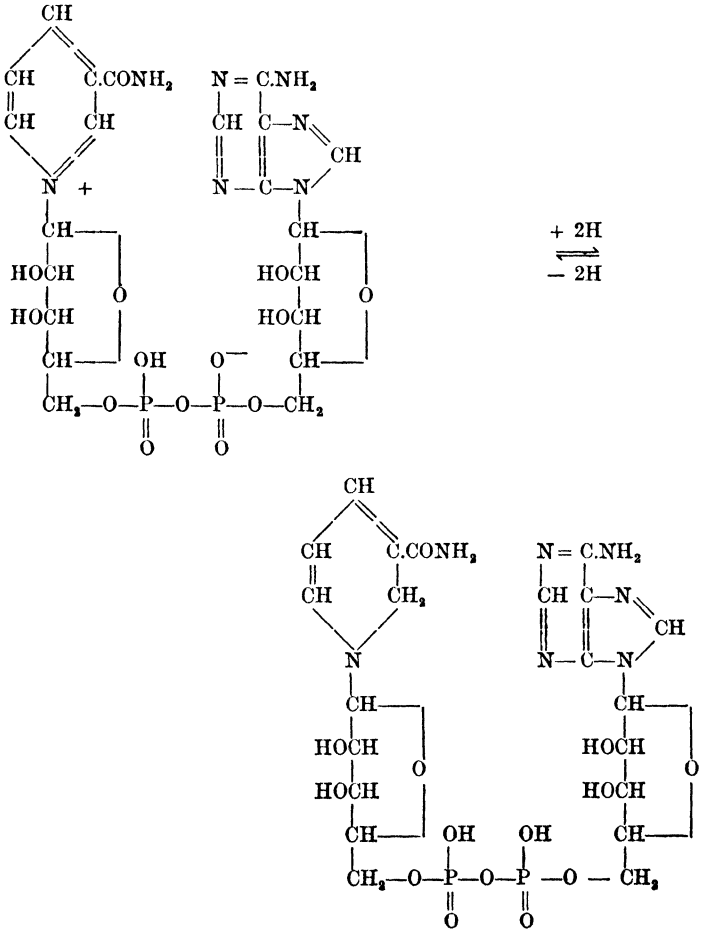
(1) 3-Phosphoglyceraldehyde + phosphate + co-enzyme I

$$\Big\Vert \text{ Triose phosphoric enzyme}$$

1 : 3 diphosphoglycerate + dihydroco-enzyme I

(2) Dihydroco-enzyme I + $CH_3CHO \longrightarrow$ Coenzyme I + C_2H_5OH.

The pyridine ring in co-enzyme I becomes reduced to give dihydroco-enzyme I which can be re-oxidised, in the presence of the specific flavoprotein acting as a dehydrogenase, to give the original co-enzyme :—



The breakdown of formic acid to hydrogen and

carbon dioxide (see p. 249) similarly takes place with co-enzyme I acting as hydrogen acceptor, and then becoming re-oxidised.

Co-enzyme I plays a similar role in the action of aldehyde mutase in producing alcohol and acetic acid from acetaldehyde :—

$$(1)\ CH_3CHO + \text{Co-enzyme I} \xrightarrow{+H_2O} CH_3COOH + \text{reduced co-enzyme I}$$

$$(2)\ \text{Reduced co-enzyme I} + CH_3CHO \longrightarrow \text{Co-enzyme I} + C_2H_5OH.$$

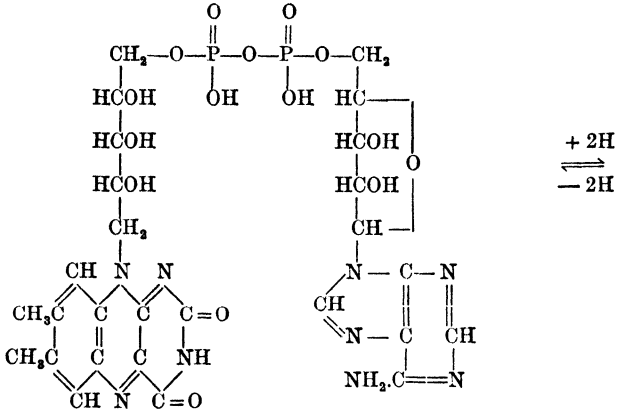
The overall effect is the coupled oxidation and reduction of acetaldehyde :—

$$2CH_3CHO + H_2O \longrightarrow CH_3CH_2OH + CH_3COOH.$$

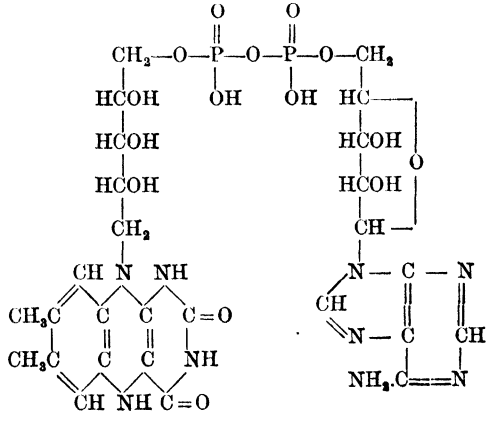
The dismutation of triose phosphate in yeast fermentation takes place by the same mechanism (see page 276).

Co-enzyme II, triphosphopyridine nucleotide, constituted similarly to co-enzyme I but containing three instead of two phosphate groups, behaves in the same way in the conversion of hexose-monophosphate to phosphohexonic acid, and in the citric acid cycle.

The flavo-protein enzymes which participate in the oxidation of dihydroco-enzymes contain the prosthetic group, riboflavin adenine di-nucleotide, which acts as hydrogen acceptor from the dihydroco-enzyme and gives rise to the colorless dihydro compound (*see opposite page*);



Yellow.



Colorless.

The dihydro- (or leuco-) flavoprotein can be re-oxidised by atmospheric oxygen to form the original enzyme and hydrogen peroxide. In some cases the leuco-flavoprotein may be re-oxidised with the intervention of yet another carrier, probably cytochrome, before

the hydrogen is finally handed over to atmospheric oxygen.

The flavin adenine dinucleotide and its carrier protein (sometimes called diaphorase) are present in animal tissues and micro-organisms and constitute the enzyme necessary to oxidise reduced co-enzyme I and co-enzyme II. Warburg's " yellow respiratory enzyme," consisting of riboflavin phosphate and protein, serves the same purpose but is not found in animal tissues.

The aerobic oxidation of a substrate such as lactate can be summarised by the following equations :—

(1) Substrate + co-enzyme I $\longrightarrow$
Oxidised substrate + dihydroco-enzyme I

(2) Dihydroco-enzyme I + flavoprotein $\longrightarrow$
Co-enzyme I + leucoflavoprotein.

(3) Leucoflavoprotein + oxidised cytochrome $\longrightarrow$
Flavoprotein + reduced cytochrome.

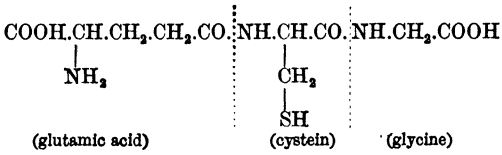
(4) Reduced cytochrome + oxygen $\longrightarrow$
Oxidised cytochrome + water.

The necessity for the long series of steps between the initial substrate and oxygen arises because the reactions must all take place under conditions of *p*H and temperature compatible with living cells, but must provide a considerable amount of energy. Obviously if the change took place in one step the reversal necessary to keep up a supply of the enzyme would involve somewhat drastic conditions. As a somewhat crude analogy the process of enzymatic oxidation of a substrate might be likened to transferring sacks full of some cargo from the deck of a ship to the hold and returning the sacks to be refilled. The sacks might be dropped straight into the hold, but the distance might well be too great to throw the empty sacks back again. If, however, the cargo were emptied from the sack at deck level into one at a slightly lower level, the empty sack could be handed back and refilled easily, and the process carried on from level to level until

the bottom of the hold was reached. The cargo would all be transferred from the deck to the hold but no sack would have to be moved through more than a small distance. The cargo obviously represents hydrogen atoms, the sacks are the enzymes, the different levels are the different co-enzymes, the deck is the substrate and the hold represents atmospheric oxygen.

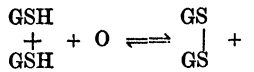
The autotrophic hydrogen bacteria contain a hydrogenase by which the reduction of such substrates as oxygen, nitrate, sulphate or fumarate by molecular hydrogen is effected, a process very similar to reduction by hydrogen in presence of platinum black as a catalyst.

It is possible to induce many anaerobes to grow in the presence of oxygen by causing the medium to have a reducing potential sufficiently high to overcome the effect of the oxygen and prevent the formation of hydrogen peroxide. This can be accomplished by the addition of strongly reducing substances like cystein or the oxidation-reduction system glutathione, both of which contain the sulphydryl group —SH. Glutathione is a tripeptide (γ-glutamylcysteylglycine) composed of glycine, cystein and glutamic acid :—



Its formula is usually written in brief as GSH.

Two molecules of this reduced form of glutathione can combine with elimination of hydrogen to give the oxidised form :—



The oxidised form can be readily reduced again if a

suitable hydrogen donator (almost invariably a protein) is present :—

$$\begin{matrix} GS \\ | \\ GS \end{matrix} + DH_2 \longrightarrow \begin{matrix} GSH \\ + \\ GSH \end{matrix} + D.$$

(protein)

Thus glutathione acts as an intermediary in hydrogen transfer from the donator to oxygen as acceptor in such a way that hydrogen peroxide is not formed.

It is very probable that glutathione is the prosthetic group of the enzyme glyoxalase which brings about the conversion of methylglyoxal to lactic acid (see p. 246) by an internal dismutation :—

$$CH_3CO.CHO + H_2O \longrightarrow CH_3CHOH.COOH \quad .$$

We have seen that when an organism grows in a medium containing an organic compound as the source of energy it usually oxidises that compound which accordingly loses a certain amount of hydrogen. If the process is aerobic the hydrogen is taken up by oxygen, but under anaerobic conditions some substance other than oxygen must act as the hydrogen acceptor. In order that it may do so it must be activated by the organism concerned. Aerobically only one compound, the hydrogen donator, has to be activated, but anaerobically both donator and acceptor have to be activated. If a medium contains two compounds which can be activated in this way (one as donator and one as acceptor) by an organism, which is normally aerobic, it will support the anaerobic growth of that organism, but not of an organism which can activate only one or neither of the compounds. The following examples illustrate this point. Each of the organisms *Esch. coli*, *Serratia marcescens*, *Proteus vulgaris* and *Alcaligenes fæcalis* has been shown by the methylene blue technique to activate lactate as a hydrogen donator. Both *Esch. coli* and *Ser. marcescens* can activate fumarate and nitrate to act as hydrogen acceptors, and in consequence these two organisms can grow anaerobically on a

medium containing either lactate and fumarate or lactate and nitrate, but not on one containing only one of these substances (see Table 17).

TABLE 17

	Activates Lactate as Donator.	Activates as Acceptor.		Anaerobic Growth.	
		Fumarate.	Nitrate.	L+F.	L+NO_3.
Esch. coli - -	+	+	+	+	+
Ser. marcescens -	+	+	+	+	+
Proteus vulgaris -	+	—	+	—	+
Alcaligenes fæcalis	+	—	—	—	—

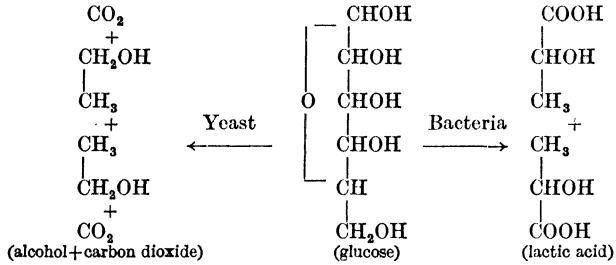
Proteus vulgaris can activate nitrate as hydrogen acceptor, but not fumarate, and therefore it is capable of anaerobic growth on a lactate+nitrate medium but not on lactate+fumarate. Finally, *Alcaligenes fæcalis*, which will activate neither fumarate nor nitrate, cannot be induced to grow anaerobically on either of the media.

Esch. coli contains enzymes which can activate glucose, glyceraldehyde, glycerol, acetate, butyrate, *l*-glutamate, lactate, malate, pyruvate and even molecular hydrogen as donators of hydrogen to fumarate as acceptor under anaerobic conditions.

The majority of bacteria can reduce nitrates to nitrites, and many of these can further reduce nitrites to ammonia; one group, the denitrifying bacteria like *Pseudomonas fluorescens*, reduces nitrites with production of gaseous nitrogen. Some organisms while unable to reduce nitrates are able to reduce nitrites. Among the organisms which are capable of reducing nitrates to nitrites but not further are the *Vibrios*; some of these, such as *V. comma*, can also produce indole from the tryptophane in peptone and use is made of this property in their diagnosis. On

addition of sulphuric acid to such a culture in peptone water a red colour develops (the so-called "cholera red reaction"), due to the nitroso-indole reaction between nitrite and indole.

In intra-molecular fermentations of Type II (see p. 197) complete oxidation does not occur as a rule. In these cases the energy is supplied by a shift of the oxygen in the molecule, usually towards the ends of the chain. This may be illustrated by a comparison of glucose and its fermentation products, alcohol and carbon dioxide, as the result of yeast fermentation, or lactic acid following bacterial fermentation :—



Compounds in which this accumulation of oxygen at the ends of the carbon chain has already occurred are not capable of serving as energy sources by intra-molecular fermentation. If they are fermented at all it is by the mechanism of Type III, in which some outside hydrogen acceptor is necessary. Thus the simple alcohols, fatty acids and dibasic acids, like oxalic, malonic or succinic acids, require either oxygen or some hydrogen acceptor like nitrate in order that they may be utilised by the organism. It is the uniform distribution of oxygen along the chain of carbohydrate molecules which renders them so valuable as nutrient materials. Hydroxy-acids, like tartaric or lactic acids and amino-acids, which yield hydroxy-acids on hydrolytic deamination (see Chapter XIII), can also be fermented anaerobically by Type II reactions.

From a consideration of all these facts it can be appreciated that it is not possible to define the classes aerobic, facultative and strict anaerobic organisms at all rigidly. For instance, it is not strictly accurate to define aerobes as bacteria which need oxygen as hydrogen acceptor, since we have seen that they will grow anaerobically if other suitable hydrogen acceptors are provided. The converse also holds; the normally strict anaerobe, *Cl. sporogenes*, can be made to grow in oxygen if a strongly reducing substance like cystein is added to the medium. The role played by the toxicity of oxygen must also be borne in mind, particularly in considering micro-aerophilic organisms which will only tolerate a lowered oxygen tension in spite of the fact that they normally use oxygen for their respiratory processes. It should be pointed out that oxygen is not itself toxic, but gives rise to hydrogen peroxide which is toxic. On the other hand, the streptococci, which grow in the presence of oxygen but do not utilise it for their respiration, are not on that account called anaerobes, although their metabolism is exactly that of the anaerobes. While oxygen may not be required for the supply of energy, it is sometimes necessary for the growth requirements of the organism. The urea bacteria afford an illustration of this; they derive their energy needs by the breakdown of urea to ammonia and carbon dioxide, but they cannot grow in the absence of oxygen, because the synthesis of their cell constituents involves dehydrogenation reactions, needing oxygen as a hydrogen acceptor, urea itself being unable to serve as such.

Strictly speaking, then, aerobes and anaerobes are not clear-cut groups, but may be more or less interchangeable under appropriate conditions. The differentiation of bacteria into aerobic, anaerobic and facultative organisms is, however, a very useful working classification for general purposes.

A list of the chief enzymes involved in the respiration

of micro-organisms is given in Table 18. The exo-cellular enzymes of the Hydrolase class are not included since their function is preparative and they are only indirectly concerned in respiration, by assisting in the supply of raw materials.

TABLE 18

ENZYME	REACTION	ORGANISM
Acetaldehyde reductase	Acetaldehyde $\longrightarrow$ Ethyl alcohol	Bacteria, yeasts.
Carboxylase	Decarboxylation of pyruvic acid	Yeast.
Catalase	Decomposition of hydrogen peroxide	Aerobic and most facultative anaerobic bacteria, yeast.
Cytochrome-oxidase	Oxidation of reduced cytochrome	All aerobic organisms.
Deaminase	*l*-Glutamic acid $\longrightarrow$ *a*-ketoglutaric acid + NH_3	Bacteria, yeast.
Enolase	2-Phosphoglyceric acid $\longrightarrow$ Phosphopyruvic acid.	Bacteria, yeasts.
Flavoproteins (Warburg's "yellow enzyme," Diaphorase)	Oxidation of reduced Co-enzymes I and II	Bacteria, yeasts.
Formic dehydrogenase	Formic acid $\longrightarrow$ Reduced carrier + CO_2	*Esch. coli.*
Fumaric hydrogenase	Fumaric acid $\longrightarrow$ Succinic acid	*Esch. coli.* Anaerobic bacteria.
Glucose dehydrogenase or Glucose oxidase	Oxidation of glucose to gluconic acid	Penicillia, Aspergilli, *B. gluconicum.*
Glyoxalase	Methyl glyoxal $\longrightarrow$ Lactic acid	Lactic acid bacteria, yeasts.
Hexose monophosphorylase	Glucose-6-phosphate $\longrightarrow$ 6-Phosphogluconic acid	*Esch. coli*, yeasts.
Hydrogenase	Reduction of oxygen, nitrate, sulphate, fumarate	*Azotobacter*, *Esch. coli*, Autotrophic bacteria.
Hydrogenlyase	Formic acid $\longrightarrow$ $H_2 + CO_2$	*Aerobacter*, *Esch. coli.*
Isomerase	Dihydroxyacetone phosphate $\longrightarrow$ Phospho-glyceraldehyde	Yeasts.
Lactic dehydrogenase	Lactic acid $\longrightarrow$ Pyruvic acid	*Esch. coli*, *N. gonorrhoeæ*, yeasts.
Lactic acid enzyme	2 Pyruvic acid $\longrightarrow$ Lactic acid + acetic acid + CO_2	Lactic acid bacteria, *N. gonorrhoeæ.*
Peroxidase	Oxidation of substrates by hydrogen peroxide	*N. gonorrhœæ*, *Acetobacter peroxidans*, Streptococci.
Phosphogluconic acid enzyme	6-Phosphogluconic acid $\longrightarrow$ 6-Phospho-2-ketogluconic acid	*Acetobacter*, yeasts.
Phosphoglycero-mutase	3-Phosphoglyceric acid $\longrightarrow$ 2-Phosphoglyceric acid	*Esch. coli*, yeasts.
Pyruvic oxidase	Pyruvic acid $\longrightarrow$ Acetic acid + CO_2	*L. delbrückii*, *N. gonorrhœæ*, *Str. pyogenes.*
Succinic dehydrogenase	Succinic acid $\longrightarrow$ Fumaric acid	*Esch. coli*, etc.
Triose phosphorylase	Phosphoglyceraldehyde $\longrightarrow$ Phosphoglyceric acid (with reduction of co-enzyme I)	*Esch. coli*, yeasts.
Zymohexase	Fructose-1 : 6-diphosphate $\longrightarrow$ Triosephosphates	*Esch. coli*, yeasts.

For further reading :—

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H. Wieland, "On the Mechanism of Oxidation." Oxford University Press. London, 1932.

CHAPTER XIII

NITROGEN METABOLISM

THE cells of bacteria, yeasts and fungi may contain as much as 87·5 per cent. of nitrogenous constituents, although about 70 per cent. is a more usual value. The greater proportion of these substances comprises the proteins of the protoplasm and nuclear material of the cells, but no less important in function, though smaller in amount, are the enzymes, which are all nitrogenous. In general the bacterial proteins are like those found in other organisms, being built up of the same amino-acid units, but individual differences occur from species to species. Many of the serological distinctions between bacteria of different species depend on differences between the proteins contained in them.

Obviously, in order that bacteria may grow and reproduce, a supply of nitrogen, as well as of other constituents, must be available from which the cells can synthesise the proteins and enzymes and other nitrogenous compounds to be incorporated in the newly formed cells. We will first consider the forms in which nitrogen is available to the organism, and then the mechanisms by which it is converted into an integral part of the structure of the cell.

Nitrogen Requirements

Elementary Nitrogen.—Free nitrogen can be utilised by certain of the soil bacteria, notably *Azotobacter* and *Rhizobium* which fix atmospheric nitrogen, probably with the intermediate formation of ammonia. There has

been some variation of opinion as to the fixation of nitrogen by yeasts. About the beginning of the century it was claimed that species of *Torula*, *Saccharomyces*, *Oidium* and *Monilia* were able to use gaseous nitrogen when grown on artificial media containing only carbohydrates and tap-water. Then followed a period during which fixation of nitrogen was denied, the growth observed in the earlier experiments being ascribed to traces of nitrogenous impurities in the sugars and to ammonia and nitrates in the water. It was shown that very small quantities of nitrogen, of the order of 0·01 per cent., would serve to support growth. Later Fulmer and Nelson showed that if *Sacch. cerevisiæ* is grown for a long period on sucrose-phosphate solutions freed from ammonia and oxides of nitrogen, fixation does occur. The gain in nitrogen is only apparent after about six weeks, an actual loss being observed during the early stages, probably due to conversion of some of the nitrogen in the yeast into undetectable compounds. This time-lag may well be the cause of the failure of earlier workers to detect fixation.

Nitrites and Nitrates.—These substances can be used as nitrogen source by bacteria of the *Azotobacter* species, ammonia probably being formed as an intermediate. The question is still not settled in the case of the yeasts, but the balance of opinion is that nitrites and nitrates are not utilised, especially under aerobic conditions. Their presence in a medium stimulates spore formation.

Ammonium Salts and Amines.—These substances are usually readily assimilable, ammonium phosphate being a good source of nitrogen for all micro-organisms except *Vibrio comma*. Soil bacteria of the *Nitrosomonas* group oxidise ammonia to nitrite. Free ammonia can be used by many organisms, but if it is present in any but very low concentrations it retards growth owing to its toxicity. The same applies to the amines, some of which, especially

hydrazines, are very toxic. In the presence of glucose, ammonium salts are more readily utilised than amines. The presence of ammonium salts stimulates the utilisation of amides; some acids, for example malic acid, which are not normally fermented can be fermented when present as the ammonium salt.

In the commercial production of protein by yeasts, using molasses as the substrate, the replacement of up to 50 per cent. of the nitrogen of the molasses by ammonium salts leads to an increased yield, but a complete replacement restricts growth; the optimum concentration of ammonium salts has been shown to be that which causes least swelling of the proteins.

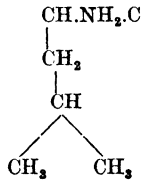
Amides.—The amides, particularly urea, can act as nitrogen source for bacteria and yeasts, probably being utilised *via* ammonia as an intermediate. Formamide appears to be more readily utilised than other amides, probably because of the constant presence of ammonia in its solutions.

Amino-Acids.—The majority of amino-acids are effective as nitrogen sources, although some, for example tryptophane and tyrosine, may be toxic if present in any great quantity owing to the end products, indole and phenol respectively, formed under appropriate conditions. The open chain amino-acids are more easily attacked than those containing ring systems (tyrosine, tryptophane, histidine). Some organisms, for example *C. diphtheriæ*, *H. influenzæ* and Lactobacilli, require complex mixtures of amino-acids for their growth, although most will grow on a single simple amino-acid. For the exacting organisms some amino acids are essential, but others are not. Thus for the growth of *L. arabinosus* on a synthetic medium arginine, cystine, glutamic acid, *iso*leucine, leucine, methionine, phenylalanine, tryptophane, tyrosine and valine are essential; in addition other amino-acids are also required but the need can be supplied by one of several, whilst omission of any one of those listed causes

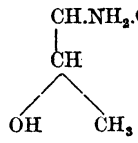
failure to grow. *Esch. coli* and "trained" *Eberthella typhosa* can synthesise tryptophane from ammonia and carbohydrates; *B. anthracis* and *Staphylococcus aureus* need amino-acids, whilst untrained *E. typhosa*, *C. diphtheriæ*, *Cl. sporogenes*, *Cl. botulinum*, etc., need tryptophane preformed. For the growth of *E. typhosa* or *C. diphtheriæ* indole can replace tryptophane, but derivatives such as indole-acrylic acid, indole-acetic acid or indole-propionic acid cannot. *Staphylococcus aureus* cannot convert indole to tryptophane.

Some amino-acids may be toxic unless an adequate concentration of others is present; for example, glycine, β-alanine, serine and threonine are toxic to *Str. lactis* in the presence of very small amounts of α-alanine, but not if larger amounts are present.

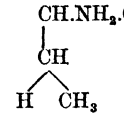
The toxic effect of leucine,



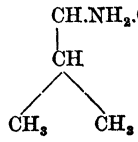
threonine,



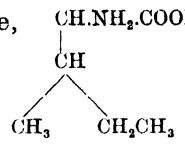
or α-aminobutyric acid,



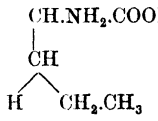
on *B. anthracis* can be eliminated by valine,



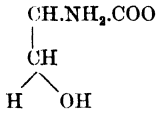
and *vice versa*. The toxicity of *iso*leucine,



norleucine,



or serine,



is removed by a mixture of valine and leucine but not by either alone. The toxicity of serine can be removed by threonine. Definite quantitative relationships between the amounts of the amino-acids exist but they are not necessarily equimolecular.

The amino-acids are less readily utilised by yeasts than are ammonium salts, which stimulate their utilisation. The amino group of asparagine, $COOH.CH(NH_2).CH_2.CONH_2$, is utilised more readily than the amide group.

It is possible that the breakdown of certain amino-acids serves as the energy source for the anaerobes *Cl. sporogenes* and *Cl. botulinum*. The reaction, described by Stickland, is between two amino-acids, one activated as hydrogen acceptor and the other as donator; glycine, *l*-proline and *l*-hydroxyproline are acceptors, and *l*-leucine, *d*-alanine, *d*-valine, *l*-phenylalanine, *l*-aspartic acid and *d*-glutamic acid serve as donators. The reaction is probably according to the equation :—

$$\begin{matrix} R_1CH.NH_2.COOH \\ + \\ RCH.NH_2.COOH \end{matrix} + H_2O \longrightarrow + \begin{matrix} R_1CO.COOH \\ \\ RCH_2COOH \end{matrix} + 2NH_3$$

Cl. tetani does not bring about the "Stickland reaction" but breaks down glutamic acid, aspartic acid or serine to give carbon dioxide, ammonia, acetic acid and butyric acid, together with some lactic acid and ethyl alcohol from aspartic acid. The same products are formed in the dissimilation of pyruvic and fumaric acids, with malic acid in addition from the latter. Glucose is not attacked by *Cl. tetani*. *Cl. tetanomorphum* and *Cl. cochlearium* can derive energy for growth from the breakdown of glutamic acid alone.

Ring Compounds.—The purines, pyrimidines and similar compounds can be used by many bacteria but not by all. Thus *Esch. coli* will not utilise uric acid or hypoxanthine, whereas *Aerobacter aerogenes* can do so. Hippuric acid can support the growth of the hæmolytic streptococci. *Cl. acidi-urici* and *Cl. cylindrosporum* can utilise uric acid and some other purines.

Proteins, Proteoses and Higher Polypeptides.—Compounds of these types are not utilised directly by bacteria or yeasts, even by the most actively proteolytic organisms. Purified proteins as a sole source of nitrogen will not permit the growth of bacteria, most probably because the cell membranes are not permeable to them. Before they can be utilised they must be broken down into simpler substances which can penetrate into the cell, where they can be acted upon by the true metabolic enzymes of the cells, the endo-enzymes. This preliminary breakdown of complex proteins to the simpler nitrogen compounds which can be used is the function of exo-cellular, hydrolytic enzymes elaborated by the bacteria. Until enough of such proteolytic enzymes are produced by an inoculum of bacteria proteins cannot be made available. Usually, of course, there is sufficient nitrogen available in a simple form in a medium to enable the culture to start growing and produce enough proteolytic enzymes to allow the organism to utilise any proteins present. Such organisms as *Esch. coli*, which do not

produce proteolytic enzymes, can never utilise proteins or proteoses and their nitrogen must be supplied already partially broken down to amino-acids or as ammonium salts. Peptone (which is protein that has been subjected to mild acid hydrolysis) in a medium serves this purpose.

Nitrogen Fixation

The phenomenon of nitrogen fixation, so important from an agricultural standpoint, has been known from time immemorial, but that it is due to micro-organisms in the soil was only established comparatively recently. Jodin in 1862 showed that certain micro-organisms (which he called "mycoderms") could grow in solutions containing sugar or tartaric acid but no organic nitrogen, and that if the cultures were allowed to stand in sealed vessels, nitrogen as well as oxygen was removed from the enclosed space. Berthelot later showed that soil which was allowed to stand underwent an increase in nitrogen content, but that this increase did not occur if the soil were first sterilised by heat. Later still he isolated organisms which could grow at the expense of atmospheric nitrogen. Much of our knowledge of nitrogen fixation is, however, due to Winogradsky, who began to study the problem in 1893; he showed that an anaerobe, *Cl. pastorianum*, very closely related to *Cl. butyricum* which also fixes atmospheric nitrogen, was capable of growth on synthetic media and could derive its nitrogen from the atmosphere. The fixation of nitrogen was proportional to the amount of glucose fermented; for each 1 gram of glucose destroyed (with formation of butyric and acetic acids together with carbon dioxide and hydrogen) approximately 2·5 mg. of nitrogen was fixed. If other sources of nitrogen, such as ammonium salts, were present fixation of atmospheric nitrogen ceased and the nitrogen of the ammonium salts was used preferentially. Winogradsky suggested that the nitrogen was reduced with formation of ammonia by nascent

hydrogen evolved during the breakdown of glucose.

Besides such anaerobic processes of nitrogen fixation, aerobic organisms are known which effect the same reaction. Beijerinck isolated organisms from soil and canal water which, when grown in a nitrogen-free medium containing an adequate carbon source, actively fixed nitrogen. The chief organism responsible is *Azotobacter chroococcum*, It is usually accompanied in nature by an organism, *Alcaligenes radiobacter*, which lives in symbiosis with it, but is itself not capable of fixing nitrogen. *A. chroococcum* ferments glucose to give mainly carbon dioxide together with lactic, acetic and formic acids and some alcohol. Stoklasa showed that when *A. chroococcum* is grown anaerobically on a medium containing nitrate it reduces the latter to nitrite and ammonia, but gives only feeble growth compared with that under aerobic conditions ; a certain amount of nitrogen fixation also occurs anaerobically. When grown aerobically on nitrate media good growth occurs, the nitrate is reduced mainly to nitrite, very little ammonia being found since it is probably used up in the synthetic processes accompanying the increased growth ; fixation of nitrogen, in this case, occurs only to a very limited extent. *Alc. radiobacter* seems to grow equally well aerobically or anaerobically and is in each case an active denitrifier, rapidly converting the nitrate to free nitrogen, which is lost from the system. When *A. chroococcum* and *Alc. radiobacter* are grown in symbiosis on nitrate media the nitrogen set free by the latter is fixed by the former and converted into cell constituents. When low concentrations of nitrate are present considerable amounts of atmospheric nitrogen are fixed, but this does not occur when high concentrations of nitrate are present in the medium.

It has been shown by other workers that when *Azotobacter* is grown on synthetic media containing glucose or mannitol as the carbon source, the organism fixes nitrogen in four to six days, and that ammonia and amino-nitrogen

can then be found in the medium after the organisms have been removed by centrifugalisation; nitrites or nitrates were not detected. These experiments support Winogradsky's theory that the nitrogen is fixed by reduction to ammonia, which is then converted to amino-compounds; further evidence is the fact that the fixation is inhibited by the presence of ammonia and of nitrate, and that nitrate is reduced to ammonia, as was shown by Stoklasa.

The fixation of nitrogen seems to be intimately bound up with the growth of the organism since the ratio of nitrogen fixed to glucose fermented (supplying the energy required) is highest in the early stages when growth is rapid, but falls off with the age of the culture until finally no more nitrogen is fixed, though glucose continues to be fermented; nitrogen is no longer fixed at this stage because there is no further growth, and therefore no synthetic requirements, but glucose continues to be fermented by the enzymes which have already been produced, and liberated by autolysis of the old cells.

The optimum *p*H for nitrogen fixation by *A. agile* is between 7·6 and 7·8. Calcium, which can be replaced by strontium which is rather less effective, is necessary for nitrogen fixation by *Azotobacter*, at a concentration of 2×10^{-4} molar. Molybdenum at a concentration of 10^{-9} molar, vanadium at 2×10^{-8} molar and iron at 10^{-3} molar stimulate growth and nitrogen fixation of *Azotobacter*.

Cl. butyricum, after continued cultivation on laboratory media, tends to lose its nitrogen fixing power. However, the power can be restored by cultivation in sterile soil, a procedure somewhat analogous to animal passage in the restoration of the virulence of pathogens. *Azotobacter* shows no tendency to such loss of activity.

Besides the free-living bacteria which fix nitrogen there is a group which live in symbiosis with the leguminous plants. The bacteria grow in the nodules on the roots of the plants which supply the carbon and energy require-

ments of the micro-organisms, which in their turn fix nitrogen. The legumes, if grown in sterile soil, require combined nitrogen for their growth, do not produce nodules and do not fix nitrogen. In non-sterile soil growth can occur in the absence of combined nitrogen, nodules are produced and atmospheric nitrogen is fixed. Beijerinck isolated the responsible organism, now known as *Rhizobium leguminosarum* and induced it to grow apart from the plant. The process of fixation of nitrogen by *Rhizobium* is considered by Virtanen and Laine to proceed through formation of hydroxylamine as a first step. They observed that when peas were grown in sterile sand inoculated with *Rhizobium* soluble nitrogen compounds were excreted by roots carrying nodules only. The nitrogen compounds were identified as the oxime of oxalacetic acid (1 to 2 per cent.), *l*-aspartic acid and β-alanine. Ammonia could not be detected. Oxalacetic acid is also an essential intermediate and is found in highest concentration at noon on bright days and lowest in the dark. The active nodules contain a red pigment which is identical with, or very similar to, hæmoglobin. Nodules may be formed by non-nitrogen fixing strains of *Rh. leguminosarum* but are not pigmented, nor are those formed by active strains in the absence of oxygen. If the plants are kept in the dark for several days the pigment becomes green, due to opening of the porphyrin ring, and the system can no longer fix nitrogen, even if the plants are transferred to the light. Virtanen suggests that the hæmoglobin acts as an oxygen transfer system, becoming oxidised to methæmoglobin with simultaneous reduction of atmospheric nitrogen to hydroxylamine :—

$$N_2 + \text{Methæmoglobin } (Fe^{III}) \rightleftharpoons NH_2OH + \text{hæmoglobin } (Fe^{II}).$$

The hydroxylamine condenses with oxalacetic acid, formed by the plant in the breakdown of sugars, to give the oxime :—

$$COOH.CO.CH_2.COOH + NH_2OH \longrightarrow COOH.C.(NOH).CH_2COOH + H_2O$$

The oxime is then reduced to give aspartic acid, $COOH.CH.NH_2.CH_2.COOH$, which can be decarboxylated to give β-alanine, $CH_2.NH_2.CH_2.COOH$. In presence of the enzyme, transaminase, aspartic acid can transfer the amino group to pyruvic acid to form α-alanine, $CH_3.CH.NH_2.COOH$, a process by which other amino-acids may be synthesised.

That nitrogen fixation is intimately connected with reduction processes involving molecular hydrogen, is shown by the fact that when nitrogen fixation by *Azotobacter* is inhibited by the presence of combined nitrogen, such as nitrate, the activity of the enzyme hydrogenase is also stopped. The adaptation of the bacteria to use nitrate more readily is accompanied by increased inhibition of both nitrogen fixation and hydrogenase activity. This occurs even in the presence of the substrate, hydrogen. Hydrogenase appears, therefore, to be an adaptive enzyme whose formation depends not on the presence of its substrate but on nitrogen fixation.

Protein and Amino-acid Breakdown

The utilisation of proteins and amino-acids by bacteria in the production of new cells during growth appears to follow somewhat the same lines as in animal nutrition. Complex proteins and polypeptides are broken down outside the cell by the proteolytic enzymes in a manner analogous to the digestion of the proteins in the stomach and intestine by the enzymes pepsin and trypsin. The breakdown products of this hydrolysis, the amino-acids and the lower peptides, are then taken into the cell, where they are either directly rebuilt into the proteins characteristic of the particular bacterial species concerned or are further broken down into ammonia and simple carbon compounds, which are then used in the synthetic processes involved in the growth of the cells.

Protein Degradation.—As indicated above, the proteins

are not available to the organism as such but must first be hydrolysed to molecules small enough to penetrate the cell wall of the bacteria. All the proteolytic organisms liberate exo-enzymes into the medium where the proteins are attacked. In other words, it is possible to obtain cell-free solutions by the filtration of fluid cultures of such organisms as *Proteus vulgaris*, *B. subtilis*, *Serratia marcescens* and most anaerobes, which will hydrolyse proteins, as shown by the liquefaction of gelatin or the breakdown of casein. The enzymes responsible are constitutive, that is, they are produced even when the organism grows on a synthetic medium containing no protein. In some cases it has been claimed that they are adaptive enzymes, only being formed when protein is present in the medium, although the evidence for this is somewhat doubtful; it is very improbable that such proteolytic enzymes could be adaptive since the protein which would provoke them cannot penetrate into the cell, where alone it could influence enzyme synthesis.

Proteolytic endo-enzymes also occur in some organisms, from which they may be extracted after destroying the cell structure by an appropriate means, such as solution in bile, or in sodium hypochlorite, or by grinding, or repeated alternate freezing and thawing. Usually these endo-enzymes attack peptones and partially degraded proteins more readily than they do the complex proteins. For instance, endo-enzymes have been obtained from the pneumococcus, which will break down peptone to amino-acids but will not attack gelatin or egg-albumin; hæmolytic streptococci yield an endo-enzyme which destroys peptone and casein but not serum albumin. Conversely the proteolytic exo-enzymes do not break down the proteins completely, but only sufficiently to enable the fragments to enter the cell, where they undergo further degradation where the products are of use to the organism. As we have already mentioned, *Esch. coli* can grow on and break down amino-acids but has no effect on proteins,

nor even on such simple compounds as the dipeptides, which are formed by the union of two amino-acids. Even the active proteolytic bacteria, like *Proteus*, cannot utilise pure protein as the sole source of carbon and nitrogen, since not enough exo-enzyme is carried over with the inoculum to break the protein down to diffusible fragments on which the cells must depend for their growth, and in the absence of growth, of course, no enzymes can be formed. The addition of small quantities of some simple nitrogen source is sufficient to allow growth to start and proteolytic exo-enzymes to be formed, and then the proteins can be hydrolysed.

Protein Sparing Action.—It is often claimed that the presence of carbohydrate in media reduces the utilisation of proteins and the production of proteolytic enzymes. Kendall states that addition of glucose to gelatin media delays the formation of the proteolytic enzymes until all the sugar has been fermented. He explains this by saying, "When the sugar is exhausted the organism is forced to derive its energy from the protein constituents, and the enzyme is then formed to bring about the necessary changes in the protein to make it assimilable." That this is not the true explanation is suggested by Berman and Rettger, who state that the inhibition is due to the acid produced by the fermentation of the sugar. In the case of *B. subtilis*, which ferments glucose only slowly, or of *Aerobacter cloacæ*, which yields products which are not strongly acid, the presence of sugar has little or no effect on the breakdown of peptone or protein. With *Esch. coli* or *Proteus*, which give much acid, the growth and chemical activities are quickly brought to a standstill unless the medium is so heavily buffered that the *p*H value never falls low enough to inhibit the breakdown of protein. When such buffering is employed the breakdown of protein proceeds as vigorously as in the absence of glucose.

It has also been shown that in the case of *Proteus* there is an optimum *p*H at 8·0 for the production of pro-

teolytic enzymes, that good aeration favours production of the enzymes, and that on synthetic media, even though good growth may occur in their absence, no proteolytic enzymes are formed unless small quantities (0·025 per cent.) of calcium and magnesium are present.

Another possible explanation of apparent protein sparing action is that the methods of estimating protein utilisation are fallacious. Normally the extent of protein degradation is estimated by the amount of ammonia and amino-nitrogen appearing in the medium as a result of the breakdown of the protein. In the presence of sugar less of these breakdown products appear than is the case when little or no sugar occurs in the medium. Now the amount of ammonia and amino-nitrogen found in the medium is that which is left over after the organism has taken what it needs to build up its protoplasm and other nitrogenous constituents. The more rapid and profuse the growth the more of such raw materials will it require and the less will be left over in the medium. One of the chief effects of a plentiful supply of easily assimilated carbon, such as glucose or other sugar, is to increase the growth of the organism in such a medium. That is, in presence of glucose more growth occurs, more amino-nitrogen is used and less remains in the medium ; in other words, there appears to be less production of amino-nitrogen and consequently less breakdown of protein, whereas in reality there may be just as much protein breakdown, or even more, in presence of sugars.

Amino-acid Degradation.—The amino-acids resulting from hydrolysis of the proteins find their way into the cell where they are acted upon by the endo-enzymes to give a variety of products, the nature of which depends on the amino-acid, the organism and the condition of the medium. The earliest work on this subject was of little scientific value since it was done with mixed cultures of putrefactive organisms on mixtures of proteins. This period was followed by one in which the action of mixed

cultures on pure amino-acids was investigated, but still it was impossible to connect the action of any one organism, for instance, with the formation of any particular type of product. Finally, Harden and Ehrlich instituted the present stage by studying the effect of pure cultures of a single organism on single pure amino-acids.

If we take $R.CH_2.CH.NH_2.COOH$ as representing the structure of an amino-acid, the various types of breakdown which it may undergo can be summarised as follows :—

A. Decarboxylation to give the **Amine.**

$$R.CH_2.CH.NH_2.COOH \longrightarrow R.CH_2.CH_2.NH_2 + CO_2.$$

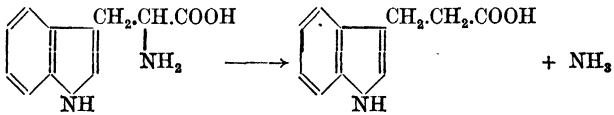
This type of breakdown is apparently only accomplished by bacteria, and is favoured by anaerobic conditions. As an example, *Ps. fluorescens* breaks down glycine to give methylamine.

B. Deamination.

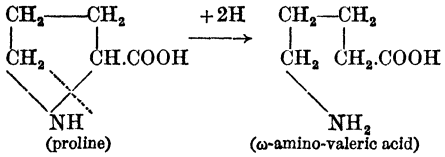
(*a*) **Reductive** to give **Saturated Acids,**

$$R.CH_2.CH.NH_2.COOH \xrightarrow{H_2} R.CH_2.CH_2.COOH + NH_3.$$

The products will be seen to be substituted propionic acids ; tryptophane gives indole-propionic acid :—



The hydrogen accepting amino-acid in the " Stickland reaction " (see p. 218) undergoes reductive deamination, at the expense of the second amino-acid which undergoes oxidative deamination (B.(*d*), p. 230). Thus glycine gives acetic acid and ammonia, ornithine gives ω-amino-valeric acid and ammonia whilst *l*-proline also gives ω-amino-valeric acid but no free ammonia. Here the opening of the ring is equivalent to deamination, the difference being that the freed amino group is held by the other end of the chain :—

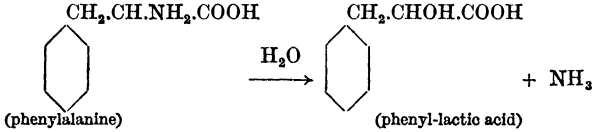


Anaerobic conditions are essential, and bacteria are particularly active in effecting this type of breakdown, although yeasts and moulds are also capable of it. Almost all amino-acids are liable to reductive deamination.

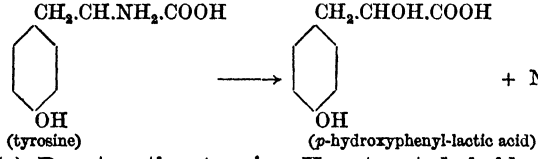
(*b*) **Hydrolytic** to give **α-Hydroxy-acids,**

$$R.CH_2.CH.NH_2.COOH \xrightarrow{H_2O} R.CH_2.CHOH.COOH + NH_3.$$

The products are substituted lactic acids ; indole-lactic acid from tryptophane, and phenyl-lactic acid from phenylalanine are examples :—



Different bacteria may attack different optical isomers ; thus *Proteus* destroys *d*-tyrosine and leaves the lævo-isomer and produces the *d*-hydroxy-acid, whilst *B. subtilis* attacks the lævo-isomer and leaves *d*-tyrosine unharmed, the product being the *l*-hydroxy-acid.



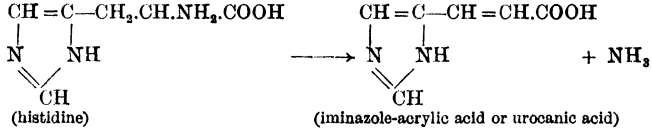
(*c*) **Desaturative** to give **Unsaturated Acids,**

$$R.CH_2.CH.NH_2.COOH \longrightarrow R.CH{=}CH.COOH + NH_3.$$

The α-β linkage is attacked with formation of substituted acrylic acids. This type of breakdown is observed with the coli-typhoid group of organisms. As examples may be quoted the formation of fumaric acid from aspartic acid :—

$$\underset{\text{(aspartic acid)}}{COOH.CH_2.CH.NH_2.COOH} \longrightarrow \underset{\text{(fumaric acid)}}{COOH.CH{=}CH.COOH} + NH_3,$$

and of iminazole-acrylic acid or urocanic acid from histidine :—



(*d*) **Oxidative** to give **α-Keto-acids,**

$$R.CH_2.CH.NH_2.COOH \longrightarrow R.CH_2.CO.COOH + NH_3.$$

Here the products are substituted pyruvic acids. This type of degradation is not easily detected since the α-keto-acids are unstable and readily undergo further breakdown, particularly under the action of yeasts. It is favoured by aerobic conditions.

The breakdown of *l*-glutamic acid and *l*-aspartic acid by *H. parainfluenzæ* to acetic acid, carbon dioxide and ammonia follows this type of degradation, the steps being α-ketoglutaric acid, succinic acid, fumaric acid, malic acid, oxalacetic acid, pyruvic acid, acetaldehyde and acetic acid from *l*-glutamic acid and oxalacetic acid, pyruvic acid, acetaldehyde and acetic acid from *l*-aspartic acid.

The hydrogen donating amino-acid in the " Stickland reaction " (see p. 218) undergoes this type of breakdown. Alanine, for instance, yields acetic acid, carbon dioxide and ammonia :—

$$\begin{aligned} CH_3CH.NH_2.COOH &\xrightarrow{H_2O} CH_3.CO.COOH + NH_3 + 2H \\ CH_3CO.COOH &\longrightarrow CH_3CHO + CO_2 \\ CH_3CHO &\longrightarrow CH_3COOH \end{aligned}$$

All the above types of deamination yield products which have the same number of carbon atoms as the original amino-acid. The α-keto-acids, owing to the ease with which they lose carbon dioxide by the action of the enzyme carboxylase (see Chapter XV), give rise to a series of products with fewer carbon atoms than the parent amino-acid, thus :—

C. With One Carbon Atom Less.

(*a*) Aldehydes,

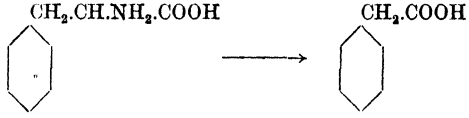
$$R.CH_2.CO.COOH \longrightarrow R.CH_2.CHO + CO_2.$$

The aldehydes are usually not end products since they undergo further reactions :—

(*b*) Saturated Acids,

$$R.CH_2.CHO \xrightarrow{\text{oxidation}} R.CH_2.COOH.$$

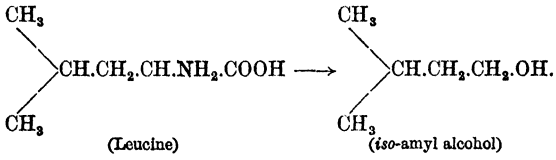
The aldehyde is oxidised to give substituted acetic acids, particularly by bacteria under aerobic conditions. For example, phenylalanine gives phenyl-acetic acid :—

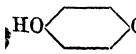


(*c*) Saturated Alcohols,

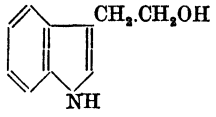
$$R.CH_2.CHO \xrightarrow{\text{reduction}} R.CH_2.CH_2.OH.$$

This type of breakdown of amino-acids is very common among the yeasts, and is, in fact, the mode of origin of the higher alcohols which constitute the fusel oil produced during alcoholic fermentation, as was shown by Ehrlich. Fungi, *Proteus*, and the lactic acid bacteria can also bring about this type of change. As examples may be mentioned the formation of *iso*-amyl alcohol from leucine :—



Tyrosine gives tyrosol (*p*-hydroxyphenyl-ethyl alcohol), 

and tryptophane gives tryptophol

(β-indole-ethyl alcohol), 

(*d*) "Hydrocarbons,"

$$R.CH_2.CH.NH_2.COOH \longrightarrow R.CH_2.CH_2.COOH \longrightarrow R.CH_2.CH_3 + CO_2$$

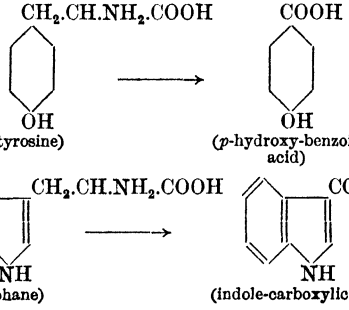
These products arise as the result of reductive deamination followed by decarboxylation, that is, by decarboxylation of the substituted propionic acid. This type of breakdown occurs by the action of putrefactive bacteria under anaerobic conditions. Glycine, for example, gives methane.

D. With Two or Three Carbon Atoms Less.

(*a*) Acids,

$$R.CH_2.CH.NH_2.COOH \longrightarrow R.CH=CH.COOH \xrightarrow{H_2O} R.CO.CH_2.COOH \longrightarrow R.COOH + CH_3COOH$$

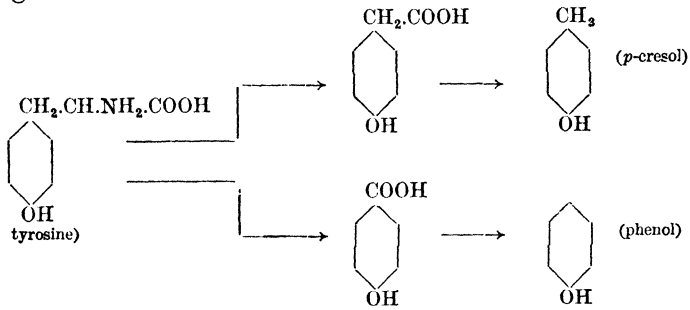
The substituted formic acids, R.COOH, with two carbon atoms less than the original amino-acid arise by decomposition of the unsaturated acid *via* the β-keto-acid. For example, *Salmonella paratyphi* and *Sal. schottmülleri* (*B. paratyphosus-A* and *-B*) under aerobic conditions produce *p*-hydroxy-benzoic acid from tyrosine and indole-carboxylic acid from tryptophane :—



(*b*) "Hydrocarbons,"

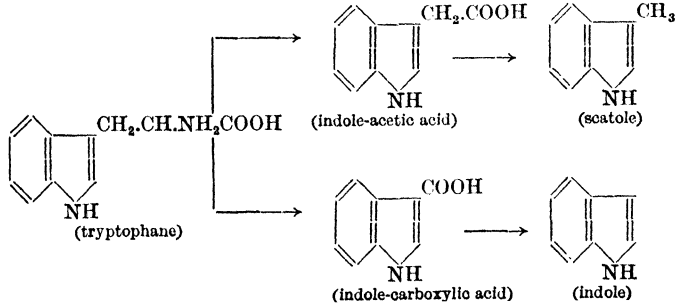
$R.CH_2.COOH \longrightarrow RCH_3 + CO_2$ (2 carbon atoms less).
$R.COOH \longrightarrow R.H + CO_2$ (3 carbon atoms less).

These products arise by the decarboxylation of the substituted acetic and formic acids produced as above. This type of breakdown appears to occur only with amino-acids like tyrosine and tryptophane, which contain ring structures. Tyrosine gives *p*-cresol (corresponding to $R.CH_3$) by the action of putrefactive organisms under anaerobic conditions, and phenol (RH) by the same organisms under aerobic conditions :—



As much as 0·8 gram of phenol per litre may be formed by certain organisms isolated from fæces.

Tryptophane gives scatole by the anaerobic action of *Esch. coli* and indole by its aerobic action, but only if sugars are absent from the medium.



Whether or not an organism decarboxylates the acid R.COOH to R.H depends on whether or not it possesses the enzyme carboxylase. *Esch. coli* does possess it and can therefore form indole from tryptophane, but *Sal. paratpyhi* and *Sal. schottmülleri* have no carboxylase and therefore produce no indole but stop at indole-carboxylic acid.

The following general scheme of amino-acid degradation (pp. 235, 236) was proposed by Raistrick, as a result of his observation that histidine under the action of organisms of the coli-typhoid group gave rise to the unsaturated acid, urocanic acid (see p. 230). All the other products of amino-acid degradation are readily accounted for by reactions of the unsaturated acid, which is formed as the primary intermediate.

The Factors Influencing the Type of Breakdown.

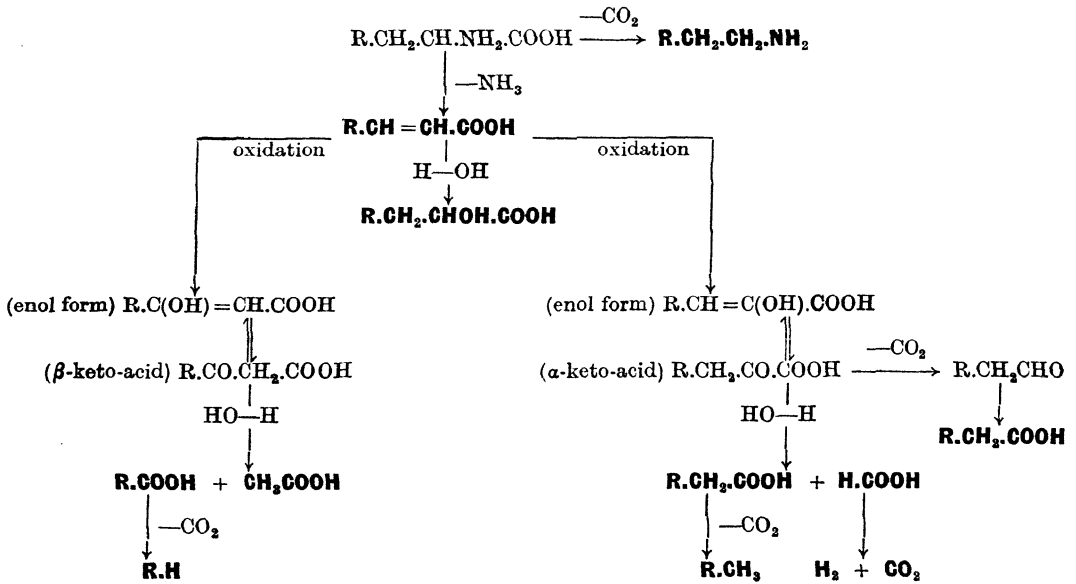
1. **The Organism.**—(*a*) **Yeasts.**—The yeasts usually cause hydrolytic deamination followed by decarboxylation and reduction to give rise to the alcohols, as in the production of fusel oil.

(*b*) **Moulds.**—The moulds usually give hydrolytic deamination, but do not cause decarboxylation, the product being the hydroxy-acid. Sometimes the hydroxy-acid may be completely oxidised.

(*c*) **Bacteria.**—The bacteria can bring about any of the types of reaction described, with formation of a correspondingly much greater variety of products. Decarboxylation does not occur so frequently as with yeasts.

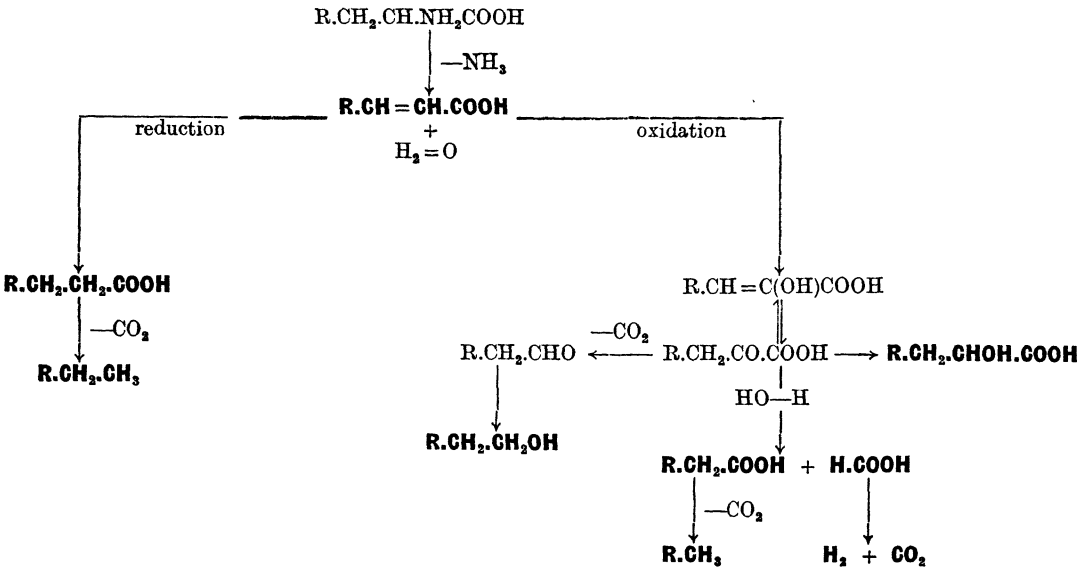
2. **The Condition of the Medium.**—Anaerobic conditions are naturally usually accompanied by reduction with accumulation of the saturated acids and the " hydrocarbons." Aerobic conditions favour the production of other types of product. Generally speaking, the presence of a readily available source of carbon inhibits the formation of indole, possibly by changing the course of the breakdown.

Aerobic



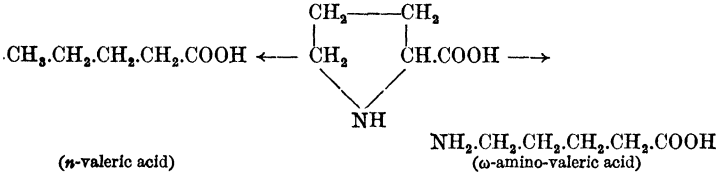
The compounds in heavy type are those which have actually been isolated.

Anaerobic



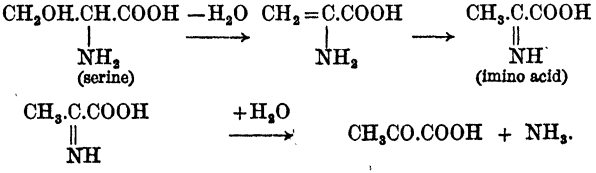
The compounds in heavy type are those which have actually been isolated.

3. The Character of the Group R.—Long chain amino-acids are more easily attacked than those containing ring structures. The ease of attack appears to increase with the length of the chain ; glycine, the simplest amino-acid, $CH_2.NH_2.COOH$, is very resistant to bacterial action, but alanine, $CH_3.CH.NH_2.COOH$, is much less resistant. Ring structures containing nitrogen can be attacked by some organisms but not others. Putrefactive organisms can open the ring of proline with formation of ω-amino-valeric acid and of n-valeric acid :—



The iminazole ring of histidine is opened by *Esch. coli*, *Sal. paratyphi* and *Sal. schottmülleri* and *Ps. fluorescens*, but not by *Proteus vulgaris*. Tryptophane is similarly attacked. Moulds can break down any type of nitrogen ring if no other source of nitrogen is available.

The deamination of serine by *Esch. coli*, which can occur aerobically or anaerobically, appears to involve the hydroxyl group since if it is masked by substitution deamination does not occur. The suggested mechanism is *via* the imino acid and pyruvic acid :—



The sulphur containing amino-acids like cysteine, $CH_2.SH.CH.NH_2.COOH$, appear to undergo reductive

deamination accompanied by further breakdown with liberation of hydrogen sulphide, the products of the action of *Proteus vulgaris* on cysteine being hydrogen sulphide, ammonia, carbon dioxide, hydrogen and acetic acid.

The course of the breakdown of amino-acids can be followed by analysing the resulting solutions for the following fractions :—

1. Total nitrogen (by Kjeldahl's method).
2. Amino-nitrogen (by van Slyke's method).
3. Synthetic nitrogen in proteins and cellular material (by precipitation with colloidal iron and Kjeldahl estimation).
4. Ammonia nitrogen (by vacuum distillation).
5. Non-amino-nitrogen, that is nitrogen in the ring, is given by 1—(2+3+4).

An increase in the amino-nitrogen figure indicates breakdown of the side chains. A decrease in the non-amino-nitrogen follows a breakdown of the ring structure. An increase in the " synthetic nitrogen " value is usually accompanied by a reduction of the amino-nitrogen and ammonia nitrogen values since the proteins are built up at the expense of such compounds. When an alternative source of carbon, for example glucose or glycerol, is present there is usually an increased utilisation of amino- and ammonia nitrogen and a corresponding increase in the " synthetic nitrogen," which is the probable explanation of the so-called " protein-sparing " action of carbohydrates (see p. 226).

When organisms act on racemic amino-acids both isomers are usually attacked, but the naturally occurring one more readily, so that an optically active mixture results. Three possibilities arise : (*a*) The natural component is attacked so rapidly compared with the other one that an almost optically pure amino-acid results

(*b*) both enantiomorphs are attacked at the same rate, giving an inactive residue, and (*c*) the rates are different but not very widely so, resulting in an optically active but impure mixture of the acids. The method can be used as a means of resolution of the isomers, but has the disadvantage of giving a maximum possible yield of 50 per cent. and of destroying the naturally occurring isomer.

The problem of protein synthesis by micro-organisms will be considered in Chapter XVIII.

For further reading :—

E. F. Gale, "Enzymes Concerned in the Primary Utilisation of Amino Acids by Bacteria." *Bact. Reviews*, **4** (1940), 135.

P. Hirsch, "Einwirkung von Mikro-organismen auf die Eiweisskörper." Die Biochemie in Einzelldarstellungen. IV.

H. Raistrick, Papers in *Biochem. J.*, **11** (1917), 71; **13** (1919), 446; **15** (1921), 76.

M. Stephenson, "Bacterial Metabolism," Chapter V. 2nd Edition. Longmans, Green & Co. London, 1939.

CHAPTER XIV

CARBON METABOLISM

AS we have seen, many organisms are capable of building up all their cell constituents and can maintain and reproduce themselves, using only a single organic substance and ammonium salts as the sources of raw materials and energy. Substances which can serve in this way to support growth may belong to almost any type of compound, saturated and unsaturated fatty acids, hydroxy-acids, keto-acids, di- and tri-baics acids, alcohols, carbohydrates, amines, amino-acids, amides, and aromatic compounds among others. Generally speaking, only a comparatively small proportion of the compound destroyed ultimately finds its way into the composition of the cell; the bulk of the compound is more or less profoundly altered during the processes by which energy is obtained. This altered part of the substrate accumulates in the medium as the products of fermentation which are characteristic of the various organisms. Some organisms, the yeasts, for instance, break up sugars with formation of alcohol and carbon dioxide, others break up sugars with production of such substances as lactic acid, acetic acid, butyl alcohol, or acetone. All these fermentation products are, from the point of view of the organism, waste products, although they may be very valuable to mankind.

The part of the compound which is converted into cell material and taken into the composition of the newly produced cells is said to be **assimilated.** The portion which is broken down to provide the energy

requirements is said to be **dissimilated.** Comparatively little is known about the processes involved in assimilation, but dissimilation has been more intensively studied.

Dissimilation, particularly under aerobic conditions, is often a catalytic oxidation process. The oxidation of ethyl alcohol to acetic acid by the various vinegar organisms is a case in point ; it is a partial oxidation, for the organism is not able to carry the reaction further to form carbon dioxide and water. Wieland, it will be remembered, proposed that these oxidations were in reality dehydrogenations, since it was possible to replace free oxygen by reducible substances like methylene blue or quinone which can act as hydrogen acceptors. Bacteria and other organisms are able to bring about these dehydrogenations by means of the enzymes, dehydrogenases, dehydrases or hydrogen transportases, which they produce and which activate the hydrogen atoms of the substrate, ethyl alcohol, acetaldehyde, etc., which is to be oxidised. The vinegar organisms, species of the genus *Acetobacter*, usually oxidise substances other than alcohols completely to carbon dioxide and water ; but one species at least is known, *A. suboxydans*, which is capable of oxidising partially many other substances, of which the following examples will serve as illustrations :—

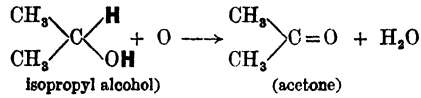
1. (*a*) $CH_3.C(OH)(H)(H) + O \longrightarrow CH_3.C(=O)(H) + H_2O$

(ethyl alcohol) (acetaldehyde)

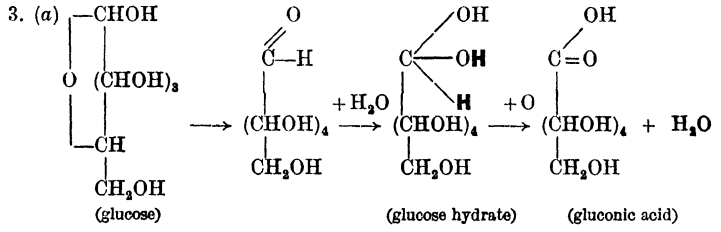
(*b*) $CH_3.C(=O)(H) + H_2O \longrightarrow CH_3.C(OH)(OH)(H) + O \longrightarrow CH_3.C(OH)(=O) + H_2O$

(acetaldehyde hydrate) (acetic acid)

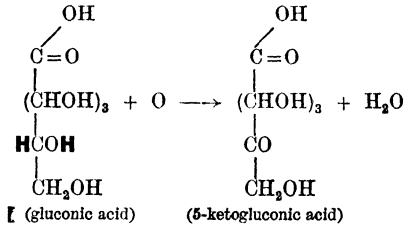
2.



3. (*a*)

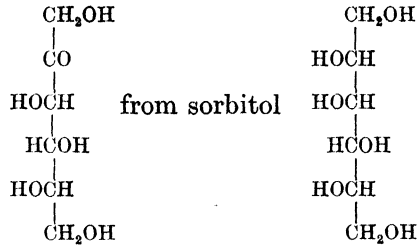


(*b*)

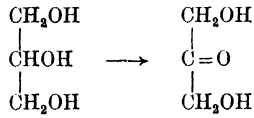


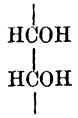
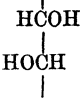
A. suboxydans oxidises glucose to gluconic acid and 5-ketogluconic acid in an acid medium ; if the medium is kept natural by carrying out the fermentation in presence of calcium carbonate, 2-ketogluconic acid is formed.

The point to be noted in each of these examples is the activation of two hydrogen atoms (shown in bold type in the formulæ) and their transfer to an oxygen atom. The reactions can all be induced to occur anaerobically if a suitable hydrogen acceptor like methlyene blue is provided in place of the oxygen. *A. suboxydans* restricts its activities to the oxidation of the secondary alcohol group, $\rangle$CHOH, to the keto group, $\rangle$C=O, but *A. xylinum*, Bertrand's sorbose bacillus, which also oxidises the same group in the same way, producing *l*-sorbose,



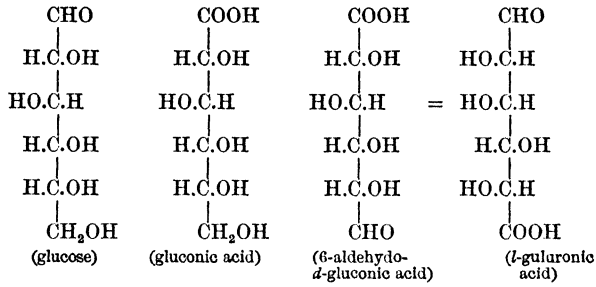
and dihydroxyacetone from glycerol,



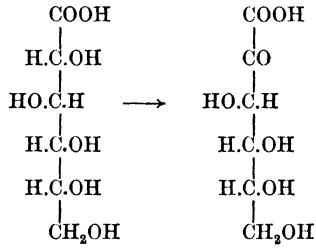
for example, can, under conditions of vigorous aeration, carry the oxidation to completion, forming carbon dioxide and water, probably by a chain of such reactions. *A. xylinum* only oxidises the >CHOH group when the hydroxyl group is adjacent to a primary alcohol group and to a second hydroxyl group, that is, alcohols with the *cis*-configuration,  , are oxidised but not those with the *trans*-configuration,  .

The potential aldehyde group of sugars is oxidised to a carboxyl group.

Both *A. xylinum* and a similar organism called *Bact. gluconicum* in addition to gluconic acid and 5-ketogluconic acid oxidise glucose to 6-aldehydo-gluconic acid which is identical with *l*-guluronic acid :—

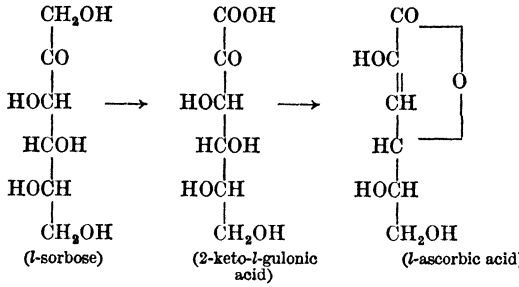


Bact. gluconicum, in neutral solution in presence of calcium carbonate, was also found to oxidise gluconic acid to 2-ketogluconic acid, which is in contradiction of Bertrand's rule, since the two hydroxyl groups are in the *trans*-position :—



Two points should be noticed, however; first, that a different organism is involved and second, that a carboxyl and not a primary alcohol group is adjacent.

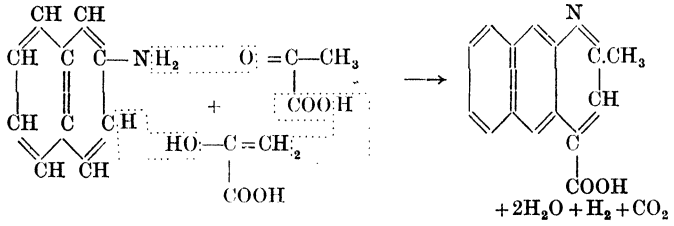
Like *A. xylinum*, *Bact. gluconicum* and *Bact. xylinoides* oxidise sorbitol to *l*-sorbose, the yields being 59, 76 and 60 per cent. respectively. *A. xylinum* is used commercially in the production of ascorbic acid to convert sorbitol, obtained by the reduction of glucose, into *l*-sorbose, which is oxidised to 2-keto-*l*-gulonic acid whose methyl ester is readily transformed to ascorbic acid :—



Acid Fermentation by Bacteria.—The acids most usually found as a result of bacterial fermentation are formic, acetic and lactic acids, but propionic, succinic and butyric acids with some others are also found with certain organisms and under appropriate conditions. Various theories to account for their production have been proposed, and a number of intermediate compounds suggested. The chief among the latter are acetaldehyde, pyruvic acid and methylglyoxal.

Acetaldehyde, like aldehydes in general, forms a water-insoluble compound with sulphites or bisulphites. If, then, bacterial fermentation is allowed to proceed in the presence of sulphite (bisulphites are usually poisonous to bacteria) any acetaldehyde formed as an intermediate will be " trapped " as the insoluble compound and will play no further part in the process but will accumulate. In this way the formation of acetaldehyde has been detected during the fermentation of glucose, mannitol and glycerol by members of the *Esch. coli* group ; among the products of all organisms giving a positive Voges-Proskauer reaction ; in acetic acid fermentation ; in acetone fermentation ; in the fermentation of sucrose by *Aerobacter aerogenes* and in the fermentation of pentoses by *B. aceto-ethylicus*. Acetaldehyde appears, therefore, to be a very general, if not universal, intermediate in bacterial fermentations.

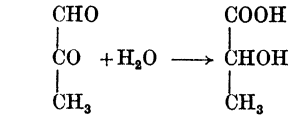
Pyruvic acid (which is, as we shall see, the normal precursor of acetaldehyde) has also frequently been isolated from bacterial fermentation solutions by appropriate "trapping" methods, for example, by the use of β-naphthylamine with which it forms the insoluble compound, α-methyl-β-naphthocinchoninic acid :—



(β-naphthylamine) (2 pyruvic acid) (α-methyl-β-naphthocinchoninic acid)

In this way pyruvic acid has been identified as an intermediate in the fermentation of lactic and fumaric acids by *Esch. coli*, and of glucose, maltose and glycerol by *B. aceto-ethylicus*. It has been shown, too, that pyruvic acid can be utilised by bacteria.

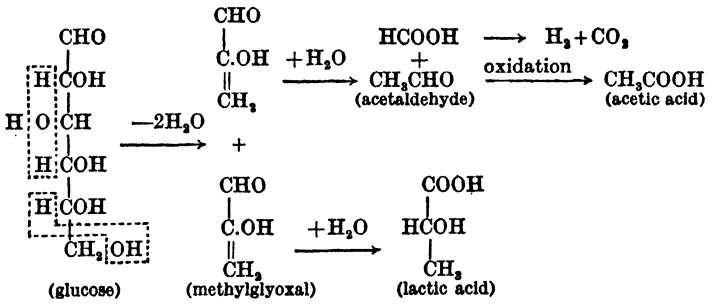
Methylglyoxal is converted into lactic acid by the action of the enzyme glyoxalase, which occurs in the liver, muscle tissues and many bacteria including *Esch. coli*, *Str. lactis*, *L. casei*, and *Acetobacter* :—



(methylglyoxal) (lactic acid)

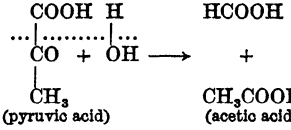
Methyl glyoxal has been detected following the action of *Esch. coli* or of *A. xylinum* on magnesium hexose phosphate in presence of toluene, and in the fermentation of glycerol by the propionic acid bacteria.

Lusk suggested that glucose is fermented to give lactic, acetic and formic acids according to the scheme shown at the top of the following page.



This is an over simplified expression of the mechanism of lactic acid fermentation by such organisms as *Esch. coli*, but obviously does not account for the fermentation by the homofermentative lactic acid bacteria which give almost 100 per cent. lactic acid. Almost certainly phosphorylation reactions are involved in lactic acid fermentation by all bacteria (see p. 249).

In alcoholic fermentation by yeast, pyruvic acid is decarboxylated to give acetaldehyde and carbon dioxide (see Chapter XV), but this mechanism cannot apply to the bacterial fermentations since it does not account for the formic acid and hydrogen found in many such fermentations. Possibly with some bacteria the pyruvic acid is broken down in another way with formation of formic and acetic acids :—



There is evidence that the reaction occurs through phosphopyruvic acid and is reversible, for when pyruvic acid is dissimilated by *Esch. coli* in presence of formic acid containing "heavy" carbon, C^{13}, the residual pyruvic acid contains C^{13} in the carboxyl group and the rate of transfer is accelerated by the addition of inorganic phosphate. Heavy carbon is also found in the

carboxyl group of pyruvic acid when *Esch. coli* acts on pyruvic acid and "labelled" sodium bicarbonate, $NaHC^{13}O_3$.

Some bacteria, such as *Lactobacillus delbrückii*, *Neisseria gonorrhœæ* and *Streptococcus hæmolyticus*, contain pyruvic oxidase which catalyses the conversion of pyruvic acid to carbon dioxide and acetic acid :—

$$CH_3CO.COOH \longrightarrow CO_2 + CH_3COOH.$$

Probably acetaldehyde is formed as an intermediate step and its oxidation takes place with flavine adenine dinucleotide as co-enzyme or carrier; the decarboxylation and oxidation are linked processes and do not occur independently. Pyruvic oxidase is inhibited by cyanide or fluoride, but yeast carboxylase is not.

The anaerobic dismutation of pyruvic acid to lactic and acetic acids and carbon dioxide probably also involves pyruvic oxidase and a carrier :—

$$\begin{array}{ccccc} CH_3CO.COOH & & H_2 & & CH_3CHOH.COOH \\ + & + & \| & \longrightarrow & + \\ CH_3CO.COOH & & O & & CH_3COOH + CO_2 \end{array}$$

Esch. coli yields a certain amount of ethyl alcohol during fermentation as well as the main acid products. It is thought that the alcohol arises as the result of a **dismutation** (the term applied to the enzymatic equivalent of the Cannizzaro reaction) of acetaldehyde :—

$$\begin{array}{ccccc} CH_3CHO & & O & & CH_3COOH \text{ (acetic acid)} \\ & & \| & & \\ + & + & \longrightarrow & + & \\ & & H_2 & & \\ CH_3CHO & & & & CH_3.CH_2OH \text{ (ethyl alcohol)} \end{array}$$

Before the end of the nineteenth century it was shown by Hoppe-Seyler that the gas production by bacteria was almost certainly due to the breakdown of formic acid or of formates. He showed that organisms producing gas from glucose all fermented formates, whilst those which were not gas producers did not ferment formates. Harden showed that *Esch. coli* and *Eberth. typhosa* when grown anaerobically on glucose broke down half the sugar to

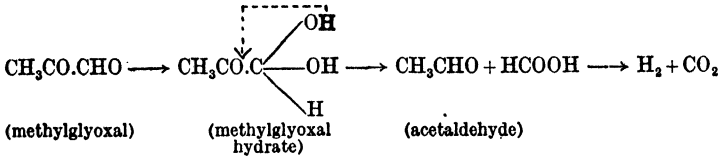
lactic acid and the other half to alcohol, acetic acid and formic acid. The formic acid was further broken down to hydrogen and carbon dioxide by *Esch. coli* (to give the typical "acid and gas" fermentation), but it was not attacked by *Eberth. typhosa* ("acid, no gas" fermentation).

The lactic acid fermentation seems to be more or less independent of the other acid fermentations, in that its formation may be stopped without affecting that of the other products. For instance, Virtanen has shown that washed suspensions of *Esch. coli*, which are deprived of cozymase (see Chapter XV) in this way, no longer produce lactic acid from glucose, though the other products are formed as usual. Virtanen considers that the first stages of lactic acid fermentation are identical with those of alcoholic fermentation by yeast and involve phosphorylation of the glucose, for which process cozymase is essential; methylglyoxal, the precursor of lactic acid, is then formed. That phosphorylation does play a part in lactic acid fermentation is shown by the fact that the addition of inorganic phosphates to such a fermentation brings about an acceleration of the process just as it does in alcoholic fermentation. Bacterial cozymase can replace that from yeast in alcoholic fermentation. Virtanen claims that cozymase does not play a part in the formation of the other products. It has been shown that the propionic acid bacteria behave similarly; washed suspensions no longer produce propionic acid, but still give rise to the formation of alcohol, acetic acid, succinic acid and carbon dioxide.

By grinding *Esch. coli* with powdered glass cell free extracts can be obtained which contain enzymes which are capable of converting phosphoglyceric acid to phosphopyruvic acid, as occurs in yeast fermentation (see p. 276). The equilibrium between 3-phosphoglyceric acid and 2-phosphoglyceric acid also occurs in the presence of the bacterial enzymes as well as in the yeast and

muscle systems. The transfer of phosphate from phosphopyruvic acid *via* adenylic acid to glucose, similarly, takes place under appropriate conditions. Enzymes have been obtained from *Staph. albus* which can bring about all the reactions of the Embden-Meyerhof scheme (see p. 275). Lactic acid is only produced under anaerobic conditions by this organism. There is, therefore, very considerable evidence that the initial stages of bacterial fermentation are very similar to, if not identical with, those of yeast fermentation, and that the variations producing the additional acids and other substances arise in the later stages of fermentation.

Kluyver, also, regards methylglyoxal as the intermediate in these acid fermentations :—



The acetaldehyde gives acetic acid by direct oxidation aerobically, or possibly by dismutation, anaerobically. Kluyver considers that there are three types of fermentation brought about by organisms of the *Esch. coli* group :—

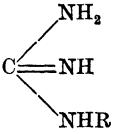
1. Succinic acid fermentation, which occurs in the absence of cozymase, and therefore in the absence of phosphorylation. It takes place by a splitting of the glucose molecule into a four-carbon atom fragment and a two-carbon atom fragment giving tartaric dialdehyde and ethylene glycol respectively, which in turn give rise to succinic acid and to acetaldehyde, as shown in the scheme below.

Virtanen also suggested that succinic acid arose by splitting of hexoses into four-carbon and two-carbon fragments.

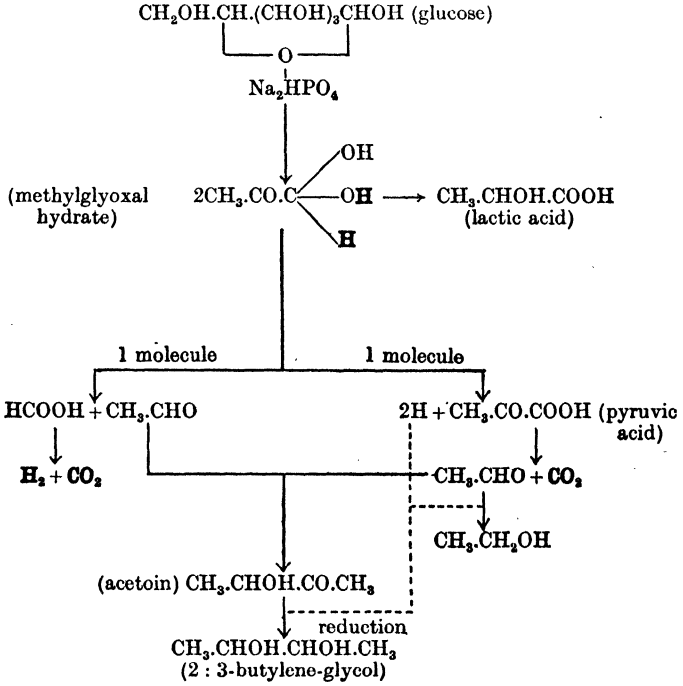
As a result of studies of the fixation of carbon dioxide

by heterotrophic bacteria it has been shown that succinic acid is formed by the condensation of pyruvic acid with carbon dioxide followed by reduction *via* malic and fumaric acids (see p. 258). It is probable, therefore, that succinic acid does not arise by the splitting of a six-carbon molecule into four-carbon and two-carbon fragments. Succinic acid may also be formed by the reductive deamination (p. 228) of aspartic acid :—

$$\begin{array}{l} COOH.CH_2.CH.COOH \longrightarrow COOH.CH_2.CH_2.COOH + NH_3 \\ \quad\quad\quad\

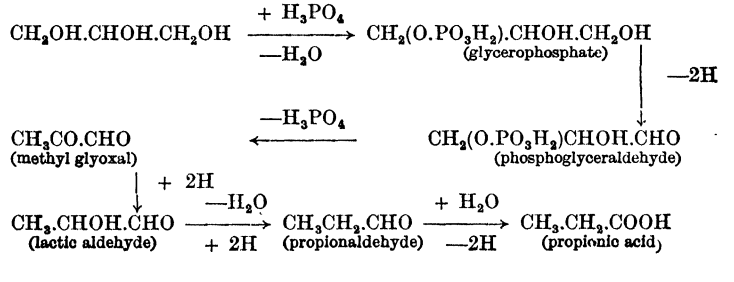
Proskauer reaction. It has been demonstrated that the Voges-Proskauer reaction depends on the production of acetyl-methyl-carbinol, or acetoin, $CH_3CO.CHOH.CH_3$, which, in the presence of potassium hydroxide becomes oxidised to diacetyl, $CH_3CO.CO.CH_3$, which reacts with some substance in the peptone containing a guanidine residue,  , to give the red-coloured compound.

The formation of acetoin by Voges-Proskauer positive organisms like *Aerobacter aerogenes* follows the scheme below, suggested by Kluyver :—



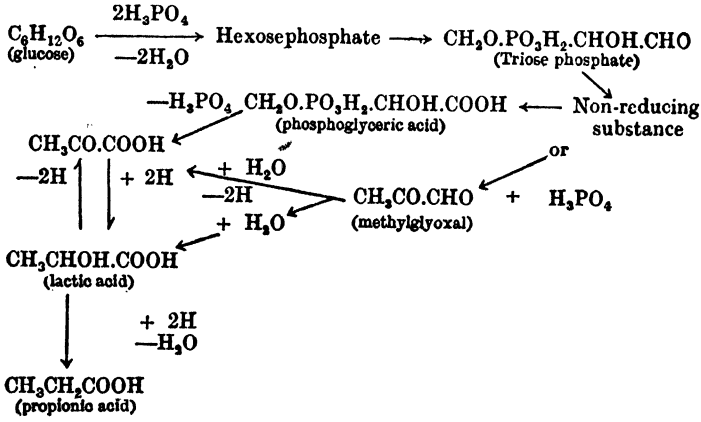
Methylglyoxal hydrate, produced as in alcoholic fermentation (see Chapter XV), is partially converted into lactic acid (type 3), partially broken down to give pyruvic acid and carbon dioxide and partially broken down to give acetaldehyde and formic acid. The formic acid gives hydrogen and carbon dioxide, as it does in the case of *Esch. coli*. The pyruvic acid is decarboxylated (see Chapter XV) to give acetaldehyde and carbon dioxide. The molecules of acetaldehyde from this source and directly from the methylglyoxal condense, under the influence of the enzyme carboligase, to form acetoin. The hydrogen evolved when methylglyoxal hydrate yields pyruvic acid is partly taken up in reducing some acetaldehyde to alcohol and partly in reducing some of the acetoin to 2 : 3-butylene glycol, which is almost invariably found among the products of the Voges-Proskauer positive organisms. The 2 : 1 ration of carbon dioxide to hydrogen which is associated with the Voges-Proskauer reaction follows from the mechanism suggested, carbon dioxide arising from two sources and hydrogen from one. The formation of acetoin is favoured by conditions, such as aeration or the presence of other hydrogen acceptors, which restrict the reduction of acetaldehyde to alcohol.

The formation of propionic acid from glycerol by the propionic acid bacteria proceeds without any production of gas. Propionaldehyde and pyruvic acid have been detected in fermenting cultures. Wood and Werkman suggest that the steps in the fermentation are :—



An alternative route is that glycerophosphate is oxidised to phosphoglyceric acid which gives pyruvic acid which is reduced to propionic acid.

When glucose is dissimilated by propionic acid bacteria, phosphoglyceric acid is produced and can be isolated if toluene and sodium fluoride are present (the latter inhibiting further breakdown of the phosphoglyceric acid). Pyruvic acid can be fixed by using sodium sulphite, but acetaldehyde cannot be detected. This suggests that the propionic acid bacteria have no carboxylase and do not split pyruvic acid to give acetaldehyde and carbon dioxide, as do the yeasts. Some strains also produce lactic acid, but others do not. Lactic acid, however, is fermented by all strains with formation of propionic acid. Succinic and acetic acids are also formed, but undergo further breakdown, the ratio of propionic acid to acetic acid increasing during the fermentation. If the culture is buffered by sodium bicarbonate the ratio of propionic acid to acetic acid remains approximately constant. Wood, Stone and Werkman suggested that propionic acid was formed by the following scheme :—



The triose phosphate, derived *via* phosphorylation of glucose, gives a non-reducing substance of unknown structure which may give phosphoglyceric acid by oxidation, or which may give methylglyoxal by loss of the phosphate group. Phosphoglyceric acid is converted to pyruvic acid which is reduced *via* lactic acid to propionic acid. Methylglyoxal may be converted to lactic acid directly (presumably by the action of glyoxalase) or it may be oxidised to pyruvic acid ; propionic acid is then formed as in the alternative scheme. These workers postulated that part of the pyruvic acid might undergo another series of reactions to give succinic and propionic acids, pyruvic acid giving the hydrate which would split to give acetic acid and carbon dioxide ; two molecules of acetic acid then condense to give succinic acid which in its turn is decarboxylated to form propionic acid and carbon dioxide :—

$$CH_3CO.COOH \longrightarrow CH_3C(OH)(OH)COOH \xrightarrow{-2H} CH_3COOH + CO_2$$

$$2CH_3COOH \xrightarrow{-2H} COOH.CH_2.CH_2.COOH \longrightarrow CH_3CH_2.COOH + CO_2$$

In view of the recent work on the fixation of carbon dioxide it seems more probable that the succinic acid arises by way of condensation of carbon dioxide with pyruvic acid (see p. 258).

Carbon Dioxide Fixation.—Recently work on the fixation of carbon dioxide by bacteria has thrown a new light on the mechanism of acid production by bacteria. It has been known for a long time that autotrophic bacteria utilise carbon dioxide, either by photosynthetic or by chemosynthetic processes, as their sole source of carbon. It has also been known for many years that carbon dioxide plays an important role in the metabolism of some heterotrophic bacteria : thus *Brucella abortus*, when first isolated from cattle, will not grow unless the

atmosphere contains about 10 per cent. of carbon dioxide ; many bacteria, such as *Esch. coli*, fail to grow if steps are taken to remove the carbon dioxide from the medium, by vigorous aeration for example. Until a few years ago, however, it was not realised that carbon dioxide was actually assimilated by heterotrophic bacteria, the reason being that under normal conditions such micro-organisms form carbon dioxide from carbohydrates in larger amounts than they use so that the net production of carbon dioxide masks its assimilation. The first clue was given by the fact that when the propionic acid bacteria ferment glycerol they do not produce carbon dioxide and it was found that the products of fermentation contained more carbon than could be accounted for by that in the medium initially. Since then, by the use of isotopic " heavy " carbon or radioactive carbon in the carbon compounds of the medium, it has been shown that assimilation of carbon dioxide is a general phenomenon in heterotrophic as well as autotrophic bacteria. The difference appears to be that autotrophic bacteria can make use of it in conjunction with inorganic substances as sources of energy whilst the heterotrophic bacteria require compounds already containing at least one carbon atom in organic linkage.

Carbon dioxide has been shown to be fixed by barley roots, liver, yeast, *Esch. coli*, the propionic acid bacteria, *Micrococcus lysodeikticus*, *Aerobacter indologenes*, *Proteus vulgaris*, *Str. paracitrovorus*, *Staph. candidus*, *Cl. welchii*, *Cl. acetobutylicum* and *Cl. aceticum*.

The mechanism proposed by Van Niel to account for the photosynthetic reaction :—

$$CO_2 + 2H_2A \longrightarrow (CH_2O) + H_2O + 2A$$

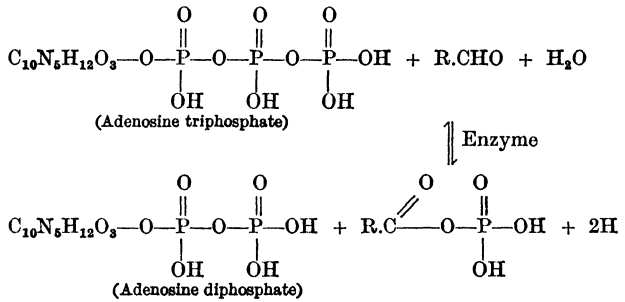
probably also holds for chemosynthetic reactions. Ruben has suggested that the fixation of carbon dioxide in the dark (that is chemosynthetically) takes place with the

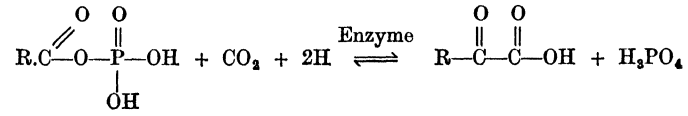
intervention of a phosphate-donor complex with a high energy content, in such a way that an aliphatic compound, probably an aldehyde, is carboxylated and reduced with the ultimate formation of carbohydrates. The general reaction is :—

$$\text{Phosphate-donor} + \text{RH} \rightleftharpoons \text{free donor} + \text{RH-phosphate}$$

$$\text{RH-phosphate} \xrightleftharpoons[\text{carboxylase}]{CO_2} \text{R.COOH} + \text{inorganic phosphate}$$

When RH is an aldehyde and if the phosphate donor is considered to be adenosine triphosphate (which is very probable, see p. 275) the reactions are :—







$$\text{R.CHOH.COOH} + \text{phosphate-donor} \xrightleftharpoons{\text{Enzyme}} \text{R.CHOH.CO.PO}_3\text{H}_2 + \text{donor}$$

$$\text{R.CHOH.CO.PO}_3\text{H}_2 + 2\text{H} \xrightleftharpoons{\text{Enzyme}} \text{R.CHOH.CHO} + \text{H}_3\text{PO}_4$$

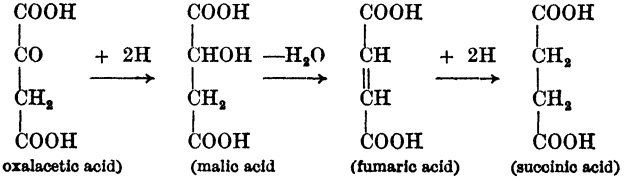
The aldehyde R.CHOH.CHO, which can be regarded as a carbohydrate, could give rise to polysaccharides

through further cycles of phosphorylation, carboxylation and reduction, or by condensation with similar molecules, in a manner analogous to the enzymatic synthesis of starch or glycogen from glucose-1-phosphate.

In heterotrophic systems the acceptor of carbon dioxide is probably a phosphorylated C_3 compound, as has been shown for the propionic acid bacteria. These organisms, which occur in dairy products (particularly in Emmenthaler cheese), silage, soil and similar situations, ferment carbohydrates with production, mainly, of propionic acid, acetic acid and carbon dioxide, with smaller amounts of lactic and succinic acids and, sometimes, acetoin. When grown on a glycerol medium containing "labelled," that is C^{13}, sodium bicarbonate, *Propionibacterium pentosaceum* utilises carbon dioxide which is found almost entirely in the carboxyl groups of the succinic acid formed. The succinic acid formed and the carbon dioxide taken up are very nearly in equimolecular proportion; in the absence of carbonate practically no succinic acid is formed. The small amounts of "labelled" carbon found in the other products of fermentation, propionic and acetic acids and propyl alcohol, are probably derived by side reactions involving succinic acid. Wood and Werkman suggested that the carbon dioxide condensed with pyruvic acid to give oxalacetic acid:—

$$CH_3CO.COOH + CO_2 \longrightarrow COOH.CH_2.CO.COOH$$

Succinic acid then arises *via* malic and fumaric acids:—

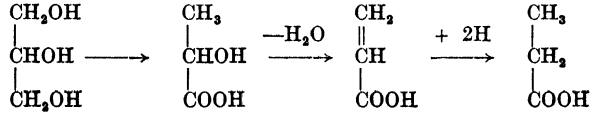


oxalacetic acid) (malic acid (fumaric acid) (succinic acid)

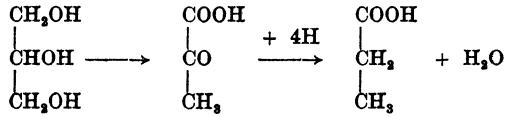
Evidence that this is so is provided by the fact that fumaric and malic acids have been detected in the metabolism solutions, and that oxalacetic, malic and fumaric acids added to the system become converted to succinic acid.

An enzyme preparation has been obtained from *M. lysodeikticus* which, in presence of magnesium or manganese, and possibly phosphate, catalyses the carboxylation of pyruvic acid to oxalacetic acid. The enzyme only decarboxylates pyruvic acid if co-carboxylase is also present. When pyruvic, lactic or oxalosuccinic acids are decarboxylated by the enzyme in presence of " labelled " carbon dioxide there is no evidence of exchange of carbon dioxide. Similarly after glucose or pyruvate have been treated with *Str. lactis* (which can convert pyruvic acid into lactic and acetic acids and carbon dioxide) in presence of " labelled " carbon dioxide, the isolated pyruvic, lactic and acetic acids do not contain " labelled " carbon.

The mechanism of formation of propionic acid from glycerol is not yet clear but it may arise *via* lactic acid :—



or *via* pyruvic acid :—



When carbon dioxide is assimilated by other bacteria it has been found to be distributed as follows :—*Esch. coli* : in formic, acetic, lactic and succinic acids ; *Aerobacter indologenes* : in acetic, lactic and succinic acids ; *Proteus vulgaris*, *Str. paracitrovorus* and *Staph. candidus* :

in lactic and succinic acids ; *Cl. welchii* : in acetic and lactic acids ; *Cl. acetobutylicum* : in lactic acid. Whenever succinic acid is formed it contains fixed carbon dioxide, but the other acids do not always contain assimilated carbon dioxide. The amount and rate of production of succinic acid formed by *Esch. coli* from glucose, galactose or pyruvic acid depends on the quantity of carbon dioxide available. When carbon dioxide is removed by aeration the yield of succinic acid is low, but when the reaction is carried out in presence of carbon dioxide the yield is high. It has been shown, again by the use of " labelled " carbon, that succinic acid can be formed by condensation of two molecules of acetic acid :—

$$\begin{matrix} CH_3COOH \\ + \\ CH_3COOH \end{matrix} \longrightarrow \begin{matrix} CH_2.COOH \\ | \\ CH_2.COOH \end{matrix} + 2H$$

and that the reaction is reversible. Thus acetic acid containing fixed carbon dioxide could arise by the breakdown of succinic acid.

Formic acid can be derived by direct reduction of carbon dioxide in presence of the enzyme hydrogenlyase :

$$CO_2 + H_2O \longrightarrow \underset{\text{(carbonic acid)}}{HO{-}\overset{\overset{OH}{|}}{C}{=}O} \xrightarrow{+H_2} \underset{\text{(formaldehyde hydrate)}}{HO{-}\overset{\overset{OH}{|}}{\underset{\underset{H}{|}}{C}}{-}OH} \longrightarrow \underset{\text{(formic acid)}}{HO{-}\overset{\underset{H}{|}}{C}{=}O} + H_2O$$

Formaldehyde has been isolated from cultures of propionic acid bacteria, by fixation with dimedon, confirming that carbon dioxide is reduced.

The precursor of lactic acid containing fixed carbon has been suggested to be a four-carbon dicarboxylic acid (other than succinic acid) formed by condensation of three-carbon and one-carbon molecules; it is decarboxylated to give lactic acid.

For further reading :—

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CHAPTER XV

ALCOHOLIC FERMENTATION

ALCOHOLIC fermentation is the most widely studied of the fermentations, partly because of its widespread industrial importance and partly because of the ease with which it can be carried out and the conditions modified. About 95 per cent. of the sugar fermented is normally recovered as equi-molecular proportions of alcohol and carbon dioxide. Gay-Lussac long ago expressed this in the form of the classical equation :—

$$C_6H_{12}O_6 \longrightarrow 2CO_2 + 2C_2H_5OH.$$

The fermentation, however, is not so simple as this equation suggests ; in reality the end products arise from a chain of reactions involving a number of intermediate compounds. Besides the alcohol and carbon dioxide there is a constant production of some 3 to 4 per cent. of glycerol, small amounts of fusel oil (derived from the yeast proteins, see Chapter XIII), and varying small proportions of hexose mono- and di-phosphates.

The first real advance in our knowledge of alcoholic fermentation was the almost simultaneous proof in 1837 by Cagniard-Latour, Schwann and Kützing that the fermentation was associated with the living organism, yeast. If sugar solutions were sterilised by boiling and only heated air admitted to them no fermentation occurred.

The next big step forward in the elucidation of the mechanism of alcoholic fermentation occurred when Buchner in 1897 succeeded in demonstrating its enzymatic nature by preparing an active cell-free extract by grinding

yeast with sand and submitting the mixture to considerable pressure. The expressed juice was capable, in virtue of the enzymes which it contained, of causing the fermentation of sugar with the production of the same products as the living yeast. Since then a number of other yeast preparations have been obtained by various workers, of which zymin (yeast dried with acetone) and "maceration juice," obtained by the autolysis of yeast in water, are the most important.

The Role of Phosphates in Fermentation.—In 1905 Harden and Young established the importance of inorganic phosphates and phosphoric esters in fermentation processes. During an investigation of anti-enzymes Harden had added a serum, prepared by injecting yeast juice into rabbits, to a fermenting sugar solution and found that an increase, and not the expected decrease, of fermentation occurred. Normal serum was found to have the same property. The addition of boiled yeast juice to yeast juice and sugar solution had a similar effect. The cause of the increase in the case of boiled yeast juice was traced to two factors : (*a*) the presence of inorganic phosphates, and (*b*) to a heat stable co-enzyme or co-zymase. In the case of serum the effect was shown to be due to the phosphates present.

It was found that the addition of inorganic phosphate to fermentations by yeast preparations caused a considerable increase in both the rate of fermentation and in the absolute amount of sugar fermented. This increase did not occur on the addition of phosphate to fermentations by living yeasts. The rate of fermentation was measured as the volume of carbon dioxide evolved every five minutes, and it was found that the extra volume of carbon dioxide evolved was proportional (molecule for molecule) to the amount of inorganic phosphate converted into esters. The normal rate of fermentation by yeast juice is about 3 ml. of carbon dioxide per five minutes, represented by the straight line (1) in Fig. 7. The rate

of fermentation is constant, and is controlled by the supply of inorganic phosphate derived from the hexose phosphate esters by the action of the enzyme phosphatase. The inorganic phosphate so formed esterifies more sugar, which is fermented with liberation of the phosphate again. There is no accumulation of phosphate esters nor of inorganic phosphate, and the reaction proceeds at a constant rate until all the sugar has been fermented, at which stage inorganic phosphate is finally set free and the sugar of the esters fermented.

If inorganic phosphate is added to such a fermenting mixture the velocity of fermentation increases temporarily,

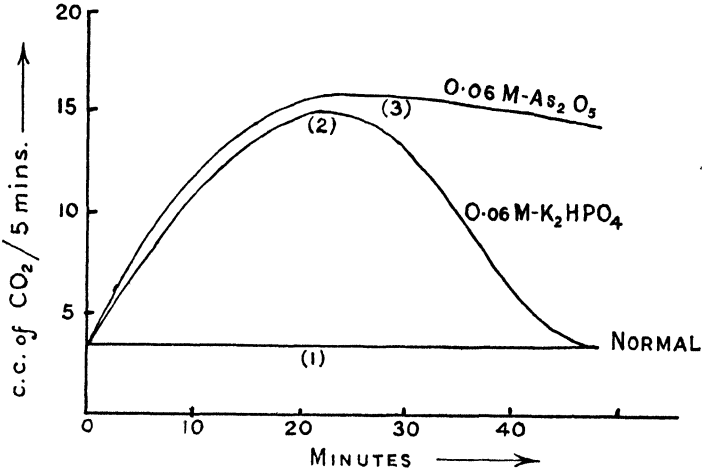


Fig. 7 (*After Harden*)

due to the increased formation of the fermentable ester which accumulates until all the added inorganic phosphate has been converted to ester, after which the rate falls back to the normal value, depending on the rate of hydrolysis of the ester. Curve (2) in Fig. 7 shows this effect.

If the enzyme phosphatase, which hydrolyses the hexose phosphate esters, is added, an increase in the rate of fermentation will occur, due to the increased rate at which the esters are hydrolysed and inorganic phosphate set free to esterify more sugar. The same effect can be brought about by the addition of arsenate, which stimulates phosphatase activity (curve (3), Fig. 7). If the phosphatase is sufficiently active to hydrolyse the esters as fast as they are formed, a rapid fermentation unaccompanied by any accumulation of ester will ensue. The rate of fermentation is about the same as the maximum obtained on addition of inorganic phosphate (curve (2)), but remains constantly high as long as sugar is available, since in effect the addition of phosphatase (or its stimulation) is the same as a continuous addition of inorganic phosphate.

To account for these facts Harden and Young suggested that sugar was fermented in two stages according to the equations :—

$$(1)\ 2C_6H_{12}O_6 + 2Na_2HPO_4 \longrightarrow C_6H_{10}O_4(Na_2PO_4)_2 + 2H_2O + 2CO_2 + 2C_2H_5OH.$$

$$(2)\ C_6H_{10}O_4(Na_2PO_4)_2 + 2H_2O \longrightarrow C_6H_{12}O_6 + 2Na_2HPO_4.$$

According to this scheme the first stage consists in the esterification of one molecule of glucose to hexose diphosphate at the same time as a second molecule is broken down to carbon dioxide and alcohol. The second stage is the hydrolysis of the hexose diphosphate to sugar and inorganic phosphate, which then go through the cycle again, with fermentation of half the sugar at each cycle, until it has all been converted into the end products. The equation (1) represents the state of affairs occurring when inorganic phosphate is added to the system (curve (2)) ; equation (2) corresponds to the normal rate of fermentation (curve (1)). The addition of arsenate causes the hydrolysis of the esters according to equation

(2) to proceed at such a rate that inorganic phosphate is supplied sufficiently rapidly to enable the reactions of equation (1) to proceed at their maximum velocity.

No such acceleration of fermentation occurs on the addition of inorganic phosphate or arsenate to living yeast fermentations, which proceed about twenty to forty times as fast as those due to yeast preparations. The addition of inorganic phosphate to the latter increases the velocity some ten to twenty times. Presumably in living yeast cells the ratio of the activities of the enzymes is at an optimum, and the balance is upset during the extraction of the yeast juice or other treatment. Harden showed that the velocity of fermentation decreased and the magnitude of the response to added phosphate increased, with greater disintegration of the cell structure.

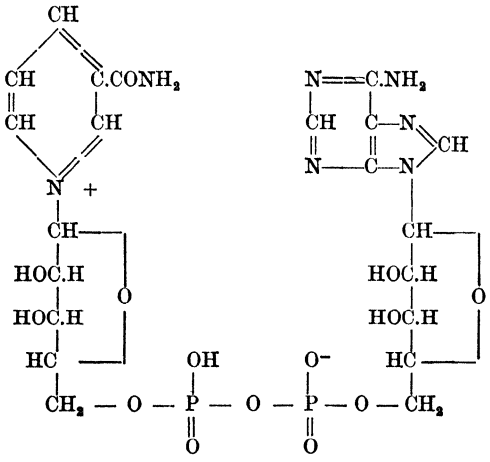
From calculations based on the phosphorus content of the yeast cell it appears that the phosphate-ester cycle must be completed once every five or six minutes in order to maintain the normal rate of fermentation. For an average yeast preparation the cycle takes approximately two hours.

Harden and Young showed that the ester which accumulated after addition of inorganic phosphate was a hexose diphosphate, later shown to be 1 : 6-fructose diphosphate, even when the sugar being fermented is glucose or mannose. Later, Robison isolated a hexose monophosphate from such solutions, which was ultimately proved to be a mixture of about 80 per cent. of 6-glucose monophosphate (known as Robison's ester) and 20 per cent. of 6-fructose monophosphate. More recently Embden and Myerhof and their co-workers have isolated a number of triose monophosphates, including α-glycerophosphoric acid, 2- and 3-phosphoglyceric acids and 2-phosphopyruvic acid, from alcoholic fermentations. Their importance will be seen at a later stage.

The ratio of mono- and di-hexose phosphates found in fermentation mixtures is very variable, and depends on the strain of yeast and on the concentration of the yeast or yeast preparation used. Living yeast is found not to ferment added hexose diphosphate, and also leads to the formation of only a small amount of it during fermentation. Consequently Harden and Young's equations are almost certainly not a true representation of the mechanism. It has been suggested by Kluyver and Struyk and by Meyerhof that the real intermediate is the monophosphate, and that the diphosphate is a by-product formed either by further phosphorylation of the monophosphate or by condensation of two molecules of a triose monophosphate, which also plays a part in the fermentation scheme. Hexose diphosphate, however, plays an essential part in the fermentation reactions.

Co-enzyme.—It was mentioned on p. 263 that the accelerating effect on fermentation of the addition of boiled yeast juice was traced to two factors, inorganic phosphates and the co-enzyme. It was found that if yeast juice was dialysed or submitted to filtration through porcelain candles it could be divided into two fractions, neither of which alone could induce fermentation of sugar, but which when remixed were once more active. The residue after dialysis or filtration was heat labile and destroyed by boiling; as was to be expected it contained the undialysable enzymes of the yeast juice. The dialysate or filtrate, on the other hand, was heat stable, and was termed co-enzyme or co-zymase. Besides the above-mentioned method for its separation, co-enzyme can also be obtained by washing acetone dried yeast, zymin, with water.

Subsequent investigations have shown that the co-enzyme, now known as co-enzyme I, consists of two parts, a magnesium salt and diphosphopyridine nucleotide :—



in which nicotinamide and adenine are joined by two molecules of ribose-5-phosphate. Its function is to act as a hydrogen carrier between phosphoglyceraldehyde and acetaldehyde, being reduced to dihydro-co-enzyme I by the former and re-oxidised by the specific flavo-protein enzyme with acetaldehyde as the hydrogen acceptor (see pp. 203 and 276). Co-enzyme I is very widely distributed and participates in the respiration of many bacteria, particularly those producing lactic acid by fermentation. It is to be found in nearly all animal tissues, in red blood corpuscles, plants, fungi and bacteria. It is a growth factor for some members of the genus *Hœmophilus*.

The closely related co-enzyme II, triphosphopyridine nucleotide containing an extra phosphate group, plays the same role of hydrogen carrier in animal tissues in the conversion of glucose to lactic acid.

In view of the discovery of the necessity of magnesium salts as well as the co-enzyme in yeast fermentation the following terminology for the enzyme systems concerned has been proposed : **Zymase** to indicate the pure enzyme

free from all activators ; **Holozymase** (or panzymase) to designate the complete system of zymase plus the activators ; and **Apozymase**, which is the holozymase free from co-enzyme but still containing the magnesium salts. That is,

$$\text{Holozymase} = \text{Zymase} + \text{Mg} + \text{Co-enzyme}$$
$$\text{Apozymase} = \text{Zymase} + \text{Mg}.$$

Zymase is, itself, a complex mixture of several enzymes (see p. 275 *et seq.*).

Mechanism of Alcoholic Fermentation.—In elaborating a mechanism to account for alcoholic fermentation the following facts have to be considered :—

(1) All the compounds which are produced in a normal alcoholic fermentation can be derived from molecules containing three carbon atoms, which may be obtained by a preliminary split of the glucose molecule into two such fragments following phosphorylation as an essential step.

(2) Acetaldehyde has been demonstrated, by fixation with sulphite or dimedon, as an intermediate.

(3) Pyruvic acid is also regularly formed, as shown by its fixation by the use of β-naphthylamine.

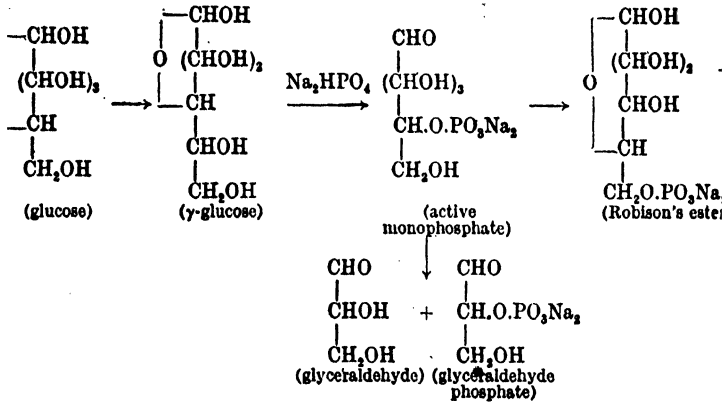
(4) Neuberg isolated from yeast the enzyme carboxylase, which splits pyruvic acid (and α-keto-acids in general) into carbon dioxide and acetaldehyde.

(5) Glycerol is always formed to the extent of 3 to 4 per cent. in normal fermentations, and under certain conditions large quantities of it are formed.

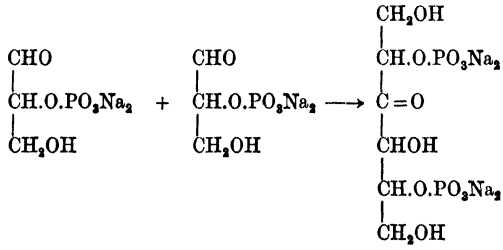
(6) Usually none of the intermediates is left, and the end products are almost entirely carbon dioxide and alcohol in equivalent quantities.

A considerable number of theories have been proposed to account for all these facts, of which the three most important are those due to Kluyver, Neuberg, and Meyerhof ; the last of these is the one now generally accepted.

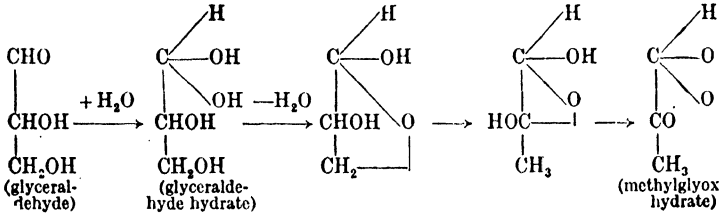
Kluyver considers that the first step in the fermentation of glucose is the formation from γ-glucose of a reactive hexose monophosphate which then splits into two C_3 fragments, one of which is still phosphorylated :—



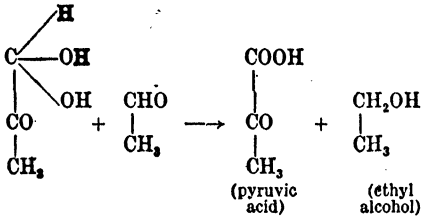
The triose fragments he considers to be glyceraldehyde and glyceraldehyde phosphate. Two molecules of the latter may condense to give hexose diphosphate as a side product :—



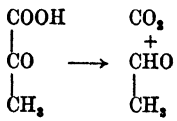
Normally the glyceraldehyde phosphate is hydrolysed to glyceraldehyde and phosphate. The glyceraldehyde undergoes a series of hydrogen transfer reactions to give methylglyoxal hydrate :—



The methylglyoxal hydrate loses hydrogen to acetaldehyde (or some other hydrogen acceptor at the beginning of the fermentation, before acetaldehyde is formed) to form pyruvic acid, the acetaldehyde being at the same time reduced to alcohol.



The pyruvic acid is decarboxylated to give carbon dioxide and acetaldehyde, which acts as hydrogen acceptor for the production of further supplies of pyruvic acid from methylglyoxal hydrate :—

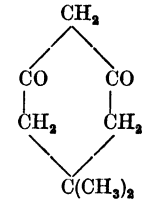


The only enzymes involved in this scheme are phosphatase, hydrogen transportase and carboxylase.

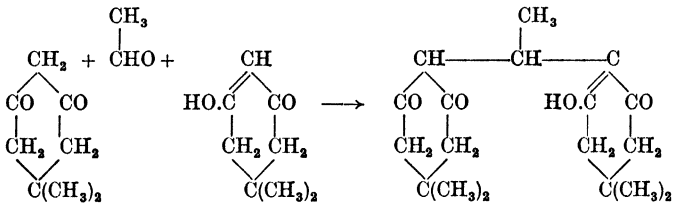
The evidence for this scheme is based, in the main, on analysis of the proportions of the various products formed and misses many of the steps which are now known to take place ; it does not take account of the

action of co-enzyme, for instance. Moreover, methylglyoxal has only been isolated following the action of an extract of dried bottom yeast on magnesium hexose diphosphate, and is not itself fermented.

Neuberg showed that acetaldehyde was an intermediate in alcoholic fermentation as a result of "fixation" experiments using calcium sulphite or dimedon

(dimethyl-cyclohexane-dione),  , which re-

move the acetaldehyde from further participation in the fermentation either as the insoluble acetaldehyde bisulphite complex or according to the reaction :—



in the case of dimedon fixation, with a consequent loss of alcohol production. He also showed that yeast contained the enzyme carboxylase, which could break down α-keto-acids to the aldehyde and carbon dioxide, and, in particular, pyruvic acid, which was also shown to be an intermediate by "fixation" experiments. As a result of these findings he put forward the following scheme for the mechanism of alcoholic fermentation; glucose is converted into two molecules of methylglyoxal by steps involving phosphorylation :—

$$C_6H_{12}O_6 \xrightarrow[\text{phosphorylation}]{-2H_2O} \underset{\text{(enolic form)}}{2CH_2{=}C(OH).CHO} \longrightarrow \underset{\text{(methylglyoxal)}}{2CH_3CO.CHO}.$$

These two molecules of methylglyoxal undergo dismutation (under the influence of the enzyme mutase) to give one molecule of glycerol and one molecule of pyruvic acid :—

$$\begin{array}{l} CH_2{=}C(OH).CHO \\ \quad + \\ CH_3.CO.CHO \end{array} \quad \begin{array}{l} \\ +H_2O + \\ \\ \end{array} \quad \begin{array}{l} H_2 \\ \| \\ O \end{array} \longrightarrow \begin{array}{l} CH_2OH.CHOH.CH_2OH \text{ (glycerol)} \\ \\ CH_3.CO.COOH \text{ (pyruvic acid).} \end{array}$$

The pyruvic acid is then decarboxylated by the enzyme carboxylase to give acetaldehyde and carbon dioxide :—

$$CH_3.CO.COOH \longrightarrow CH_3CHO + CO_2.$$

In the subsequent dismutation of methylglyoxal, one molecule of the latter is replaced by acetaldehyde, which becomes reduced to alcohol, whilst the molecule of methylglyoxal is oxidised to pyruvic acid :—

$$\begin{array}{l} CH_3.CO.CHO \\ \quad + \\ CH_3CHO \end{array} \quad + \quad \begin{array}{l} O \\ \| \\ H_2 \end{array} \longrightarrow \begin{array}{l} CH_3.CO.COOH \\ \quad + \\ CH_3CH_2OH \end{array}$$

This pyruvic acid in its turn is decarboxylated to give more acetaldehyde, and so the cycle goes on until all the sugar is fermented.

This, Neuberg's *First Form of Fermentation*, accounts for the small amount of glycerol, always found in a normal fermentation, as being formed by the reduction of methylglyoxal as hydrogen acceptor before the usual acceptor, acetaldehyde, is formed. If the acetaldehyde is trapped and prevented from acting as hydrogen acceptor no alcohol will be formed, and methylglyoxal will continue to act as hydrogen acceptor and yield a molecule of glycerol for every molecule of carbon dioxide formed and every molecule of aldehyde fixed. This is Neuberg's *Second Form of Fermentation*. Neuberg also established a *Third Form of Fermentation*, which takes place if the

fermenting solution is made alkaline by addition of sodium bicarbonate. These conditions favour a Cannizzaro reaction of the acetaldehyde with formation of acetic acid and alcohol :—

$$\begin{array}{ccccc} CH_3CHO & & O & & CH_3COOH \\ + & + & \| & \longrightarrow & + \\ CH_3CHO & & H_2 & & CH_3CH_2OH \end{array}$$

Since some of the aldehyde is removed in this way it can no longer act as hydrogen acceptor in the dismutation of methylglyoxal, and an equivalent amount of the latter is reduced to glycerol. Since two molecules of acetaldehyde, by the Cannizzaro reaction, give one molecule each of alcohol and acetic acid, and one molecule of glycerol is formed for every molecule of acetaldehyde diverted from acting as hydrogen acceptor, it is obvious that for each molecule of alcohol produced under these conditions there will be two molecules of glycerol formed :—

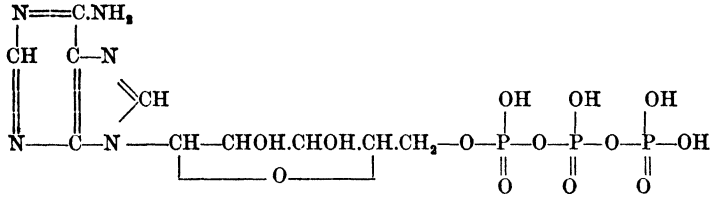
$$2C_6H_{12}O_6 \longrightarrow \mathbf{2CO_2} + 2CH_3CHO + \mathbf{2CH_2OH.CHOH.CH_2OH.}$$
$$2CH_3CHO + H_2O \longrightarrow \mathbf{CH_3COOH} + \mathbf{CH_3CH_2OH.}$$

The chief deficiencies of Neuberg's theories are that no account is given of the phosphorylation processes, nor of the influence of co-enzyme, and that the chief intermediate, methylglyoxal, has not been detected in normal fermentations, and is not fermented when added. It has the advantage that it accounts for the constant small amount of glycerol in normal fermentation and explains the increased yields in special circumstances.

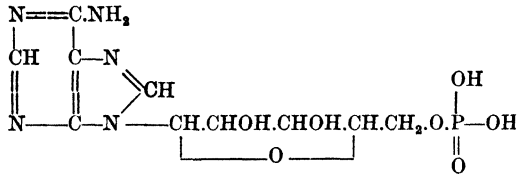
Meyerhof's theory has points of similarity with both Kluyver's and Neuberg's schemes but is based on more complete experimental evidence. Some of the evidence is derived from the work of Embden, Lohmann and others on the course of muscle glycolysis, many of the steps in which have been shown also to occur in yeast fermentation. The main evidence in addition to that mentioned on p. 269 is that hexose diphosphate, dihydroxyacetone phosphate and 3-glyceraldehyde phosphate have

been isolated from fermentations of glucose by yeast extract in presence of mono-iodoacetate which inhibits their further breakdown; equilibrium is established between them when the appropriate enzymes are present; they are rapidly fermented by yeast extracts. The breakdown of phosphoglyceric acid to pyruvic acid and phosphoric acid is inhibited by sodium fluoride so that phosphoglyceric acid accumulates when fermentations are conducted in its presence. Both 2- and 3-phosphoglyceric acids have been isolated from muscle extracts and so also has phosphopyruvic acid. Adenylic acid and adenosine triphosphate have been isolated from yeast juice and shown to react with fructose-1:6-diphosphate.

According to Meyerhof's scheme glucose is phosphorylated by a transfer of phosphate groups from adenosine triphosphate :—

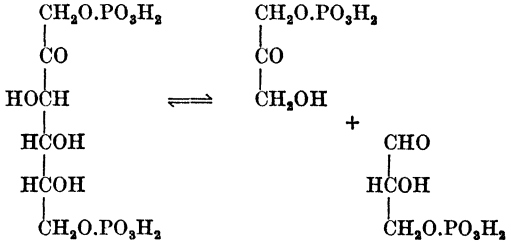


to give first hexosemonophosphate and then hexose diphosphate. At the same time the adenosine triphosphate is converted through the diphosphate to adenosine monophosphate or adenylic acid :—

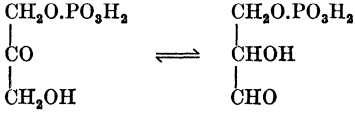


The hexose diphosphate (fructose- 1 : 6 -diphosphate), under the influence of the enzyme zymohexase, breaks

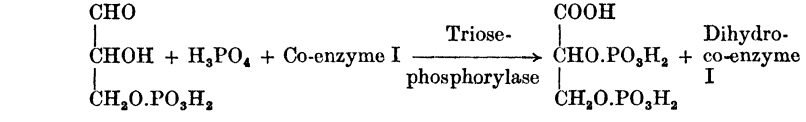
down to give a mixture of dihydroxyacetone monophosphate and 3-phosphoglyceraldehyde :—



The dihydroxyacetone phosphate is converted into 3-glyceraldehyde phosphate by the enzyme isomerase :—



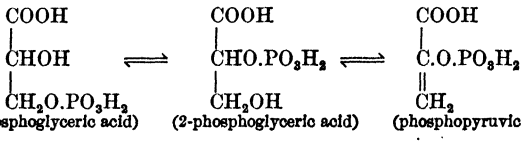
The 3-glyceraldehyde phosphate, reacting with inorganic phosphate and co-enzyme I, is oxidised to 2 : 3-diphosphoglyceric acid whilst the co-enzyme is reduced (see p. 203) :—



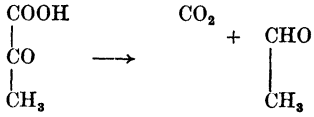
2 : 3-Diphosphoglyceric acid then loses phosphate to adenosine diphosphate :—

2 : 3-diphosphoglyceric acid + adenosine diphosphate ⟶ 3-phosphoglyceric acid + adenosine triphosphate

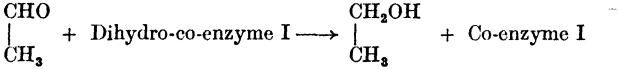
3-Phosphoglyceric acid is converted by the enzyme, phosphoglyceromutase, to 2-phosphoglyceric acid and this by the enzyme, enolase, to phosphopyruvic acid :—



Phosphopyruvic acid is now dephosphorylated by adenosine diphosphate to give pyruvic acid and adenosine triphosphate which hands on its phosphate groups to fresh hexose molecules. Pyruvic acid is decarboxylated in presence of the enzyme, carboxylase, to give acetaldehyde and CO_2 :—



The acetaldehyde is reduced by dihydro-co-enzyme I to give ethyl alcohol :—



The co-enzyme I thus becomes available again to oxidise 3-phosphoglyceraldehyde to more 3-phosphoglyceric acid, and the cycle is maintained.

The reactions outlined above represent the " Stationary condition," in which acetaldehyde acts as hydrogen acceptor from dihydro-co-enzyme I. In the " Initial phase," as Meyerhof calls it, before any acetaldehyde is available 3-phosphoglyceraldehyde is dismuted with formation of α-glycerophosphate and 3-phosphoglyceric acid. The latter is involved in the series of reactions already described with the ultimate production of CO_2 and alcohol whilst the α-glycerophosphate is hydrolysed to form glycerol and phosphate. It is in this way that the 2 to 3 per cent. of glycerol produced in a normal alcoholic fermentation arises. The initial phase can be inhibited by fluoride which prevents the utilisation of the 3-phosphoglyceric acid. The effect of fluoride can be overcome by adding acetaldehyde so that the initial phase is " by-passed." If acetaldehyde is fixed by addition of sulphite or dimedon, or if the stationary condition is

inhibited by mono-iodoacetate which blocks the conversion of glyceraldehyde phosphate to phosphoglyceric acid, the system continues in the initial phase and glycerol, *via* α-glycerophosphate, accumulates at the expense of alcohol.

This scheme differs from Neuberg's in that glyceraldehyde phosphate and not methylglyoxal is the intermediate in the formation of pyruvic acid (compare Kluyver's scheme). The formation of glycerol under alkaline conditions is explained in the same way as in Neuberg's scheme, the acetaldehyde undergoing a Cannizzaro reaction rather than being reduced to alcohol at the expense of dihydro-co-enzyme I.

Biological Reduction.—It will have been gathered from what has gone before that reduction plays an important part in alcoholic fermentation, particularly the reduction of acetaldehyde to alcohol and of glyceraldehyde to glycerol. If other hydrogen acceptors are introduced into the system they will compete for the hydrogen available and themselves become reduced. For instance, Neuberg showed that if an excess of acetaldehyde was introduced into a fermenting mixture it first of all underwent condensation, as a result of the action of the enzyme carboligase, present in yeast, to give acetoin, $CH_3CO.CHOH.CH_3$; the acetoin could act as a hydrogen acceptor, and in doing so became reduced to give 2 : 3-butylene glycol, $CH_3.CHOH.CHOH.CH_3$. If the aeration of the solution was increased, or if other hydrogen acceptors such as methylene blue or sulphur were added, the yield of butylene glycol was lessened and acetoin accumulated, because oxygen or the other substances diverted a considerable portion of the available hydrogen.

A great variety of compounds can be reduced in the presence of actively fermenting yeast. Thus nitrobenzene gives aniline, benzaldehyde is converted to benzyl alcohol, and the ketone, methylheptanone, gives

the alcohol, methylheptanol; the process is not restricted to organic compounds, but inorganic substances, too, can be reduced in this way; sodium thiosulphate, for example, yields hydrogen sulphide and sodium sulphite, and elementary sulphur gives hydrogen sulphide.

The fact that yields of optically active alcohols of considerably more than 50 per cent. can be obtained from optically inactive starting material indicates that true reduction occurs and not a Cannizzaro reaction,

$$2R.CHO + H_2O \longrightarrow R.CH_2OH + R.COOH,$$

which would give a maximum possible yield of 50 per cent. For instance, aldol, $CH_3.CHOH.CH_2.CHO$, gives 63·5 per cent. of optically active 1 : 3-butylene glycol, $CH_3.CHOH.CH_2.CH_2OH$.

The hydrogen used in these reductions is in all probability that produced during the formation of the pyruvic acid from the three-carbon intermediate, since in one case at least, when methylheptanone is reduced to methylheptanol, an amount of acetaldehyde can be isolated corresponding to the amount of methylheptanol produced. Moreover, these reductions occur only during active fermentation and do not take place in the presence of yeast suspensions alone, which indicates that the reductions are coupled with the normal fermentation reactions.

These reductions are frequently called phytochemical reductions, yeast being regarded as a plant.

Fusel Oil.—Besides the glycerol, alcohol and carbon dioxide, which are the main products of the action of yeast on sugar solutions, small quantities of other products are also formed. The chief among these are the higher alcohols constituting the high boiling fraction, fusel oil, which represents 0·1 to 0·7 per cent. of the products. It was originally thought that the higher alcohols originated as by-products of the sugar breakdown, but it was proved by Ehrlich that their real source is the amino-acids derived from the medium or from the autolysis of yeast

cells. He showed that leucine, $(CH_3)_2CH.CH_2.CHNH_2.COOH$, was converted by yeast in sugar solution to *iso*-amyl alcohol, $(CH_3)_2CH.CH_2.CH_2OH$, and that *iso*leucine, $CH_3.\underset{\displaystyle C_2H_5}{\underset{|}{CH}}.CH.NH_2.COOH$, gave rise to *d*-amyl alcohol, $CH_3.\underset{\displaystyle C_2H_5}{\underset{|}{CH}}.CH_2OH$, by loss of carbon dioxide and ammonia.

Other amino-acids were found to undergo the same sort of conversion giving rise to the corresponding alcohol (see p. 231).

The production of these alcohols is only brought about by living cells which are actively growing in a sugar medium. Yeast juice and yeast preparations, like zymin, do not lead to the conversion of the amino-acid to the alcohol, nor does living yeast in the absence of sugar. It seems that the process is bound up with the life of the cell, and is the mechanism by which the organism obtains the nitrogen for its synthetic processes. All the ammonia produced is assimilated by the cell, and practically none accumulates in the medium. Yeast juice or zymin, which do not produce new cells, do not assimilate ammonia and the reaction does not occur; living yeast in the absence of sugar does not grow and reproduce, and again there is no demand for ammonia for synthesis. This dependence of fusel oil production on the nitrogen metabolism is further illustrated by the fact that if a readily available source of ammonia is present in the form of salts, the production of the higher alcohols is very much lowered because the requisite ammonia is supplied more readily from the salts.

It is very probable that the flavours and bouquets of fermented drinks depend largely on the proportions and natures of the various alcohols and esters produced

in this way, these proportions depending in their turn on the amino-acids present in the liquors being fermented.

For further reading :—

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CHAPTER XVI

THE FERMENTATION PRODUCTS OF THE LOWER FUNGI

SINCE Wehmer's classical work on the production of oxalic acid by fungi in 1891 a vast bulk of data on mould fermentations has accumulated, as a result of which it emerges that there are three main differences between bacterial and mould fermentations. The first of these is that the moulds appear to have considerably greater and more diverse synthetic powers than the bacteria. They are able to produce a large variety of compounds, aromatic as well as aliphatic, besides the normal cell constituents, proteins and nucleic acids. The range of these synthetic powers is indicated by the following examples of some of the types of product encountered: Benzopyrone, quinones, phenolic acids, heterocyclic compounds, pyrones, long chain fatty acids, fats, polysaccharides and sterols. Secondly, and perhaps most important from a practical point of view, the fungi produce non-volatile, often polybasic, acids, of which oxalic, citric and gluconic acids are the most important. They usually produce about 0·1 per cent., and never yield more than 1 per cent., of volatile acids. Lactic acid has only been reported as a mould product from *Monilia*, *Mucor rouxii*, *Rhizopus oryzæ* and *Rhizopus chinensis*. If volatile acids are formed during the metabolism of the fungi they are immediately further broken down; indeed, it has been shown that the volatile acids are even more readily attacked by fungi than are sugars. Bacteria, on the other hand, produce large quantities of such volatile acids as acetic, propionic, butyric and lactic acids. Thirdly, the moulds never seem to give rise to hydrogen or methane among their

products, whilst hydrogen is a very common product of bacteria and methane is formed comparatively frequently.

The function that these very varied compounds perform is still in very large measure unknown. The carbohydrates and fats probably serve as storage or reserve materials as also may such acids as gluconic and citric acids. Some products, such as the yellow pigment, citrinin, may play the part of oxidation-reduction systems. Other substances may exert a protective effect by depressing or inhibiting the growth of other organisms. For example, many moulds develop such acid conditions (*p*H 1 to 2) that bacterial growth is stopped. An interesting case is the production by *Penicillium notatum* and *P. chrysogenum* of the antibiotic penicillin which has marked specific bactericidal powers ; in very high dilution it entirely suppresses the growth of the pyogenic cocci and organisms of the diphtheria group, but it is easily tolerated in high concentration by other organisms such as those of the coli-typhoid group,the influenza bacillus and the enterococci. Many other antibiotic substances are now known (see Chapter XI).

In considering the metabolic products of the lower fungi it must be remembered that they are essentially aerobic organisms. If an adequate supply of air is available they usually completely oxidise, to carbon dioxide and water, not only the carbohydrate originally present in the medium but also the metabolic products which they themselves have formed. Consequently the incubation periods allowed for formation of the products must not be too long or low yields result ; the fermentations are usually stopped just before all the initial nutrient substance has been used up.

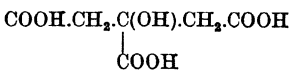
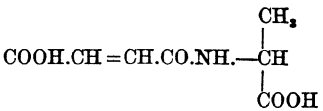
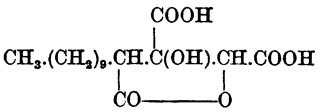
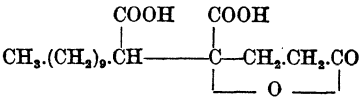
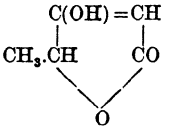
Acid Production by Fungi

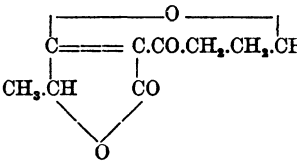
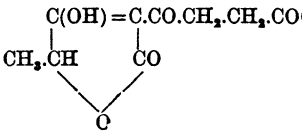
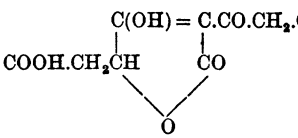
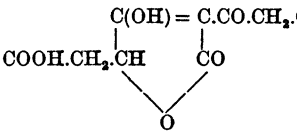
Acids of a wide variety of structures are synthesised by the lower fungi from glucose as the sole source of carbon. A list of them is provided in Table 19.

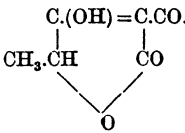
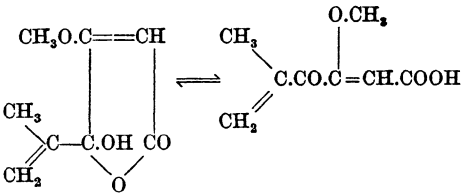
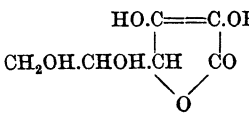
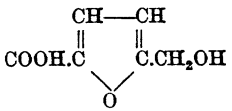
TABLE 19

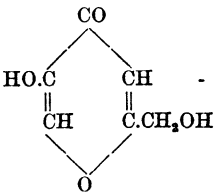
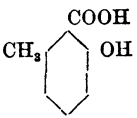
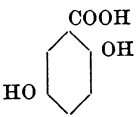
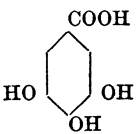
ACID	STRUCTURE	PRODUCED BY
Simple Aliphatic Acids—		
Glycollic acid	$CH_2OH.COOH$	*A. niger*
Glyoxylic acid	$CHO.COOH$	*A. niger*
Oxalic acid	$COOH.COOH$	*A. niger*
Pyruvic acid	$CH_3.CO.COOH$	*A. niger*
Lactic acid	$CH_3.CHOH.COOH$	*Mucor rouxii, Rhizopus chinensis, Rhizopus oryzæ*
Malonic acid	$COOH.CH_2.COOH$	*P. funiculosum*
Fumaric acid	$COOH.CH=CH.COOH$	*Aspergillus, Mucor, Rhizopus nigricans*
Succinic acid	$COOH.CH_2.CH_2.COOH$	*Fusarium, Mucor, Rhizopus*
Malic acid	$COOH.CHOH.CH_2.COOH$	*A. fumaricus, A. niger*
Ethylene oxide-$\alpha\beta$-dicarboxylic acid	$COOH.\overset{O}{\overset{\diagup\diagdown}{CH.CH}}.COOH$	*Monilia formosa, P. viniferum*
Acetone dicarboxylic acid	$COOH.CH_2.CO.CH_2.COOH$	*A. niger*

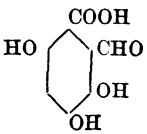
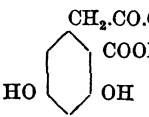
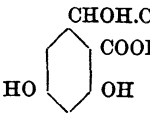
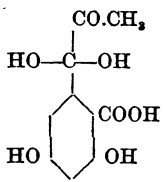
Acid	Structure	Produced By
Gluconic acid	$CH_2OH(CHOH)_4COOH$	*A. niger*, *P. chrysogenum*, *P. luteum* var. *rubrisclerotium*
Mannonic acid	$CH_2OH(CHOH)_4COOH$	*P. purpurogenum* var. *rubrisclerotium*
Glucuronic acid	$CHO.(CHOH)_4.COOH$	*Ustulina vulgaris*
Saccharic acid	$COOH.(CHOH)_4.COOH$	*A. niger*
γ-Ketopentadecoic acid	$CH_3(CH_2)_9.CH_2.CO.CH_2.CH_2.COOH$	*P. spiculisporum*
Branched Chain Acids—		
Dimethylpyruvic acid	$(CH_3)_2CH.CO.COOH$	*A. niger*
Itaconic acid	$COOH.CH_2.C(=CH_2).COOH$	*A. itaconicus*, *A. terreus*
Aconitic acid	$COOH.CH_2.C(COOH)=CH.COOH$	*A. itaconicus*

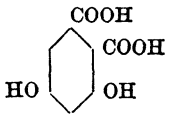
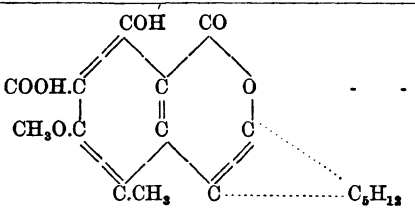
Acid	Structure	Produced By
Citric acid		*A. niger*, *A. itaconicus*, *Penicillium* species, *Monilia formosa*
Fumaryl-*dl*-alanine		*P. resticulosum*
Miniolúteic acid ($\alpha\beta$-dihydroxy-$\beta\gamma$-dicarboxy-*n*-tetradecoic-γ-lactone)		*P. minioluteum*
Spiculisporic acid (γ-hydroxy-$\gamma\delta$-dicarboxypentadecoic lactone)		*P. crateriforme*, *P. minioluteum*, *P. spiculisporum*
Tetronic Acids— γ-Methyl tetronic acid		*P. charlesii*

Acid	Structure	Produced By
Carolic acid (anhydro form) (α-[γ-hydroxybutyryl]-γ-methyl tetronic lactone)		*P. charlesii*
Carolinic acid (α-succinyl-γ-methyl tetronic acid)		*P. charlesii*
Carlic acid (hydrated form) (α-[γ-hydroxybutyry]-γ-carboxymethyl tetronic acid)		*P. charlesii*
Carlosic acid (α-butyryl-γ-carboxymethyl-tetronic acid)		*P. charlesii*

Acid	Structure	Produced By
Terrestric acid (ethyl carolic acid)		*P. terrestre*
Penicillic acid (β-methoxy-γ-hydroxy-γ-*iso*propylidene tetronic acid)		*P. cyclopium*, *P. puberulum*
Ascorbic acid (or a substance reducing 2 : 6-dichlorophenolindophenol)		*Aspergillus*, *Penicillium*
2-Hydroxymethyl-5-carboxyfurane		*A. clavatus*, *A. glaucus*, *A. niger*, *A. oryzæ*, *A. wentii*

Acid	Structure	Produced By
Kojic acid (α-hydroxymethyl-δ-hydroxy-γ-pyrone)		*A. flavus*, *A. oryzæ*, *A. parasiticus*
Phenolic acids—		
6-Methyl-salicylic acid		*P. griseo-fulvum* *P. flexuosum*
Gentisic acid (5-hydroxy-salicylic acid)		*P. brevi-compactum* *P. patulum*
Gallic acid (3:4:5-trihydroxy-benzoic acid)		*A. niger*

Acid	Structure	Produced By
Puberulic acid		*P. puberulum* *P. aurantio-virens*
(3:5-Dihydroxy-2-carboxy-benzyl) methyl ketone		*P. brevi-compactum*
(3:5-Dihydroxy-2-carboxy-phenyl) acetyl carbinol		*P. brevi-compactum*
(3:5-Dihydroxy-2-carboxy-benzoyl) methylketone (hydrated)		*P. brevi-compactum*

Acid	Structure	Produced By
3:5-Dihydroxyphthalic acid -		*P. brevi-compactum*
Mycophenolic acid - - -		*P. brevi-compactum* *P. stoloniferum*
Unknown constitution—		
Byssochlamic acid - -	$C_{18}H_{20}O_6$	*Byssochlamis fulva*
Fulvic acid - - - -	$C_{14}H_{12}O_8$	*P. griseo-fulvum, P. flexuosum, P. brefeldianum*
Glaucic acid - - - -	$C_{17}H_{22}O_5$	*A. glaucus*
Glauconic acid I - - -	$C_{18}H_{20}O_5$	green *Penicillium* species
Glauconic acid II - -	$C_{18}H_{20}O_6$	green *Penicillium* species
Stipitatic acid (possibly a dihydroxyformyl benzoic acid)	$C_8H_6O_5$	*P. stipitatum*

Oxalic Acid.—Oxalic acid was recognised quite early in the form of crystals of calcium oxalate in many moulds. Wehmer in 1891 showed that it was a fermentation product and could be produced by *Aspergillus niger* from a variety of substrates, including glucose. He demonstrated that large yields of calcium oxalate can be obtained by maintaining the medium more or less neutral by the addition of calcium carbonate, which precipitates the oxalic acid as fast as it is formed. Other species of *Aspergillus* and some species of *Penicillium* also give oxalic acid in good yield.

Citric Acid.—Citric acid was first described as a mould product by Wehmer in 1893, who claimed that it was produced only by two species of *Penicillium*-like organisms for which he created the genus *Citromyces*. However, he himself showed later that citric acid formation is not so restricted, but is, in fact, a common characteristic of many species of *Penicillium* and *Aspergillus*, particularly of the black species of the latter. By suitable adjustment of the conditions of fermentation, namely, by growth in a medium at *p*H 1 to 2 instead of the more usual *p*H 6 to 7, it is possible to suppress almost entirely the production of oxalic acid by *A. niger* and to obtain citric acid as almost the sole product. The strong acidity inhibits the growth of bacteria, yeasts and most other fungi, which makes the process very useful and easy to control industrially. It is used in America for the large-scale production of citric acid.

Gluconic Acid.—Gluconic acid seems to be an almost constant product of fermentation by *A. niger*. It was first reported as such by Molliard in 1922. He showed in subsequent work that it was possible to produce oxalic, citric or gluconic acids at will as the main product by varying the proportion of the mineral constituents of the medium. Reduction of the amount of phosphate and nitrogen to a minimum gave greatly increased yields of gluconic acid, as much as 80 to 90 per cent. being

obtained. Lowering the nitrogen supply raises the yield of citric acid, whilst high nitrogen and phosphate with low potassium gives rise to good yields of oxalic acid. The process is worked industrially in the United States, using *P. luteum* (variety *rubrisclerotium*).

In view of the high yields obtained from glucose it is considered that the formation of gluconic acid is a direct oxidation of glucose by the enzyme glucose-oxidase and is quite independent of the reactions by which citric acid is formed. This is further borne out by the facts that there is no correlation between the times of maximum formation of the two acids and that there is no formation of citric acid from gluconic acid. Some, but not all, other sugars are oxidised in a similar way by *Penicillium luteum* to give the corresponding acids ; thus mannose is oxidised to mannonic acid and xylose to xylonic acid, but galactose and arabinose are not affected, although they may be oxidised by other fungi.

Lactic Acid.—*Rhizopus oryzæ*, when grown with urea as a source of nitrogen in surface culture or as an aerated, submerged growth in a rotating drum, may convert up to 75 per cent. of the glucose utilised into *d*-lactic acid. Other species of *Rhizopus* produce large quantities of *l*-lactic acid from glucose, together with small quantities of formic acid, acetic acid, fumaric acid, *l*-malic acid, succinic acid and ethyl alcohol.

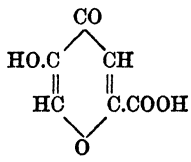
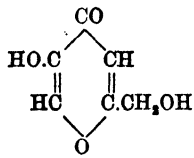
Fumaric and Malic Acids.—These acids are not very common mould products but are formed in comparatively low yields by the action of one or two species of *Rhizopus* and *Aspergillus* from glucose, fructose, galactose or arabinose. It is possible that they are normally intermediates in the formation of other acids.

Succinic Acid.—Succinic acid is a rare product of mould metabolism, although it is fairly commonly produced by yeasts and bacteria. It is probably formed by all three types of organism by the breakdown of the amino-acid, glutamic acid, $COOH.CH_2.CH_2.CHNH_2.COOH$,

derived from degradation of the cell proteins, by the mechanism of oxidative deamination followed by decarboxylation and oxidation (see p. 230) :—

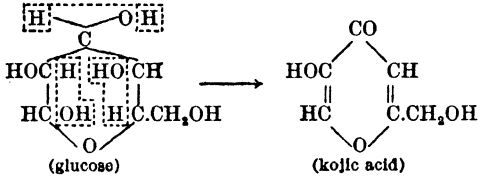
$$\underset{\text{(glutamic acid)}}{\underset{\displaystyle NH_2}{\overset{|}{COOH.CH_2.CH_2.CH.COOH}}} \longrightarrow \underset{\text{(α-ketoglutaric acid)}}{COOH.CH_2.CH_2.CO.COOH} \longrightarrow \underset{\text{(succinic acid)}}{\underset{\displaystyle COOH.CH_2.CH_2.COOH}{\overset{\downarrow}{COOH.CH_2.CH_2.CHO}}}$$

It is known that *A. niger* and *Rhizopus nigricans* fix carbon dioxide and it is possible that succinic acid may be formed by this process (see p. 258). The largest yields of succinic acid are given by species of *Fusarium*, *Mucor* and *Rhizopus*.

Kojic Acid.—Kojic acid was first isolated from the mycelium of *Aspergillus oryzæ*, an organism used in Japan to ferment steamed rice, koji, to produce the alcoholic beverage, saké. It has been since shown to be produced by a number of other related *Aspergilli* and by one species of *Penicillium*, Some acetic acid bacteria, for example *Bact. xylinoides*, produce small yields of kojic acid from mannitol and fructose. The closely related comenic acid,  , is also produced by the gluconic acid bacteria. The constitution of kojic acid was established by Barger and Yabuta as the γ-pyrone derivative, 

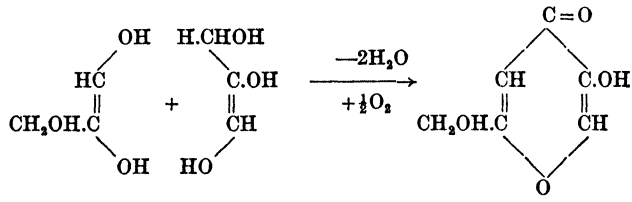
It is produced in 10 to 20 per cent. yields by the fermentation of glucose, fructose, sucrose, galactose, lactose, xylose, arabinose, glycerol, mannitol and starch. The yield of the six-carbon compound, kojic acid, is just as

high from pentoses and glycerol as it is from hexoses, which suggests that it is not formed by a series of oxido-reductions from glucose, for instance, as its structure would at first sight indicate :—

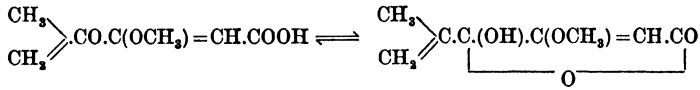


It appears more likely that the sugars first of all undergo breakdown to a common intermediate which is then built up into kojic acid. The intermediate appears not to be acetaldehyde since the formation of kojic acid is not hindered by fixation of acetaldehyde by dimedon or sulphite. *Aspergillus flavus* and *A. parasiticus*, which both produce kojic acid from glucose in culture, when plasmolysed by chloroform or toluene, convert starch, maltose, sucrose or glucose into glucosone, $CH_2OH.(CHOH)_3CO.CHO$. *A. flavus* converts glucosone into kojic acid in normal culture, suggesting that glucosone may be an intermediate between glucose and kojic acid. A less likely alternative is that all sugars are first converted into a single reserve polysaccharide and that this is the source of the sugar which is the immediate precursor of the kojic acid ; thus five hexose units might unite to give a C_{30} polysaccharide, and six pentose units or ten triose units condense to give the same polysaccharide. The difficulty is that the number of oxygen bridges would be different in each case. A further objection to the suggestion that reserve carbohydrates are involved is that kojic acid is not formed when dry mycelium is used as carbon source for the mould.

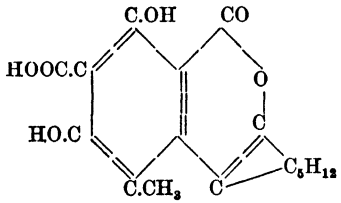
Challenger has suggested that kojic acid may arise by the condensation of two molecules of dihydroxyacetone and oxidation :—



Penicillic and Mycophenolic Acids.—In 1890 Gosio isolated moulds from diseased maize which gave rise to products, giving a blue colour with ferric chloride, which were thought to be toxic substances causing pellagra, now known to be a deficiency disease due to lack of nicotinamide. Later *P. puberulum* giving an acid, penicillic acid, $C_8H_{10}O_4$, and *P. stoloniferum* giving mycophenolic acid, $C_{17}H_{20}O_6$, were isolated from mouldy maize. Penicillic acid has been shown to have the constitution γ-keto-β-methoxy-δ-methylene-Δα-hexenoic acid which exists in both the keto and lactone forms, the latter being β-methoxy-γ-hydroxy-γ-*iso*propylidene tetronic acid :—



The constitution of mycophenolic acid has not yet been fully worked out, but it contains the following nucleus which represents the demethylated acid :—

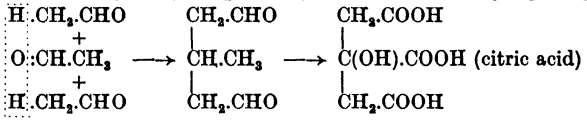


Tetronic Acids.—Carolic, carolinic, carlic and carlosic acids, produced by *P. charlesii* when grown on glucose as the sole source of carbon, are condensation products

of butyrolactone and succinic acid with γ-methyltetronic acid and of butyrolactone and butyric acid with γ-carboxy-methyltetronic acid respectively, as shown by the formulæ given in Table 19, (p. 287).

It will be seen that they have structures bearing a close resemblance to that of ascorbic acid, vitamin C, which is also said to be produced by some *Aspergillus* and *Penicillium* species, although the evidence rests on the demonstration of a substance reducing 2 : 6 dichlorophenolindophenol and not on isolation of ascorbic acid. Terrestric and penicillic acids are also shown to be derivatives of tetronic acid.

Theories of Acid Production by Fungi.—There is still a considerable amount of controversy as to the mechanism of the production of acids by fungi, particularly as regards the commercially important oxalic and citric acids. Ehrlich, in 1911, suggested that citric acid was formed as the result of the condensation of three molecules of acetaldehyde followed by oxidation of a methyl group and the aldehyde groups to give the carboxyl groups :—

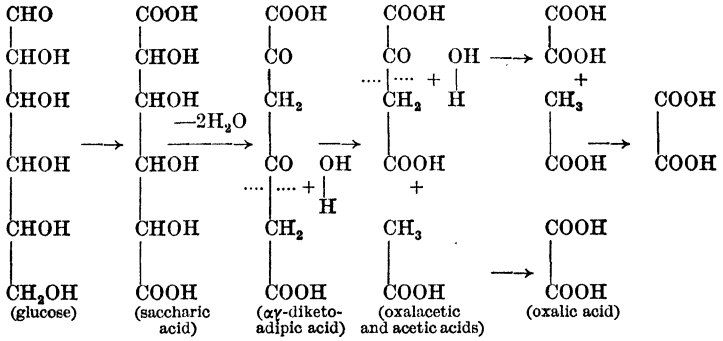


This cannot be the correct mechanism, however, since the maximum yield of citric acid from a hexose would be 71 per cent., whereas in practice yields as high as 95 per cent. can be obtained readily.

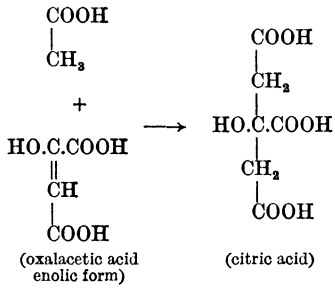
The theory of Chrzaszcz and Tiukow, according to whom acetic acid, derived by steps like those in alcoholic fermentation, condenses *via* succinic, fumaric and malic acids to give citric acid ; that of Bernhauer, according to which acetic acid condenses with succinic acid to give aconitic acid which in turn yields citric acid and that of Emde which suggests that citric acid arises by oxidation of quinic acid are all subject to the same objection that they do not allow for yields of citric acid as high as those

obtained in practice. This limitation obviously applies to any scheme which involves decarboxylation. That the same initial steps as occur in alcoholic fermentation leading to the formation of acetaldehyde (see p. 275) are not involved in citric acid production is shown by the fact that formation of the acid is not inhibited by the presence of mono-iodoacetic acid, but actually accelerated. Phosphorylation however, is involved since inorganic phosphate and glucose disappear from the medium, with formation of organic phosphate esters, as *A. niger* produces gluconic and citric acids. Inorganic phosphate is liberated again as gluconic and citric acids are further broken down to oxalic acid in older cultures.

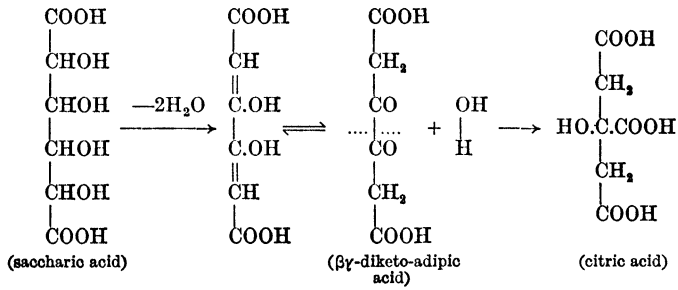
Raistrick and Clark in 1919 investigated the production of oxalic acid from a large number of acids and showed that it was not formed from any three-carbon acid, such as propionic, pyruvic or lactic acids, nor from any mono-basic four-carbon acid, like butyric acid, but that the dibasic four-carbon acids, malic or succinic acids, and also acetic acid, gave rise to good yields of oxalic acid. They considered that the mechanism of its formation from glucose was *via* $\alpha\gamma$-diketo-adipic acid which split to oxalacetic acid and acetic acid; the oxalacetic acid split down further to give oxalic and acetic acids, whilst acetic acid was oxidised to oxalic acid, according to the scheme :—



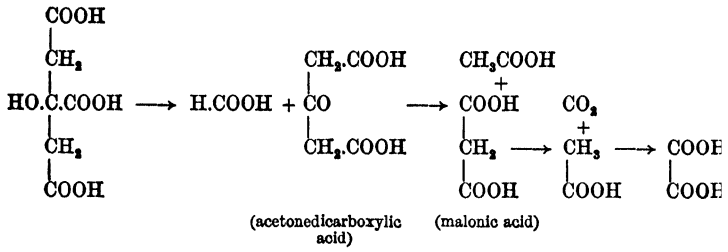
This mechanism accounts for the high yields of oxalic acid which can be obtained and also for the fact that it is formed from the four-carbon dibasic acids, which can all give rise to oxalacetic acid, and from acetic acid, but not from the three-carbon acids. They consider that citric acid is formed by the same mechanism as far as the breakdown into oxalacetic and acetic acids, which then recondense to give citric acid :—



Challenger has suggested that citric acid may arise *via* saccharic acid and βγ-diketo-adipic acid, which undergoes a benzilic acid transformation to give citric acid (a process which occurs in presence of alkali *in vitro*) :—



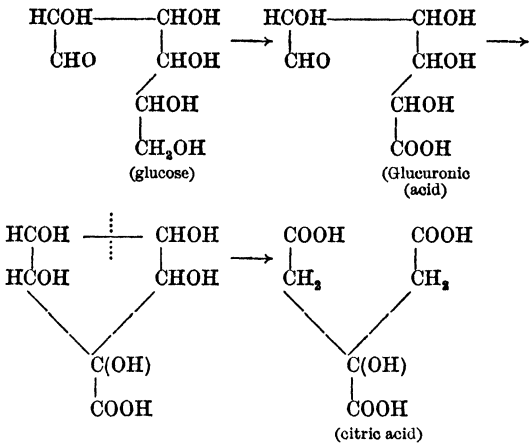
He regards citric acid as being the source of oxalic acid *via* acetic acid, which is formed by decarboxylation of malonic acid derived from acetonedicarboxylic acid :—



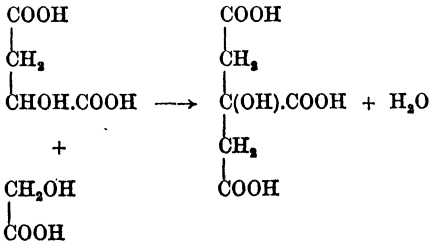
The evidence for these reactions is somewhat meagre ; small amounts of saccharic, acetonedicarboxylic and malonic acids were isolated, but $\beta\gamma$-diketo-adipic acid could not be detected as an intermediate nor could added $\beta\gamma$-diketo-adipic acid be fermented to citric acid.

Citric acid can be formed from glycerol and pentoses as well as from glucose, fructose and sucrose. This suggests that the carbon source is broken down to a common intermediate, which is then built up into a reserve carbohydrate characteristic of the particular fungus, and that this in its turn breaks down to give a hexose which is the immediate precursor of the citric acid. The same explanation may be offered of the formation of gluconic acid from the pentose, arabinose, of kojic acid from pentoses, of mannitol from glycerol and pentoses, and of polysaccharides composed of hexose units from glycerol and pentoses.

Assuming the production of a common hexose precursor the theories of Butkewitsch, of Gudlet and of Ciusa and Brüll become possible. Butkewitsch suggested that glucose was oxidised to glucuronic acid which underwent intramolecular aldol condensation to give a five-membered ring compound which was subsequently split and the terminal C atoms oxidised with formation of citric acid :—



Gudlet suggested that glucose split to give succinic acid and acetaldehyde. The former was transformed through fumaric acid to malic acid, whilst the acetaldehyde was oxidised to acetic acid which condensed with the malic acid to give citric acid. Ciusa and Brüll found that increased yields of citric acid were obtained if glycollic and malic acids were added to *A. niger* fermentations, and suggested that citric acid was formed by their condensation :—



This suggestion resembles that of Kluyver and of Virtanen for the formation of succinic acid (see p. 250) by bacteria.

It is possible that citric acid may be formed by a series of reactions analogous to part of the tricarboxylic

acid cycle involved in muscle metabolism. Oxalacetic acid can be formed by the condensation of carbon dioxide and pyruvic acid (see p. 258). Oxalacetic acid condenses with a further molecule of pyruvic acid to give *cis*-aconitic acid which gives rise to citric acid :—

$$CO_2 + CH_3CO.COOH \longrightarrow \underset{\text{(oxalacetic acid)}}{COOH.CH_2.CO.COOH} \rightleftharpoons COOH.CH=C(OH)COOH$$

$$COOH.CH=C(OH)COOH + CH_3.CO.COOH \xrightarrow{-2H} COOH.C(CH_2.COOH)=CH.COOH + CO_2$$

$$COOH.C(CH_2.COOH)=CH.COOH \xrightarrow{+H_2O} COOH.C(CH_2.COOH)(OH)—CH_2.COOH$$

(*cis*-aconitic acid) (citric acid)

Neutral Products of Fungi

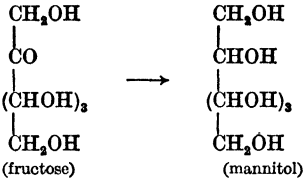
Ethyl Alcohol.—Many species of *Aspergillus* and *Penicillium* give more or less yields of alcohol, the most effective being *A. oryzæ* which is used in Japan for the production of alcoholic beverages. Many species of *Mucor* produce alcohol, whilst most *Fusarium* species give large yields, equivalent to those given by yeasts. Phosphorylation reactions are known to be involved and it is highly probable that the mechanism is very similar to that of ordinary alcoholic fermentation by yeasts.

Acetaldehyde.—Acetaldehyde has been detected by fixation methods in the case of moulds which produce alcohol. It is very probable that it is an intermediate in other fermentations as well.

Glycerol.—Glycerol has been found among the products of *Mucor*, *Aspergillus* and *Penicillium* species and seems to be a normal fermentation product. It probably arises by a mechanism similar to that by which it is formed in yeast fermentations.

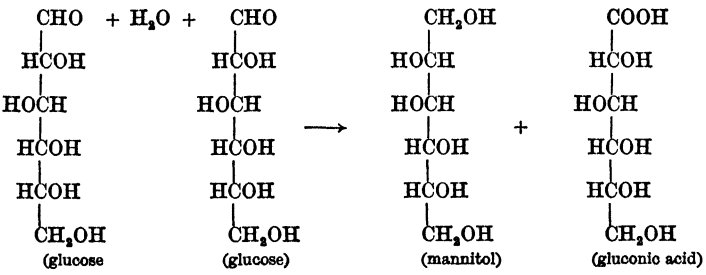
Ethyl Acetate.—*P. digitatum*, which causes the olive-green rot of citrus fruits, produces ethyl acetate, which appears to be characteristic of the species.

Mannitol.—Mannitol is produced in yields as high as 50 per cent. of the sugar fermented by several species of *Aspergillus*, notably *A. nidulans*, *A. elegans* and some white species, and also by *Byssochlamys fulva*, *Clasterosporum*, *H. geniculatum* and *P. chrysogenum*, from all sugars except fructose. On the other hand, when mannitol is formed as a bacterial product it is derived only from fructose and not from any other sugar. The reaction is, apparently, a direct reduction :—



When mixtures of fructose and glucose are fermented by bacteria the former disappears rapidly with formation of mannitol and acetic acid, whilst the glucose is fermented more slowly to lactic acid.

In the case of the moulds it has been suggested that two molecules of glucose, for instance, undergo a Cannizzaro reaction with formation of gluconic acid and mannitol :—



If this were so it would be expected that the reduction product would be sorbitol, $CH_2OH.HCOH.HOCH.HCOH.HCOH.CH_2OH$, corresponding in configuration to glucose, and not mannitol, which corresponds to mannose. Sorbitol, however, has never been detected in these fermentations. It is possible that the conversion does not involve glucose as such but the phosphorylated sugar, shown to be fructose diphosphate in the case of yeast fermentations. It has been shown that yields of 15 to 35 per cent. of mannitol, calculated on the sugar fermented, are formed by the action of a white species of *Aspergillus* on mannose, galactose, arabinose and xylose, but not from fructose. No acid is formed.

According to the Cannizzaro reaction theory of mannitol and gluconic acid production, the nature of the product which accumulates depends on the optimum *p*H conditions for the organism. Thus the white *Aspergilli* thrive in a neutral medium of about *p*H 6 to 7 and utilise the gluconic acid, leaving mannitol in solution. *A. niger* on the other hand, favours an acid medium and utilises the neutral product mannitol and allows gluconic acid to accumulate.

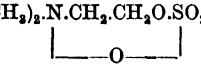
The moulds afford a better commercial source of mannitol than bacteria, since they ferment the cheap sugar glucose and not the expensive fructose, and do not require organic nitrogen, in the form of peptone or yeast extract, as do the bacteria. In addition the moulds produce mannitol in almost pure solution with very few by-products. Mannitol is used in the form of its hexanitrate as a detonator.

Products containing Sulphur, Arsenic or Selenium.—The cases of arsenical poisoning which used to occur as a result of the action of fungi on wallpapers printed with arsenic containing substances have long been considered as due to the formation of volatile substances of the type trimethyl arsine, $(CH_3)_3As$, which has been shown

to be a product of *P. brevicaule* when grown on bread containing inorganic arsenic compounds. Dimethyl selenium and dimethyl tellurium were similarly formed from sodium selenide and potassium tellurite respectively by *P. brevicaule, P. chrysogenum* and *P. notatum*. Dimethyl ethyl arsine, $(CH_3)_2.C_2H_5.As$, arises from sodium ethyl arsonate and methyl diethyl arsine, $CH_3.(C_2H_5)_2.As$, from diethyl arsenic acid; *n*-propyl arsonic acid gives rise to dimethyl *n*-propyl arsine, $(CH_3)_2.C_3H_7.As$, in the same way. Similar derivatives of antimony could not be obtained by the growth of *P. brevicaule* in potassium antimony tartrate nor does the mould methylate sulphur. Challenger has suggested that the methylation is through the agency of choline or betaine which contains a mobile methyl group capable of being transferred under biological conditions, as shown by experiments using choline or betaine containing methyl groups " marked " with heavy hydrogen.

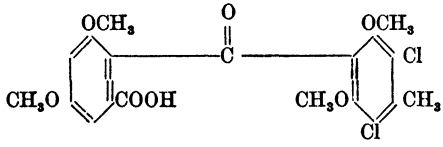
The wood rotting fungus, *Schizophyllum commune*, when grown on a synthetic medium containing inorganic sulphates produces methyl mercaptan, $CH_3.SH$.

A. sydowi, which can also produce volatile arsenic compounds, is capable of incorporating the inorganic sulphur of the medium into cyclic choline sulphate,



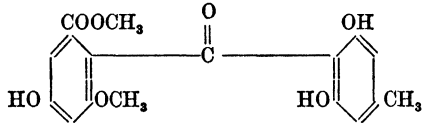
, which is an anhydride of choline sulphate. It has been shown that *Verticillium albo-atrum* and *Botrytis cinerea*, when grown on a medium containing asparagine and ammonium salts as sources of nitrogen, produce thiourea. The production of biotin (see p. 103) and of penicillin (see p. 171) obviously also involves the metabolism of inorganic sulphate with formation of organic sulphur compounds, as does, of course, the synthesis of proteins which all contain sulphur in some of their amino acids and as SH groups, and which are formed by all micro-organisms.

Chlorine containing Products.—Almost all the inorganic chlorine of Czapek-Dox medium is removed during the growth of *A. terreus* and is converted into geodin, $C_{17}H_{12}O_7Cl_2$, and erdin, $C_{16}H_{10}O_7Cl_2$. Although the constitution of the products themselves is not yet known, dihydroerdin (obtained by catalytic reduction of erdin) is methylated by diazomethane to give 3′: 5′-dichloro-4 : 6 : 2′: 6′-tetramethoxy-4′-methylbenzophenone-2-carboxylic acid :—



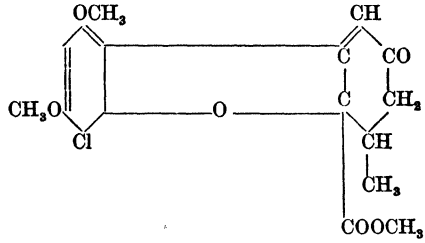
Dihydrogeodin is the methyl ester of dihydroerdin. Replacement of the potassium chloride in the medium by bromide or iodide did not give rise to the corresponding compounds although the organism grew normally.

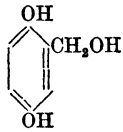
The yellow crystalline compound sulochrin,

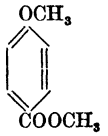


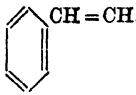
which occurs in the mycelium of *Oospora sulphurea-ochracea*, has a benzophenone structure very like that of dihydroerdin and dihydrogeodin although it lacks the chlorine atoms. Sulochrin is also related to the pigment ravenelin (see p. 396) since treatment of demethylated sulochrin with concentrated sulphuric acid gives rise to a xanthone derivative having a methyl group, a hydroxyl group and the carbonyl group in the same positions as those in ravenelin.

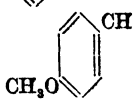
Another chlorine containing metabolic product is the strongly dextrorotatory, colourless compound griseofulvin obtained from the mycelium of *P. griseo-fulvum*, and thought to have the structure :—



Other Products.—A number of other products including polysaccharides, pigments, sterols and fats, which will be described in Chapters XIX, XX, and XXI, together with the following examples serve to emphasise the very wide range of the synthetic abilities of the lower fungi. Some of the substances which illustrate the diversity of the products of the moulds are gentisyl alcohol, 

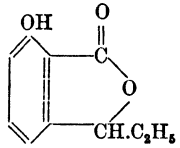
, which is produced along with the corresponding gentisic acid by *P. patulum*; methyl anisate, 

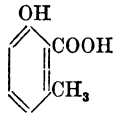
, methyl cinnamate, 

, and methyl *p*-methoxy-cinnamate, 

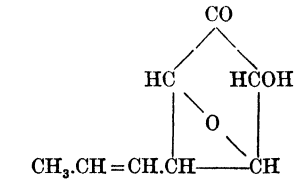
, produced by the wood rotting fungus *Lentinus lepideus*; mellein, which

has been shown to be identical with ochracin, produced by *A. melleus* and *A. ochraceus* is a lactone of 6-hydroxy-2-(α-hydroxypropyl) benzoic acid,



which, on fusion with potassium hydroxide, yields 6-methyl salicylic acid, 

, which is a product of *P. griseo-fulvum*; terrein, which is obtained from *A. terreus* and has the structure, 4-propenyl-2-hydroxy-3 : 5-oxido*cyclo*pentane-1-one



palitantin, $C_{14}H_{22}O_4$, is a colourless, crystalline, unsaturated dihydroxyaldehyde formed by *P. palitans*.

It has been noted that in many cases the same products may be produced from several sugars, including those with fewer carbon atoms, besides glucose as carbon source. This suggests, as has been pointed out above, that they are synthesised from either a common simple intermediate, possibly acetaldehyde, or else from a common reserve carbohydrate which is formed irrespective of the sugar with which the organism is supplied; this may be represented schematically in the case of a pentose as :—

6 Pentose ⟶ Reserve Carbohydrate ⟶ 5 Hexose ⟶ Other Products.

In view of the very wide variety of products formed it seems almost certain that a simple building stone like acetaldehyde must be involved.

For further reading :—

F. Challenger, "Biological Methylation. VIII. A Summary of recent work on Biological Methylation and some Hypotheses regarding its Mechanism." *Chemistry and Industry*, **61** (1942), 397, 413, 456.

H. Raistrick, *et alia*, "Studies in the Biochemistry of Micro-organisms." *Phil. Trans. Roy. Soc. B.*, **220** (1931), 1. See also numerous papers in the *Biochemical Journal*.

H. Raistrick, (*a*) "Biochemistry of the Lower Fungi." *Ergebnisse der Enzymforschung*, **1** (1932), 345.

(*b*) "The Biochemistry of the Lower Fungi." *Ann. Review of Biochemistry*, **9** (1940), 571.

CHAPTER XVII

INDUSTRIAL FERMENTATIONS

IN recent years there has been a very considerable expansion of the use of micro-organisms in industrial processes, largely due to a realisation of the variety of catalytic properties possessed by their enzyme systems. Many uses of bacteria and yeasts in industry, it is true, date back to time immemorial; it is only necessary to mention the production of alcoholic beverages of all sorts, baking, tanning and the retting of flax and hemp in order that this may be realised.

The Production of Glycerol by Fermentation.—As we have seen, glycerol is a normal product in alcoholic fermentation, where it occurs to the extent of about 3 to 4 per cent. During the 1914-1918 war period Connstein and Lüdecke, in Germany, added sulphite and bisulphite to sugar solutions fermented by *Saccharomyces cerevisiæ* and obtained much increased yields of glycerol, which ran parallel with the amount of acetaldehyde fixed by the sulphite (which is probably converted to bisulphite by the action of carbon dioxide). As the sulphite solutions are strongly alkaline, and since the alkalinity increases during fermentation by production of sodium bicarbonate, the medium soon becomes unsuitable for continued fermentation, and recovery yields of about 25 per cent. of glycerol are obtained. This difficulty was overcome to a certain extent in Great Britain by the use of a mixture of approximately equimolecular proportions of sodium sulphite and sodium bisulphite which buffers the solution at about *p*H 7. The yield is about 30 to 35 per cent.

In America glycerol was produced by maintaining the medium alkaline with sodium bicarbonate, conditions under which acetaldehyde tends to undergo dismutation with formation of acetic acid and alcohol. Glycerol is formed in amounts corresponding to the quantity of acetaldehyde diverted from its normal function of acting as hydrogen acceptor in the formation of pyruvic acid (see p. 277).

The British method gives the best yields, but the recovery of glycerol is difficult owing to the interference of the sulphite. Moreover, there is little or no market for the acetaldehyde produced as a by-product. The American method, although giving lower yields, is the best commercially, since alcohol is also formed and the proportion of glycerol and alcohol produced can be easily varied according to demand by adjustment of the degree of alkalinity.

Molasses or hydrolysed wood pulp or similar vegetable wastes serve as an effective sugar source for the growth of the yeast in glycerol fermentation.

Power Alcohol Production.—The production of alcohol for fuel purposes and for use in the chemical and other industries has grown very largely within the past ten to fifteen years. The fermentation is generally carried out by the use of an appropriate strain of yeast to ferment molasses, which provides an excellent source of carbohydrate, nitrogen and mineral salts in immediately available condition. An inoculum is built up from a pure culture of the yeast, maintained in the laboratory, by successive transfers to increasing quantities of molasses until a " seed " is obtained, about 5 per cent. of the final volume to be fermented. The molasses is maintained at *p*H 5, which allows good growth of the yeast but inhibits the growth of most bacteria which might act as contaminants. After fermentation for about forty-eight hours the alcohol, present to the extent of 5 to 10 per cent., is distilled off from the mash.

A more recent development is the use of vegetable wastes as a source of the necessary sugars for fermentation. Practically all vegetable waste materials, such as straw, grass, maize cobs and husks, and sawdust contain insoluble hemicelluloses, built up largely of pentosans. The pentosans are broken down by a preliminary acid treatment to give the soluble pentose sugars. The mash so produced is fermented by yeast.

Ethyl Alcohol-Acetone Fermentation.—Acetone and ethyl alcohol are produced from maize, potatoes, molasses and various vegetable waste materials by the action of *B. aceto-ethylicus*, which is probably identical with the starch fermenting organism, *B. macerans*. The fermentation proceeds best at a temperature between 40° and 43°C. and in presence of calcium carbonate, to prevent development of excessive acidity, the mash after preliminary acid treatment being brought to a *p*H value about 8. The chief products are acetone and ethyl alcohol corresponding to about 8 to 10 per cent. and 20 to 25 per cent., respectively, of the carbohydrate fermented; acetic, lactic and formic acids are also formed together with hydrogen and carbon dioxide. The solvents are separated by fractional distillation.

Speakman suggested the following scheme as the mechanism of fermentation. Glucose is broken down, probably in a way similar to that in alcoholic fermentation, to give pyruvic acid :—

$$C_6H_{12}O_6 \longrightarrow 2CH_3.CO.COOH + 4H$$

The pyruvic acid is then transformed in three ways, (1) to give lactic acid :—

$$CH_3CO.COOH + 2H \longrightarrow CH_3CHOH.COOH$$

(2) to give carbon dioxide and acetaldehyde, the latter then giving alcohol :—

$$CH_3CO.COOH \longrightarrow CO_2 + CH_3CHO \xrightarrow{+\ 2H} CH_3CH_2OH$$

(3) to give acetic and formic acids :—

$$CH_3CO.COOH + HO.H \longrightarrow CH_3COOH + HCOOH.$$

The balance between the three modes of fermentation will depend on the conditions prevailing. At a later stage in the fermentation acetone and ethyl alcohol are formed together, probably as a result of the condensation of acetaldehyde to give aldol,

$$2\,CH_3.CHO \longrightarrow CH_3CHOH.CH_2.CHO,$$

which then undergoes dismutation with a further molecule of acetaldehyde to give β-hydroxybutyric acid and ethyl alcohol :—

$$\begin{array}{rcl} CH_3.CHOH.CH_2.CHO & & CH_3.CHOH.CH_2.COOH \\ + & \longrightarrow & + \\ CH_3.CHO & & CH_3.CH_2.OH \end{array}$$

The β-hydroxybutyric acid undergoes an oxidation-reduction with acetaldehyde to form acetoacetic acid and ethyl alcohol :—

$$\begin{array}{rcl} CH_3.CHOH.CH_2.COOH & & CH_3.CO.CH_2.COOH \\ + & \longrightarrow & \\ CH_3.CHO & & + \quad CH_3.CH_2.OH \end{array}$$

Finally acetoacetic acid is decarboxylated with formation of acetone :—

$$CH_3.CO.CH_2.COOH \longrightarrow CH_3.CO.CH_3 + CO_2$$

Bakonyi has suggested that aldol undergoes an internal oxidation and reduction and splitting to give acetic acid and ethyl alcohol :—

$$\begin{array}{c} CH_3CHOH.CH_2.CHO \\ + H_2O \end{array} \longrightarrow CH_3.CH_2.OH + CH_3.COOH$$

Two molecules of acetic acid condense to give acetoacetic acid which is then decarboxylated to give acetone and carbon dioxide :—

$$\begin{array}{c} CH_3.COOH + CH_3.COOH \longrightarrow CH_3.CO.CH_2.COOH + H_2O \\ CH_3.CO.CH_2.COOH \longrightarrow CH_3.CO.CH_3 + CO_2 \end{array}$$

Although acetoacetic acid may be derived by condensation of acetic acid it seems unnecessary to postulate that the acetic acid is formed *via* aldol when it is known that acetaldehyde can give rise to it and ethyl alcohol by dismutation in presence of the enzyme aldehydemutase.

Butyl Alcohol-Acetone Fermentation.—The production of acetone by fermentation processes was initiated during the 1914-1918 war period, when it was required in large quantities for the manufacture of explosives. The fermentation has undergone even greater development since that time as a result of the greatly increased use of butyl alcohol and acetone as solvents for lacquers and in the form of esters as plasticisers in the cellulose paints and varnishes and in cellophane preparation. When the process was developed in this country and the United States in 1915 the butyl alcohol was a by-product for which no market could be found; nowadays it is the more valuable of the two products.

The organisms responsible for the production of these solvents from starch and sugars are spore-bearing soil organisms of the type *Cl. aceto-butylicum*, which are similar to the flax-retting organisms. Two groups of these organisms are recognised. One group produces the organic acids, butyric and acetic acids, but cannot ferment them further to the neutral products butyl and ethyl alcohols and acetone; to this group belong the true butyric acid bacteria such as *Cl. butyricum*. The second group comprises the butyl alcohol bacteria which, under favourable conditions of the medium, reduce the intermediately formed acetic and butyric acids to ethyl alcohol, acetone and butyl alcohol. Some of these organisms, for example, *Cl. butyricum*, require the medium to be maintained neutral by the addition of calcium carbonate in order that they may bring about fermentation. For others, like *Cl. aceto-butylicum*, the addition of calcium carbonate is unnecessary or even undesirable.

One of the chief contaminants producing unsatisfactory fermentation is a lactic acid organism which can bring the butyl alcohol fermentation to a complete standstill in a few hours. A bacteriophage has also been incriminated as the cause of faulty fermentations. The early method of sterilisation of the mash in large tanks prior to fermentation had to be abandoned in favour of the use of a number of small tanks in order to ensure thorough penetration of the heat. The grain, usually maize, to be fermented is freed from the embryo (from which an edible oil is expressed, leaving a cake used as cattle food), sterilised as a mash in water by steam under pressure at 130° to 140° C., and transferred through sterile pipe lines to a large fermenting tank holding as much as 50,000 gallons. A seed mash, built up from pure laboratory cultures, or withdrawn from a previous fermentation, is introduced and fermentation allowed to proceed at 55° C. The production of a maximum yield of products in the minimum of time depends largely on the preparation of the seed mash. This must bear the correct ratio to the total mash to be fermented (usually about 12 per cent.), and the number of subcultures used to build the seed up to the requisite amount is kept as low as possible in order to maintain the activity of the organism. The seed is withdrawn after the initial acidity of the fermentation has begun to decline, since the main contaminant to be expected is the lactic acid organism, *B. volutans*, whose presence is indicated by the maintenance of a high acidity; hence a sample taken from a mash whose acidity is declining is more likely to be pure.

Anaerobic conditions develop automatically; the fermentation starts in five to six hours and is complete in forty-eight hours. In addition to butyl alcohol and acetone, carbon dioxide, hydrogen and ethyl alcohol are also formed, together with small amounts of *iso*-propyl alcohol. The chief products occur in the proportions :—

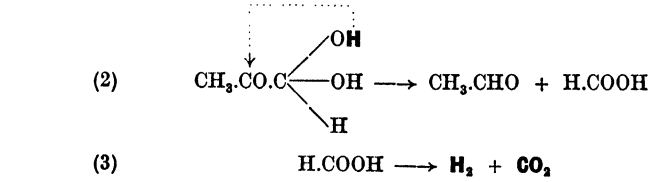
Carbon dioxide	60	per cent.
n-Butyl alcohol	25	,,
Acetone	10·5	,,
Ethyl alcohol	3	,,
Hydrogen	1·5	,,

The gaseous products are scrubbed to remove ethyl alcohol and acetone, and the carbon dioxide is removed by water under pressure, followed by dilute sodium hydroxide. The residual hydrogen is converted into ammonia by catalytic combination with nitrogen under pressure. A recently developed alternative is to remove only three-quarters of the carbon dioxide and to convert the remaining mixture of carbon dioxide and hydrogen into methyl alcohol by catalysis. The excess of carbon dioxide is solidified and used for refrigeration. The solvents are separated by fractional distillation.

In the early stages of the fermentation acidity due to the production of acetic and butyric acids develops, but later falls again as the neutral products appear. If acetic or butyric acids are added to a fermenting mash, increased yields of acetone and butyl alcohol, respectively, are obtained. It is suggested that the butyl alcohol is formed by reduction of butyric acid and that the acetone arises from acetic acid *via* aceto-acetic acid which is decarboxylated. The scheme is summarised by the following equations given by Kluyver :—

(1) $C_6H_{12}O_6 \longrightarrow 2CH_3.CO.C\begin{cases} OH \\ OH \\ H \end{cases}$ (methylglyoxal hydrate).

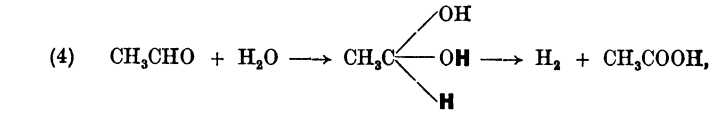
Glucose breaks down to give methylglyoxal hydrate, probably by the same mechanism as in alcoholic fermentation. The methylglyoxal hydrate then splits to give acetaldehyde and formic acid, which in turn yields hydrogen and carbon dioxide :—



(2) $CH_3.CO.C(OH)(OH)(H) \longrightarrow CH_3.CHO + H.COOH$

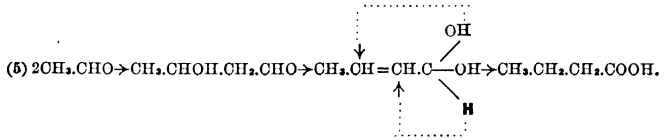
(3) $H.COOH \longrightarrow \mathbf{H_2 + CO_2}$

Part of the acetaldehyde is oxidised to acetic acid :—



(4) $CH_3CHO + H_2O \longrightarrow CH_3C(OH)(OH)(H) \longrightarrow H_2 + CH_3COOH,$

and part condenses to give aldol, which by internal rearrangement gives butyric acid :—

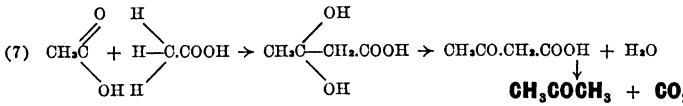


(5) $2CH_3.CHO \rightarrow CH_3.CHOH.CH_2.CHO \rightarrow CH_3.CH{=}CH.C(OH)(H) \rightarrow CH_3.CH_2.CH_2.COOH.$

The butyric acid is reduced by hydrogen formed in the production of acetaldehyde and acetic acid, to give butyl alcohol :—

(6) $CH_3.CH_2.CH_2.COOH + 4H \longrightarrow \mathbf{CH_3.CH_2.CH_2.CH_2OH} + H_2O.$

Two molecules of acetic acid (formed according to equation 4) condense to give aceto-acetic acid, which is decarboxylated to give carbon dioxide and acetone :—



(7) $CH_3C(=O)(OH) + H{-}C(H)(OH).COOH \rightarrow CH_3C(OH)(OH){-}CH_2.COOH \rightarrow CH_3CO.CH_2.COOH + H_2O$

$\mathbf{CH_3COCH_3 + CO_2}$

The ethyl alcohol is formed as a side reaction by reduction of acetaldehyde :—

(8) $CH_3CHO + 2H \longrightarrow \mathbf{CH_3CH_2OH,}$

and similarly the small amount of *iso*-propyl alcohol arises by reduction of acetone :—

(9) $CH_3.CO.CH_3 + 2H \longrightarrow CH_3.CHOH.CH_3$.

Kluyver's scheme outlined above should be modified so that the preliminary stages, represented by equations (1) to (3), agree with modern knowledge of the mechanism of alcoholic fermentation. In other words the various phosphorylation reactions leading to the formation of pyruvic acid (see p. 275) are very probably those really involved, rather than the formation and breakdown of methylglyoxal. In the present state of our knowledge there seems to be no reason to change the suggestions concerning the later stages.

Lactic Acid Fermentation.—Lactic acid is one of the commonest of bacterial products and is produced by a wide variety of bacteria and yeasts. The earlier work was largely done in connection with the dairy industry and more particularly cheese manufacture. The first fermentation method for the production of lactic acid was described in 1841 by Boutron and Frémy before, however, it was realised that the action was bacterial. When it was established by Pasteur that this was the case, improvements were made, although it was not until 1896 that pure cultures of lactic acid bacteria were used.

The lactic acid bacteria were classified by Orla-Jensen in 1919 into two main groups :—

1. *The Homofermentative or True Lactic Acid Bacteria* which produce almost pure lactic acid from sugars. They are Gram positive, non-sporing, non-motile rods and cocci which give no surface growth on liquid media. They will not grow in the absence of organic nitrogen compounds ; they produce no catalase and do not reduce nitrates. The true lactic acid bacteria are further subdivided into :—

(*a*) Rods (i) thermophilic, e.g. *Lactobacillus bulgaricus* *L. delbrückii*,
(ii) mesophilic, e.g. *L. casei*.

(*b*) Cocci, e.g. *Streptococcus lactis*.

(*c*) An intermediate group, e.g. *Str. cremoris* which gives some volatile acids (acetic and propionic) in addition to lactic acid; *Leuconostoc citrovorum* (*Str. citrovorus*) and *Leuc. dextranicum* (*Str. paracitrovorus*) which give volatile acids and acetoin from lactic and citric acids.

2. *The Heterofermentative or Pseudo-lactic Acid Bacteria* which produce volatile acids, carbon dioxide and hydrogen, as well as lactic acid, which rarely exceeds half the sugar fermented. They exhibit surface growth on liquid media, reduce nitrates and produce catalase. The group includes organisms of the coli-aerogenes type and a number of pathogenic organisms.

At the present time thermophilic organisms like *L. delbrückii* (*B. acidificans longissimus*) or *L. bulgaricus* are used to ferment the maltose and sucrose in molasses, or glucose derived from the starch of potatoes, maize or other cereals (which are first submitted to acid hydrolysis or to the action of malt diastase), or the lactose in skim milk or whey. The optimum temperature for fermentation is about 45° C., at which temperature the growth of most contaminating bacteria, especially the butyric acid bacteria, is inhibited. Yields of lactic acid of about 98 per cent., calculated on the sugar fermented, are obtained. The sterilised mash, containing about 10 to 15 per cent. of fermentable sugar, is inoculated under aseptic conditions with a pure culture of the appropriate organism, and fermentation allowed to proceed at the optimum temperature, which is maintained by steam pipes distributed in the tanks. Calcium carbonate or lime is introduced at intervals in order to maintain the solution

neutral or faintly acid ; the fermentation is complete in four to six days. The calcium lactate is recovered from the mash by filtration, evaporation of the filtrate and crystallisation. The calcium is removed as calcium sulphate by addition of sulphuric acid and the lactic acid solution concentrated to about 50 per cent.

Lactic acid is largely used for " deliming " hides, that is, for removing the lime employed in the " dehairing " process preparatory to tanning, and for softening hides. It is used extensively in the textile industry as a mordant in dyeing and for the acid dyeing of wool. It is much used in the form of its ethyl ester as a plasticiser in resins and lacquers and as a solvent for cellulose finishes. Lactic acid is finding increasing use also as a flavouring agent and acidulant in the preparation of " soft " drinks and food products.

The initial stages of lactic acid fermentation are very probably the same as those of alcoholic fermentation, involving phosphorylation and co-enzyme I. If co-enzyme I is removed from lactic acid bacteria by washing they no longer produce lactic acid. Lactic acid fermentation is accelerated by the addition of phosphates in a manner analogous to that of alcoholic fermentation. Two mechanisms are possible for lactic acid fermentation, the first A, going through pyruvic acid and the second, B, through methylglyoxal.

A. (1) Hexosediphosphate $\xrightarrow{\text{zymohexase}}$ Dihydroxyacetone phosphate + 3-phospho-glyceraldehyde.

(2) Dihydroxyacetone phosphate $\xrightarrow{\text{isomerase}}$ 3-phosphoglyceraldehyde

(3) 3-Phosphoglyceraldehyde + phosphate + co-enzyme I $\xrightarrow{\text{triosephosphorylase}}$ 1 : 3-diphosphoglyceric acid + dihydrocoenzyme I.

(4) 1 : 3-Diphosphoglyceric acid + adenosine diphosphate ⟶ 3-phosphoglyceric acid + adenosine triphosphate.

(5) 3-Phosphoglyceric acid $\xrightarrow[\text{and phosphoglyceromutase}]{\text{enolase}}$ phosphopyruvic acid.

(6) Phosphopyruvic acid + adenosine diphosphate $\longrightarrow$ pyruvic acid + adenosine triphosphate.

(7) Dihydroco-enzyme I + $CH_3.CO.COOH$ (pyruvic acid) $\xrightarrow[\text{enzyme}]{\text{lactic}}$ Co-enzyme I + $CH_3.CHOH.COOH$ (lactic acid)

Alternatively :—

B. (1) Hexose diphosphate $\longrightarrow$ 2-glycerophosphate.

(2) Glycerophosphate + 2 cytochrome $\xrightarrow{\text{dehydrogenase}}$ glyceraldehyde-phosphate + 2 reduced cytochrome.

(3) 2 Reduced cytochrome + O_2 $\xrightarrow[\text{oxidase}]{\text{Cytochrome}}$ 2 cytochrome + $2H_2O$.

(4) Glyceraldehyde phosphate $\longrightarrow$ methylglyoxal + phosphate.

(5) $CH_3CO.CHO$ (methylglyoxal) + H_2O $\xrightarrow{\text{glyoxalase}}$ $CH_3.CHOH.COOH$ (lactic acid).

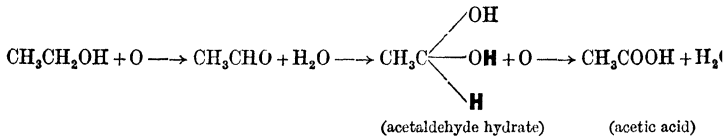
Glyoxalase was first isolated from dog's liver by Dakin and Dudley, who showed that it converted methylglyoxa quantitatively to lactic acid. Glyoxalase has also been shown to be present in yeast and it has been shown that *L. delbrückii* and *Aerobacter aerogenes* convert methylglyoxal quantitatively to racemic lactic acid, whilst *L. delbrückii* converts hexose-diphosphate into methylglyoxal and the latter to lactic acid.

Some organisms, for example *L. pentoaceticus*, produce optically inactive lactic acid, whilst others, for example *Str. lactis*, yield the dextro-rotatory acid, and yet others, for example *Leuconostoc mesenteroides*, the lævo-rotatory isomer. It has been shown that those organisms which yield inactive lactic acid contain an enzyme, racemiase, which racemises the *d*- or *l*- forms of the acid.

The industrial production of lactic acid by fungi is possible using species of *Rhizopus* or *Mucor* in surface culture or in aerated submerged culture.

Acetic Acid Fermentation. Vinegar Fermentation.—The production of vinegar from plant sugars *via* alcohol is one of the oldest fermentation industries. It involves two stages—the conversion of the sugar to alcohol by the action of yeasts, and secondly, the oxidation of the alcohol to acetic acid by various bacteria of the genus *Acetobacter*. The latter process is strictly aerobic and is accomplished in practice by trickling the alcohol solution over wood shavings impregnated with the bacteria. If too little alcohol is present the acetic acid formed is further oxidised to carbon dioxide and water and lost. The aeration has to be adequate or the oxidation of alcohol stops at the acetaldehyde stage.

In the ordinary vinegar process the acetic acid is probably formed by direct oxidation of the alcohol *via* acetaldehyde :—



The organisms *A. ascendans*, *A. pasteurianum* and *A. xylinum* have been shown capable of converting acetaldehyde anaerobically to acetic acid by dismutation. The reaction is catalysed by the enzyme aldehyde mutase with the intervention of co-enzyme I as hydrogen carrier, the aldehyde acting as both donator and acceptor of hydrogen :—

$$CH_3CHO + \text{Co-enzyme I} \longrightarrow CH_3COOH + \text{reduced Co-enzyme I}$$
$$CH_3CHO + \text{reduced Co-enzyme I} \longrightarrow CH_3CH_2OH + \text{Co-enzyme I}$$

It is therefore possible that some of the acetic acid in vinegar fermentation is formed by dismutation of the acetaldehyde :—

$$2CH_3CHO \longrightarrow CH_3CH_2OH + CH_3COOH.$$

The alcohol so formed is then oxidised to acetaldehyde,

which again undergoes dismutation ; the process continues until all the alcohol has been converted to acetic acid. Under normal aerobic conditions, however, this reaction is much slower than the direct oxidation and contributes but a small proportion of the yield.

The activity of the acetic acid bacteria in bringing about the oxidation of glucose to gluconic acid and keto-gluconic acid, of glycerol to dihydroxyacetone, and of secondary alcohol groups to keto groups has already been described (see p. 241 *et seq.*).

Methane and Hydrogen (Power Gas) Fermentation.—In comparatively recent years the use of methane and hydrogen produced by the fermentation of cellulose wastes has developed considerably. The study of cellulose fermentation has been largely the work of Omelianski. There are three main types of cellulose degradation :—

1. *Anaerobic at* 20° *to* 37° *C.* with the production of methane and hydrogen. This fermentation is the result of the action of two organisms—one *Cl. fossicularum*, giving the hydrogen, and the other, *Cl. methanigenes*, forming methane. *Cl. fossicularum* is a long slender bacillus with terminal spores, which fails to grow on ordinary media. Its chief products are acetic, lactic and butyric acids, ethyl alcohol, carbon dioxide and hydrogen. The methane fermentation also produces much fatty acid but even more gas than the other, as much as 50 per cent. of the decomposed cellulose appearing as carbon dioxide and methane, the remaining 50 per cent. appearing mainly as acetic acid. The organism concerned is morphologically very similar to the hydrogen producing type, but the two can be separated by repeated short heatings at 75° C., by which the more rapidly developing methane-bacillus is killed off, leaving the slowly developing hydrogen-producing organism in the resistant spore stage. Conversely, the hydrogen-bacillus can be eliminated by repeated transfers whilst the methane fermentation is at its most active, the slow-growing hydrogen-

bacillus being "swamped out." These two organisms are very widely distributed in soil and mud.

It is possible that *Cl. methanigenes* does not itself attack cellulose, but that it forms methane by the reduction of products formed by *Cl. fossicularum* from cellulose.

It is claimed that in most herbivorous animals 75 per cent. of the cellulose which they digest is hydrolysed by bacteria and not by the digestive fluids.

2. *Anaerobic at High Temperature.*—A number of thermophilic organisms decomposing cellulose have been isolated from soil and rotting plant residues. MacFadyen, in 1894, isolated several such organisms from rotting straw and showed that they grew at 60° C. and produced acetic and butyric acids together with methane and carbon dioxide. They were not pure cultures. *Cl. thermocellum*, isolated in America, decomposes cellulose at 62° to 66° C. with formation of acetic acid and ethyl alcohol. *Cl. dissolvens* resembles Omelianski's *Cl. fossicularum* morphologically, but grows up to a temperature of 65° C. Cellulose, the only carbohydrate which it will attack, is broken down to acetic acid, lactic acid, butyric acid, alcohol, carbon dioxide and hydrogen. *Cl. cellulolyticum* (which may be identical with *B. thermocellulyticus*) breaks cellulose down with conversion of 64 per cent. of it to volatile acids of which 80 per cent. is formic acid and about 16 per cent. acetic acid, the remainder being propionic acid.

3. *Aerobic Fermentation at* 20° *to* 37° *C.*—A number of aerobic organisms decomposing cellulose are known, but usually they are in mixed cultures and are often symbiotic. Neither their bacteriology nor chemistry has yet been worked out satisfactorily. They produce acetic, butyric and lactic acids, which are further broken down by other organisms to water and carbon dioxide.

Cytophaga hutchinsoni converts about two-thirds of the carbon of the cellulose attacked into carbon dioxide,

most of the remainder being found in the gum which the organism produces. Organisms of the genus *Cellulomonas* (small motile or non-motile rods which may be pigmented) also attack cellulose, but may use other organic substances, though usually rather feebly. Some vibrios attack cellulose; thus *V. agar liquefaciens* (*Microspora agar liquefaciens*) produces acetic and formic acids from cellulose and also attacks agar. *V. amylocella* forms the same products from cellulose, starch and dextrin. Cellulose is also attacked by the sporing organisms *Cellulobacillus mucosus* and *C. myxogenes.*

The anaerobic fermentations are used as sources of power gas and solvents from cellulose wastes and from the decomposition of sewage sludge. Mineral salts are added to the mash of wood pulp, sawdust, corn cobs, maize or other plant residues, an inoculum of appropriate thermophilic organisms introduced, and fermentation allowed to proceed at about 65° C. The conditions in the mash soon become anaerobic. Hydrogen, methane and carbon dioxide are produced together with some alcohol and acetic acid, which is neutralised by calcium carbonate. The carbon dioxide is separated from the gases and the combustible gases used for lighting purposes or for operating gas engines. The acetic acid is either regenerated from the calcium acetate or the latter distilled with formation of acetone. This process is extensively operated in the " corn belt " of the United States, where maize stalks and straw are partially fermented by cellulose- and pectin-destroying organisms with production of power gas and solvents; the more resistant fibrous parts of the stalk composing the vascular bundles are not attacked, and are used for the manufacture of cardboard.

The cellulose in plant wastes may also be partially decomposed, giving combustible gases and leaving humin which is used as a fertiliser, and the same applies to the anaerobic fermentation of sewage, where the gaseous products are again methane, hydrogen and carbon dioxide.

In this latter case the fatty acids and proteins in the sewage also contribute their quota to the decomposition products.

The primary stages in the breakdown of cellulose are probably hydrolytic with formation, by the action of the enzyme cellulase, of the disaccharide cellobiose which in turn is hydrolysed by the enzyme cellobiase to glucose. These enzymes have been obtained in cell-free filtrates of *Cellulobacillus myxogenes* and *C. mucosus*. Cellobiose and glucose can usually only be detected as products of the action of these organisms when the air supply is restricted (preventing further degradation) or when the growth of the organisms is prevented by such substances as toluene. Dextrins may form an intermediate step between cellulose and cellobiose. Some confirmation of the intermediary nature of cellobiose and glucose is afforded by the fact that the action of *Cytophaga hutchinsoni* on cellulose is inhibited by the presence of 0·1 per cent. of glucose or 1 per cent. of cellobiose which are used preferentially. The acids and other products of these micro-organisms in all probability arise by the usual metabolic reactions from the glucose so formed. It has been claimed that the gums so often produced by cellulose decomposing organisms may consist of oxycellulose and polyuronides formed by the oxidative breakdown of the cellulose, but the evidence is not at all sound.

It has been shown that the fatty acids are broken down by spore-bearing thermophilic bacteria, with the production of methane and carbon dioxide only in yields as high as 90 per cent. The process is in effect an oxidation-reduction process involving water, as shown by the empirical equation :—

$$\underset{\text{(fatty acid)}}{C_nH_{2n}O_2} + \frac{n-2}{2}H_2O \longrightarrow \frac{n+2}{4}CO_2 + \frac{3n-2}{4}CH_4,$$

which is borne out by the gas ratios actually found. It is considered that the methane from cellulose is formed in this way from fatty acids, which are the first breakdown products of the cellulose. The amino-acids from proteins are also subject to the same sort of degradation, the amino group giving rise to ammonia.

Citric and Gluconic Acids.—The production of citric and gluconic acids from sugars by fungi has already been described (see p. 292). The processes are used on a commercial scale in the United States, making use of shallow pan fermentations, in which a felt of mycelium of *A. niger*, *P. citromyces* or *P. luteum* develops on the surface of the medium. Sometimes a continuous process is used in which fresh sugar solution is fed under the established mycelial felts as the fermented liquor is drawn off. Sometimes fermentation in rotating drums, using submerged cultures, has been found more efficacious.

For further reading :—

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CHAPTER XVIII

THE PROTEINS OF MICRO-ORGANISMS

THE proteins are essential constituents of all living cells and are perhaps the most important as well as the most complex substances synthesised by micro-organisms. In spite of this, however, they can be built up by certain organisms from the very simplest of starting materials. For instance, *B. aminovorans* can thrive on methylamine, in the absence of light, as its sole source of carbon, nitrogen and energy, producing from it complex proteins, carbohydrates and fats. The proteins, because of their complexity, their colloidal nature and the lack of any criterion of purity, are the most difficult of substances to study chemically, and we know comparatively little of their internal make-up. We know that they are built up of amino-acids joined together through the carboxyl group of one and the amino group of the next to give peptide linkages :—

$$R.CH.(NH_2).COOH + NH_2.\underset{\displaystyle COOH}{\underset{|}{CH.R}} \longrightarrow R.CH.(NH_2).CO.NH.\underset{\displaystyle COOH}{\underset{|}{CH.R.}} + H_2O.$$

The process is repeated, an amino-acid joining on to the dipeptide first formed to give a tripeptide, and this taking up another amino-acid and so on until polypeptides, protamines and, ultimately, proteins are formed. The proteins have very high molecular weights ; according to Svedberg's findings they are multiples of about 34,000 or 35,000.

By hydrolysis proteins can be split up into their constituent amino-acids and those which go to make up any particular protein identified, but as to the arrangement of the amino-acids in the protein molecule we are at present largely ignorant. As a result of the study of the proportions of various amino-acids in proteins Bergmann concludes that the total number of amino-acid residues, Nt, in any protein can be expressed by the equation $Nt = 2^n \times 3^m$ where n and m are positive whole numbers. The experimental values suggest that $Nt = 2^5 \times 3^2 = 288$ or whole number multiples of it. Since the numbers of any particular amino-acid, such as glycine or alanine or tryptophane, in the protein can also be expressed as $Ni = 2^{n'} \times 3^{m'}$ it seems plausible to regard the amino-acids as being arranged in a regular repeating pattern in the peptide chain, the pattern being characteristic of the protein. Thus in silk fibroin, for which $Nt = 2^5 \times 3^4$, half the amino-acid residues consist of glycine, $N_G = 2^4 \times 3^4$; that is each glycine unit is separated from the next by another amino-acid :—

— G — X — G — X — G — X —

The number of alanine residues is found to be $N_A = 2^3 \times 3^4$ which means that every fourth residue is alanine :—

— G — X — G — A — G — X — G — A — G — X — G — A —

Tyrosine occurs in much smaller quantities, represented by $N_T = 2^1 \times 3^4$, indicating that every sixteenth amino-acid is tyrosine :—

— G — A — G — X — G — A — G — X — G — A — G — X — G —
— A — G — T — G — A — G — X — G — A — G — X — G — A —
— G — X — G — A — G — T — G —

Similarly for the other amino-acids which fit into their places in the peptide chain, which constitutes silk fibroin,

in a rhythmic order depending on their proportion in the molecule. Other proteins, although built up of the same amino acids, differ from silk fibroin and one another in having different proportions of the amino-acids and, therefore, different periodicity or internal structure.

A certain amount of information is being obtained as a result of studying the action of specific peptidases which attack only peptide groups between particular amino-acids. By this means it has been found possible to determine whether or not certain pairs of amino-acids, for instance, occur together in a given protein. Obviously, considering the number of amino-acids available (about twenty) and the number present in a protein, the number of possible arrangements and, therefore, of possible proteins is extremely large. It is to this great variety of proteins that we owe many of the serological reactions of bacteria, the reactions of antigens and antibodies affording a means of detecting the subtle differences in arrangement of the amino-acids which are at present beyond the power of chemical methods.

As was mentioned in Chapter V, the proteins of bacteria and the yeasts are in the main very like those of plants and animals, containing the same amino-acids in much the same proportions, and falling into the globulin or albumin groups, as these are determined by solubility properties.

An interesting recent development in connection with the proteins of micro-organisms is the claim put forward by Stanley that the virus of tobacco mosaic disease is a crystalline protein. The protein can be isolated only from diseased plants. Inoculation of healthy plants with as little as 10^{-11} g. of the crystals produces the disease and gives rise to the production of large quantities of the protein. It has a molecular weight, determined by sedimentation in the ultracentrifuge and from its size according to X-ray analysis, of about 17 million; the molecular weight of the normal proteins of the healthy

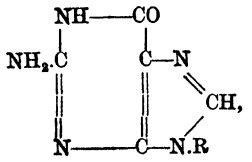
plant does not exceed about 30,000. It is inactivated by treatment with hydrogen peroxide, formaldehyde, nitrous acid or ultra-violet light, and can then no longer provoke the disease nor call forth the production of further protein; the protein is not denatured, and the molecular weight and crystalline form are not altered by this treatment, nor is the serological behaviour with antisera prepared against the active protein or the juice of infected plants. Denaturation by acid, alkali, heat or oxidation causes not only loss of activity but also loss of the other characteristic properties of the protein. Covering of up to 70 per cent. of the amino groups by acetylation or conversion to the phenylureido group does not cause loss of activity although further treatment results in inactivation of the virus. Twenty to forty per cent. of the phenolic groups of the tyrosine residues can also be masked by acetylation without destroying the activity of the virus. Inoculation of the acetyl- or phenylureido-virus into tobacco plants gives rise to the disease and to reproduction of normal virus and not acetylated virus. A similar crystalline protein having the properties of the aucuba mosaic virus has also been isolated from the juice of infected plants. It differs from the ordinary mosaic virus protein in having larger crystals (0·03 mm. long), an isoelectric point at *p*H 3·7 instead of 3·3, in being considerably less soluble and in having a sedimentation constant about 20 per cent. greater. The virus particles are believed to be thread-like macromolecules about 3000 Å units long and 150 Å units wide, but their size varies with the treatment used during isolation, suggesting that they are built up by polymerisation of smaller molecules of molecular weight about 15,000 and 150 Å units long and 15 Å wide. It has been suggested that the virus protein may be formed either by polymerisation of the normal plant proteins or by direct synthesis under the autocatalytic influence of the protein itself.

Tobacco mosaic virus has the composition of a nucleoprotein of the yeast type, containing ribose, guanine, cytosine, adenine and uridylic acid. The protein portion contains 9·0 per cent. arginine, aspartic acid, cysteine, glutamic acid, leucine, lysine, 6·7 per cent. of phenylalanine, 4·7 per cent. of proline, 6·4 per cent. of serine, 5·3 per cent. of threonine, 4·5 per cent. of tryptophane and 3·9 per cent. of tyrosine. Alanine, glycine and histidine appear to be absent. No lipoid material could be detected.

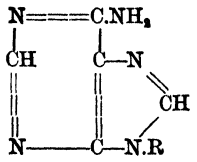
Besides the proteins, the protoplasm of micro-organisms contains the nucleoproteins and nucleins which constitute the nuclear material of the cell. This may be dispersed more or less uniformly throughout the cell contents, as in most bacteria, or it may be collected in granules as in the metachromatic or volutin granules of the diphtheria bacillus, or in true nuclei as in the yeasts.

The nucleoproteins are soluble in dilute alkali, and are precipitated from such solutions by dilute acids. They constitue about 2 or 3 per cent. of the dry weight of bacteria.

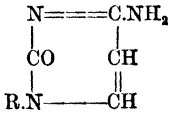
The nucleoproteins are complex molecules which break down on hydrolysis to yield a basic protein—histone or protamine—and nuclein. Nuclein on hydrolysis breaks down further to yield another protein and a nucleic acid. Nucleic acids on hydrolysis with cold alkali are degraded into nucleotides. There are two main types of nucleotide (*a*) those derived from yeasts and plants and (*b*) those derived from animals. The yeast nucleic acids are made up of guanylic acid,



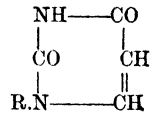
adenylic acid,



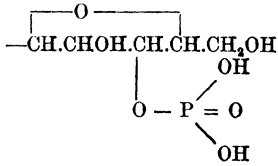
cytidylic acid,



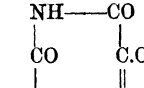
, and uridylic acid,



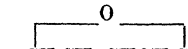
The group R is ribose-3-phosphate,



The animal nucleic acids (often called thymonucleic acids because they were first isolated from the thymus) are built up of desoxyriboguanylic acid, desoxyriboadenylic acid, desoxyribocytidylic acid and desoxyribothymosine phosphoric acid,



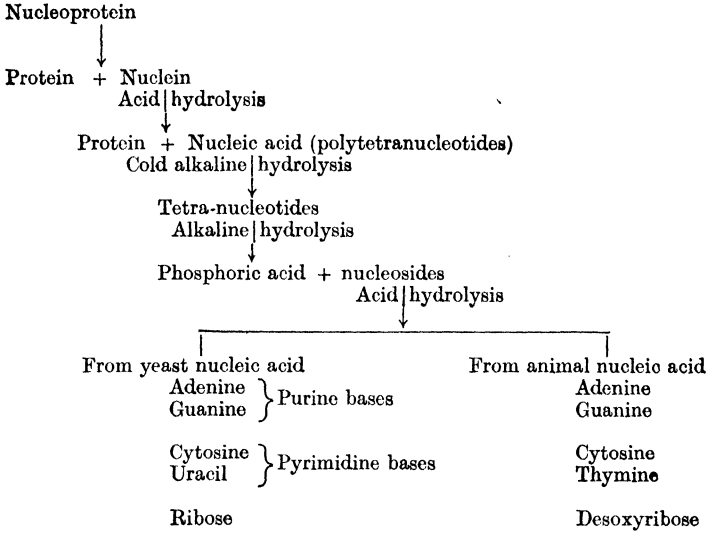
. In these nucleic acids the group R is 2-desoxyribose,



carrying a phosphate group whose position of attachment is still unknown.

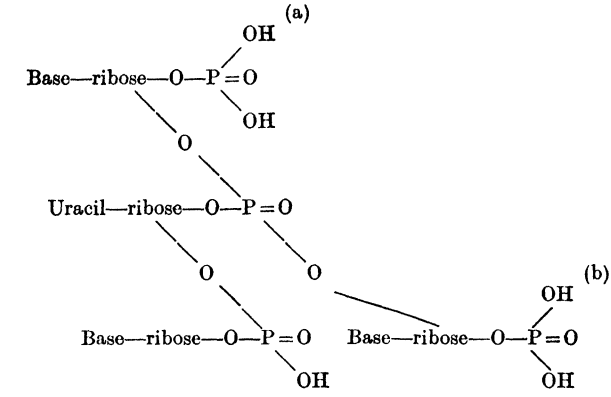
If the nucleic acids are subjected to alkaline hydrolysis the phosphate group is split off and the corresponding nucleosides are formed. The nucleosides are ribosides or desoxyribosides of guanine, adenine, cytosine, uracil or thymine (methyl uracil). It is seen, then, that the nucleic acids consist of phosphoric acid, a pentose, two purine bases, guanine and adenine, and two pyrimidine

bases cytosine and either uracil or thymine. These facts are summarised in the following scheme :—

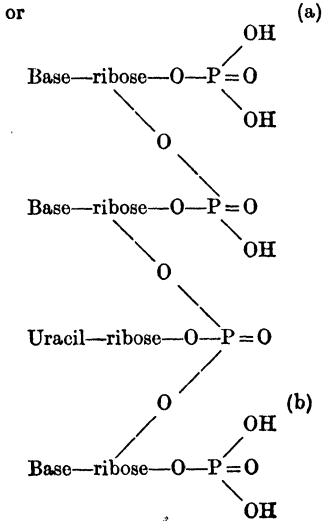


The molecular weight of the ribonucleic acids from various sources corresponds to the presence of from eight to eighteen tetranucleotide units. Careful deamination of the nucleic acids does not cause a lowering of the molecular weight, suggesting that phospho-amide groups are not involved in the linkage of the nucleotides. Electrometric titration of the poly-tetranucleotides shows that four acid dissociations are present for each tetranucleotide, three of which correspond to primary phosphoric acid dissociations and one to a secondary phosphoric acid dissociation. The deaminated nucleic acids show similar dissociation behaviour. Mild hydrolysis of the polytetranucleotides and the deaminated compounds causes a lowering of the molecular weight with the liberation of further secondary phosphoric acid groups. It is suggested, on the basis of these facts, that the

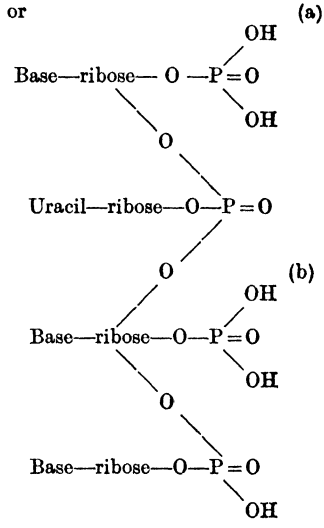
tetranucleotides are constituted as one of the following three structures :—



or



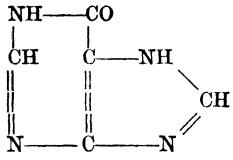
or



Polymerisation of the tetranucleotides to give the poly-tetranucleotides or nucleic acids takes place through the phosphate groups marked (a) and (b). The order in

which the bases guanine, adenine and cytosine are arranged in the above formulæ is still unknown. It is considered probable that the phosphate groups are attached at positions 2 and 3 in the ribose molecule in the ribonucleic acids. In the animal, or desoxyribonucleic acids, the phosphate linkages are probably between positions 3 and 5 of the desoxyribose, and uracil is replaced by thymine.

The tubercle bacillus gives nucleic acids of the animal, desoxyribose, type, yielding adenine, guanine, cytosine and thymine on hydrolysis, whilst *M. phlei*, the timothy grass bacillus, contains guanine, cytosine and uracil, but no thymine, correlating it with the plant nucleic acids. The nucleic acid of the diphtheria bacillus contains adenine, guanine, cytosine, uracil and thymine so that it is either a mixture of plant and animal nucleic acids or a new type. Other bacteria yield nucleic acids containing bases of the purine type only; that from *B. anthracis* gives adenine and guanine and that from *Azotobacter chroococcum* contains guanine, adenine and

hypoxanthine,  , which is derived

from adenine by deamination. The cells of streptococci contain about 80 per cent. of protein and nucleoprotein, of which nucleic acid constitutes 18 to 24 per cent. in Smooth organisms and 14 to 17 per cent. in Rough cells. The nucleic acid is a mixture of 10 to 30 per cent. desoxyribose nucleic acid from nuclear material, and the remainder ribonucleic acid from the cytoplasm. As was mentioned on p. 332 the crystalline tobacco mosaic virus consists of nucleoprotein of the plant or yeast type. The psittacosis and vaccinia viruses, on the other hand, are of the animal, desoxyribose type. Rough Type II

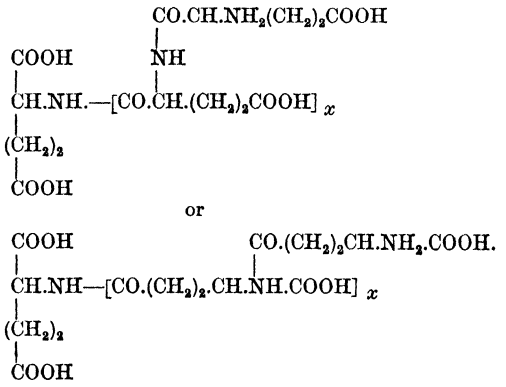
pneumococci give ribonucleic acid to the extent of 2 to 5 per cent. of their dry weight. Staphylococci also give ribonucleic acid but *Esch. coli* contains desoxyribonucleic acid.

It has been shown that the substance responsible for the Gram-positive staining reaction of *Cl. perfringens* is the magnesium salt of ribonucleic acid, which can be removed by extraction with dilute aqueous bile salt solutions leaving a Gram-negative cytoskeleton. The extract itself is also Gram-negative but can recombine with Gram-negative cell residue (but only if the latter is in a reduced condition) to give a Gram-positive complex. The ribonucleate will not combine with normally Gram-negative organisms, nor can desoxyribonucleic acid, nucleotides or nucleosides replace ribonucleic acid. *Sacch. cerevisiæ* can be similarly extracted to yield Gram-negative cells and nuclei. The extracted cells of *Cl. perfringens* and *Sacch. cerevisiæ* contain basic proteins with a high proportion of arginine and it is suggested that the Gram-positive material is a complex of reduced basic protein and magnesium ribonucleate. Gram-positive organisms often become Gram-negative in old cultures and it has been found that *Str. salivarius* becomes Gram-negative in media containing little magnesium or in media so acid that magnesium ribonucleate cannot exist.

It has very recently been shown that the substance responsible for the long known conversion of Rough pneumococci into Smooth organisms of another type is a desoxyribonucleic acid, characteristic of the type of pneumococcus from which it is derived. Thus an extract from Smooth Type III cells will cause conversion of any Rough pneumococcus to Smooth Type III but not to any other type; Smooth Type II extract gives rise to the corresponding Smooth type only. The distinguishing feature of Smooth, compared with Rough, pneumococcus organisms is a non-nitrogenous polysaccharide whose

synthesis must be initiated by the desoxyribonucleic acid. The conversion only occurs with actively growing cultures and not in resting suspensions. The desoxyribonucleic acid appears to be autocatalytic since the new Smooth organisms, even after repeated subcultures, contain it in much greater amount than that added to stimulate the change. This is analogous to the propagation of the tobacco mosaic virus, when injected into the plant.

The capsular substance of *B. anthracis*, apparently identical with that of *B. mesentericus*, which reacts with antisera in very high dilution, on hydrolysis with acid loses it serological activity and forms *d*(—)-glutamic acid. This is the first recorded natural occurrence of the lævorotatory isomer of glutamic acid. It is suggested that the capsular substance has a polypeptide-like structure :—



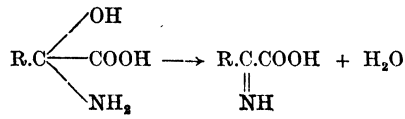
Protein Synthesis.—The synthesis of proteins by micro-organisms has been most extensively studied in the case of the yeasts. The yeast proteins, constituting approximately 50 per cent. of the dry weight of the organism, are valuable as a food since they contain all the known amino-acids. Beer yeasts have been used as a cattle fodder and even for human consumption, but

they have the disadvantage of a bitter taste, due to acid substances derived from the hops. The taste can be eliminated by washing with dilute sodium carbonate. *Saccharomyces cerevisiæ* does not grow very satisfactorily on ammonium salts as the source of nitrogen but requires expensive organic sources, so that its use for protein production is not economically sound. During the 1914 to 1918 war the use of *Torula utilis*, also known as Mineralhefe or Futterhefe, was developed in Germany. It grows much more readily on inorganic media than does *Saccharomyces*, has no bitter taste and is an effective protein source for man and animals. It is usually grown on molasses and ammonium salts under conditions of good aeration. Yields of the organism, as dry weight, up to 75 per cent. of the sugar consumed can be obtained. A similar process has been worked out recently in this country using a thermophilic variant of *T. utilis* which gives an almost theoretical conversion of the nitrogen supplied to protein. The protein content of the dried yeast is 45 to 50 per cent. The dried product also contains 20μg. per gram of aneurin, 80 to 85 μg. per gram of riboflavin and 400 to 450 μg. per gram of nicotinic acid. A fifteen-fold increase in the inoculum is obtained in 9 hours growth on molasses wastes.

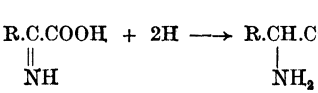
It has been shown that the amino-acid, alanine, $CH_3CH.NH_2.COOH$, in yields up to 65 per cent., can be synthesised from pyruvic acid and ammonium salts. As a result Oesterlein and Knoop suggested that amino-acids in general might be synthesised by the following route :—

$$R.CO.COOH + NH_3 \longrightarrow R.C\begin{cases} OH \\ COOH \\ NH_2 \end{cases}$$

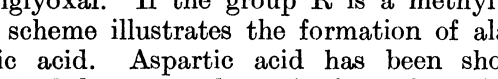
Ammonia condenses with a keto-acid to form the hydroxy-amino-acid, which loses water with formation of an imino-acid :—



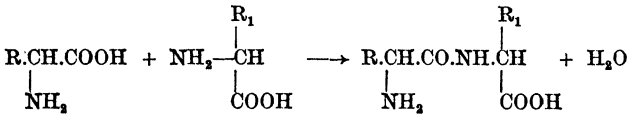
The imino-acid then becomes reduced to give the amino-acid :—



The last step is one half of a coupled oxidation-reduction process, the other half being the dehydrogenation of a sugar breakdown product, in all probability the formation of pyruvic acid from glyceraldehyde phosphate or from methylglyoxal. If the group R is a methyl group the above scheme illustrates the formation of alanine from pyruvic acid. Aspartic acid has been shown to be synthesised by many bacteria from fumaric acid and ammonium salts :—

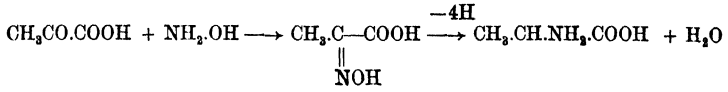


The amino-acids formed in this way condense with one another to give peptides :—

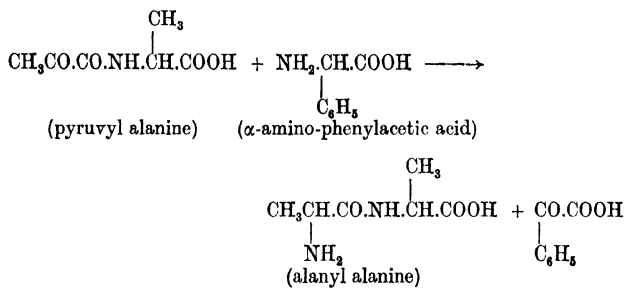


and the process is repeated with formation of polypeptides and ultimately of proteins.

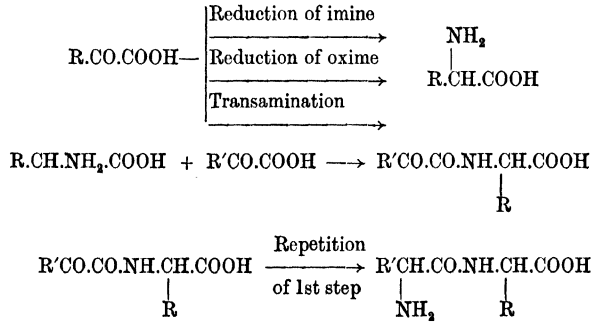
The conversion of an α-keto-acid to an amino-acid may proceed by two other routes instead of *via* the imino-acid. The keto-acid might react with hydroxylamine (which is a probable intermediate in nitrogen fixation, see p. 223) to give the oxime which, on reduction, would give an amino-acid :—



Alternatively the keto-acid may take up an amino group by transamination from another amino-acid :—



It has been suggested that the biological synthesis of peptide chains from non-amino-acid precursors may result from successive acylation and amination :—



Continued repetition gives polypeptides and finally proteins.

Probably reactions of this type are only involved in the synthesis of proteins from inorganic sources such as in the growth of autotrophic bacteria or in the fixation

of atmospheric nitrogen. When amino-acids are available, as ordinarily occurs when bacteria grow under normal conditions in normal nutrient media it is very probable that proteins are built up by the reversal of the action of proteolytic enzymes. Bergmann has shown, for instance, that papain will convert a mixture of benzoylglycine and aniline into benzoylglycine anilide :—

$$C_6H_5.CO.NH.CH_2.COOH + C_6H_5.NH_2 \longrightarrow C_6H_5.CO.NH.CH_2.CO.NH.C_6H_5 + H_2O$$

although under the same conditions it hydrolyses benzoylglycine amide to benzoylglycine and ammonia :—

$$C_6H_5CO.NH.CH_2.CONH_2 + H_2O \longrightarrow C_6H_5.CO.NH.CH_2.COOH + NH_3$$

Papain, in addition to the above reactions, also catalyses conversion reactions ; for instance it converts a mixture of benzoylglycine amide and aniline into benzoylglycine anilide and ammonia :—

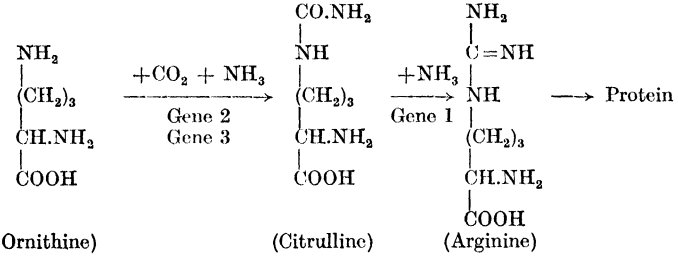
$$C_6H_5.CO.NH.CH_2.CO.NH_2 + C_6H_5NH_2 \longrightarrow C_6H_5.CO.NH.CH_2.CO.NH.C_6H_5 + NH_3$$

Bergmann pictures the *in vivo* synthesis of proteins as consisting of the action of a specific enzyme breaking down, synthesising and rearranging a number of peptide fragments until a protein is formed which is stable in the presence of the enzyme. The particular protein formed will depend on the fragments available and on the specificity of the enzyme. In any one organism or cell several enzymes may be present, resulting in the formation of a corresponding number of different proteins.

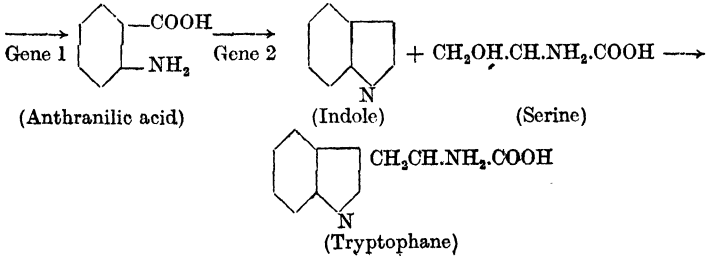
The proteinases are, in all probability, proteins themselves or at least contain protein constituents. It must be assumed, therefore, that there are proteins which are capable of multiplication or autocatalysis. Apart from the enzymes such autocatalytic proteins are to be found in the crystalline viruses and in the desoxyribonucleic acids responsible for the conversion of Rough pneumococci to Smooth organisms (see p. 337).

A recently developed method which promises to be

of great value in the study of the synthetic mechanisms of cells involves the use of mutants of the moulds *Neurospora crassa* or *N. sitophila*. When asexual spores of the moulds are treated with X-rays or ultra-violet light, germinated and crossed with the heterothallic strain of opposite sex, mutants arise which lack the ability to bring about certain syntheses which the normal strains can perform. Besides the strains which can no longer synthesise some of the growth factors, others have been produced which cannot make arginine, lysine, leucine, valine, methionine, tryptophane, proline or threonine. By using mutants in which particular stages in a synthesis are blocked it becomes possible to trace the course of synthetic processes. For instance, the cycle of formation of arginine from ornithine *via* citrulline :—



has been worked out using mutants lacking genes 1, 2 or 3 and thus incapable of carrying out the corresponding stage in the synthesis. Similarly tryptophane has been shown to arise by the following route :—



Strains which lack gene 1 will grow only if anthranilic acid, indole or tryptophane is supplied in the medium. Strains lacking gene 2 will grow if indole or tryptophane is supplied. The lack of a particular gene is specifically responsible for the absence of the corresponding enzyme.

For further information :—

G. W. Beadle, "Genetics and Metabolism in *Neurospora*." *Physiol. Rev.*, **25** (1945), 643.

M. Bergmann, "The Structure of Proteins in Relation to Biological Problems." *Chem. Rev.*, **22** (1938), 423.

R. E. Buchanan and E. I. Fulmer, "The Physiology and Biochemistry of Bacteria," Vol. I, Chapter III. Baillière, Tindall & Cox. London, 1928.

W. M. Stanley, "The Biochemistry of Viruses." *Ann. Rev. Biochemistry*, **9** (1940), 545.

CHAPTER XIX

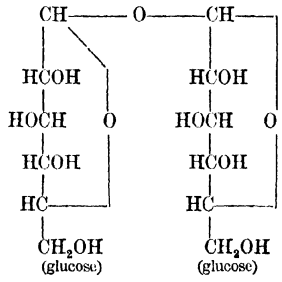
THE POLYSACCHARIDES OF MICRO-ORGANISMS

THE production of polysaccharides by micro-organisms is almost as widespread and universal as that of the proteins. Polysaccharides of a more or less degree of complexity are to be found in nearly all bacteria, yeasts and fungi. Of the common polysaccharides, starch, cellulose and glycogen are found as the result of the synthetic activities of micro-organisms, and besides these a considerable number of other polysaccharides characteristic of particular organisms is known.

The chief polysaccharide produced by yeasts is glycogen, but others comprising the various "yeast gums" have been described. Glycogen has also been isolated from the higher fungi and from certain species of *Aspergillus*. It has been claimed that it is also present in certain bacteria, including *Mycobacterium tuberculosis*, *Cl. butyricum* and *Shigella dysenteriæ*.

As was mentioned in Chapter V the presence of cellulose in micro-organisms has been established satisfactorily only in the case of *Acetobacter xylinum*, in which it was detected as early as 1886 by Brown. It is synthesised by the cell from a variety of sugars, for example glucose, fructose, sucrose and pentoses, and even more readily from such polyhydric alcohols, with three to seven carbon atoms, as glycerol, erythritol, arabitol, dulcitol, sorbitol,

human tubercle bacillus and of *M. phlei* contains fatty acids linked to the non-reducing disaccharide, trehalose,

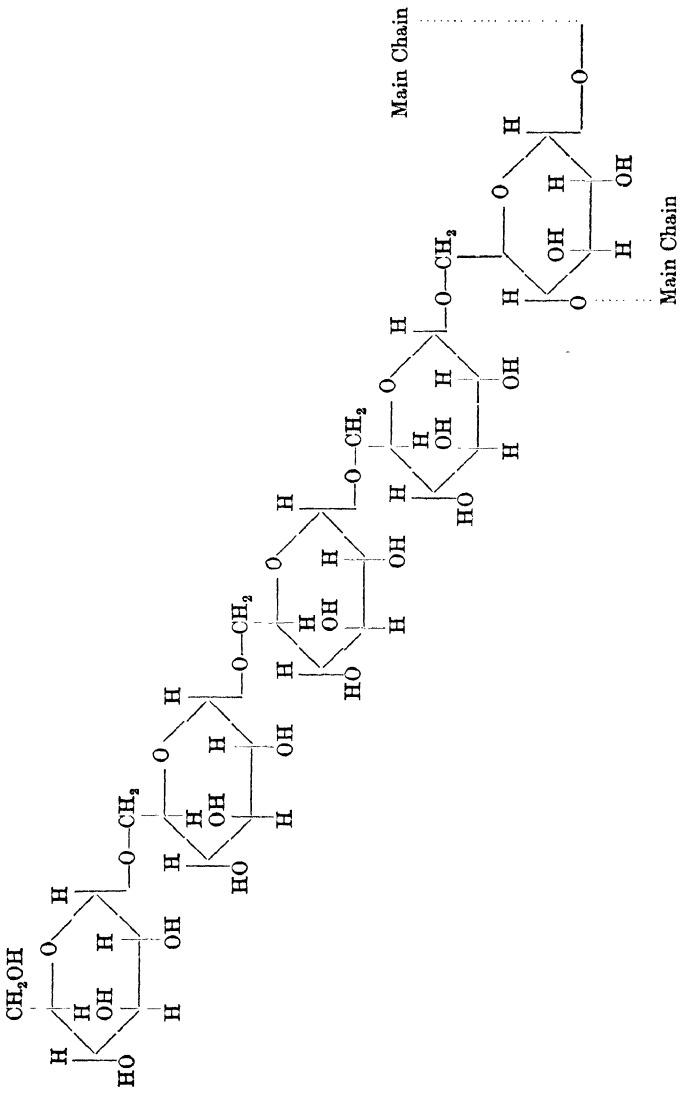


instead of to glycerol as in ordinary fats. The leprosy bacillus also contains trehalose together with a dextrorotatory polysaccharide yielding pentoses on hydrolysis. Heidelberger and Menzel have separated the polysaccharide from the human tubercle bacillus into three fractions having the properties outlined in Table 21 :—

TABLE 21

Type	Isolation	$[\alpha]_D$	Acid Equivalent	Nitrogen	Phosphorus	Products of Hydrolysis
				Per cent.	Per cent.	
A	Precipitated by $Ba(OH)_2$	+81°	1,500	0·7	1·8	...
B	Soluble in 75 per cent. methyl alcohol	+30°	2,200	0·7	1·0	61 per cent. reducing sugar, *d*-arabinose.
C	Insoluble in 75 per cent. methyl alcohol	+90°	6,700	0·1	0·2	87 per cent. reducing sugar, arabinose, mannose, magnesium palmitate.

The polysaccharide C is common to avian, bovine and human tubercle bacilli, whilst B, if present at all, occurs in only very small amount in the avian and bovine types. The removal of the magnesium palmitate from polysaccharide C does not affect its serological behaviour (compare the polysaccharide from *Salmonella typhimurium*, p. 361). An acetyl containing polysaccharide has been


Main Chain
Main Chain

in which the glucose units are united though the 1 : 6 positions.

Some strains of *Str. salivarius* and of *Str. bovis* synthesise a water insoluble dextran, $[\alpha]_D + 180°$, from sucrose or raffinose, as does *Betabacterium vermiforme* (probably a *Lactobacillus*) from sucrose but not from other sugars. This dextran, too, has $[\alpha]_D + 180°$ when dissolved in acid or alkali. It is made up of α-glucose units linked through positions 1 : 6, that is, it has the gentiobiose structure.

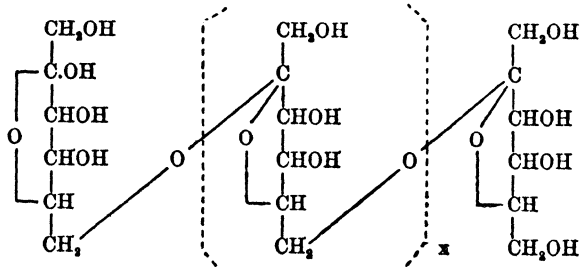
Phytomonas tumefaciens excretes into the medium a polysaccharide which has $[\alpha]_D^{25} - 9°$, yielding only glucose on hydrolysis. Its molecular weight, about 3600, indicates that it is built up of 22 glucose units, probably of pyranose form and joined by β-linkages.

Leuconostoc dextranicum, when grown on sucrose, gives a mixture of dextran and a fructosan, but when grown on glucose it yields mainly the dextran and very little fructosan.

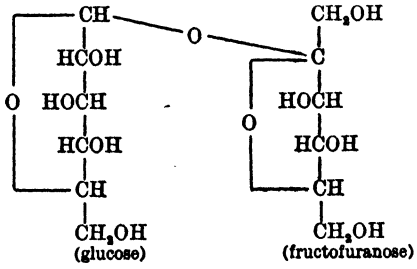
Azotobacter chroococcum gives rise to a gum which appears to consist mainly of an araban since it yields arabinose on hydrolysis. The root nodule bacteria, *Rhizobium*, also produce a gum which splits up into glucuronic acid, $\begin{matrix}\text{CHOH—(CHOH)}_3\text{—CH—COOH}\\ \text{\quad\quad\quad

which should be compared with that given for Type III pneumococcus on p. 356.

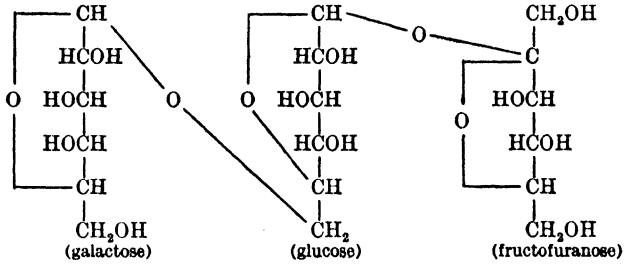
B. mesentericus and *B. subtilis* when grown on sucrose or raffinose produce levan, a lævorotatory polysaccharide, $[\alpha]_D - 45{\cdot}3°$, built up of fructofuranose units linked together through positions 2 and 6 :—



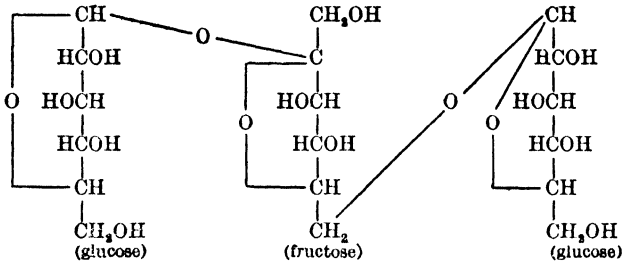
It is not formed when the organisms are grown on melezitose, maltose, lactose, glucose, xylose or fructose. That is, a terminal fructofuranose grouping, such as is present in sucrose,



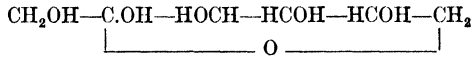
or in raffinose,



but not in melezitose,



nor in normal fructose,

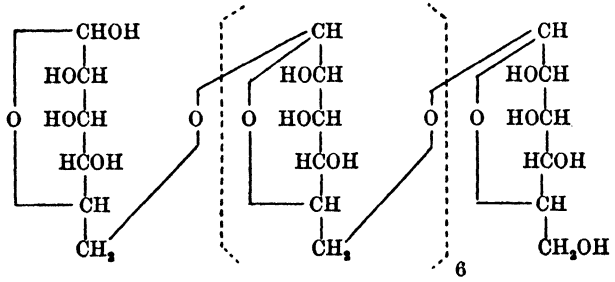


which has a fructopyranose structure, is necessary for the formation of levan. The conversion of sucrose and raffinose into levan can also be accomplished by an enzyme, levansucrase, which is secreted into the medium by *B. mesentericus* and *B. subtilis*; *B. polymyxa* and *Aerobacter levanicum* form an endocellular levansucrase which has the same effect. Levansucrase has no action on Neuberg's ester, Harden and Young's ester, methyl-γ-fructoside or inulin, all of which contain a terminal fructofuranose group. Along with levan a reducing

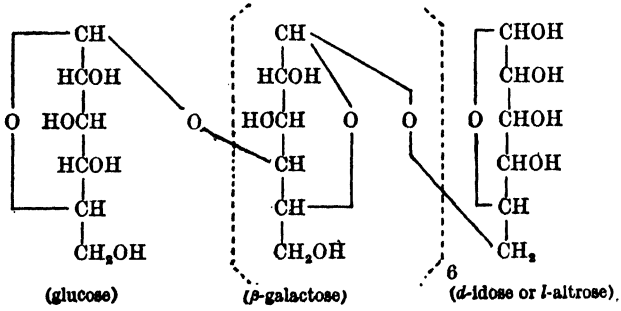
sugar is formed, glucose from sucrose, and melibiose (not galactose) from raffinose, showing that the latter is not first hydrolysed to sucrose. No carbohydrate is destroyed in the conversion, showing that the process is independent of respiration. Many strains of *Str. salivarius* synthesise levan from sucrose and raffinose. *B. lactis* produces a fructosan from sucrose only. In the sugar-refining industry contamination with *Leuconostoc* and *B. mesentericus* causes considerable loss as the result of the " viscous fermentations " to which they give rise. *B. lactis pituitosi* gives rise to the formation of a galactan which is excreted into the medium.

Mycodextran, $[\alpha]_D+251°$, a polyglucose giving no coloration with iodine, is produced by the mould *P. expansum*. A number of other glucose polysaccharides of varying degrees of molecular complexity have been described giving colours with iodine ranging from no colour through red to blue and purple. Mycogalactan, $[\alpha]_D+284°$, yielding galactose on hydrolysis, is formed by the growth of *A. niger* on a glucose medium. This production of a polysaccharide built up of sugar units different from those of the sugar on which the organism grew is not an isolated case ; a number of such conversions are now known. The formation of cellulose from a variety of carbon sources by *Acetobacter xylinum* has already been described. *P. luteum* produces an acid polysaccharide, luteic acid, when grown on glucose. Luteic acid is hydrolysed by dilute mineral acid to yield one molecule of malonic acid and two molecules of glucose. The malonic acid is linked through one carboxyl group to a neutral polysaccharide, luteose, from which it can be removed by treatment with dilute alkali. Besides luteic acid, however, the mould also produces other polysaccharides built up of fructose, galactose or mannose, even when it is grown on glucose as the sole source of carbon. The converse of this phenomenon also occurs ; when *P. luteum* is grown on galactose, mannose, fructose,

pentoses or glycerol it produces the same luteic acid, together with the other products, as it does when grown on glucose. Some moulds produce polysaccharides which are built up of more than one carbohydrate unit. Thus *P. charlesii* gives a polysaccharide containing glucose and galactose as well as a second polysaccharide, mannocarolose, $[\alpha]_{5870}+66°$, which consists of eight or nine units of *d*-mannose linked together through the 1:6 positions :—



P. varians gives rise to an even more complex polysaccharide, varianose, $[\alpha]_D+15°$, which on hydrolysis yields three sugars, galactose, glucose and either *d*-idose or *l*-altrose, in the proportions 6 : 1 : 1. They are linked together with the glucose molecule at one end and the galactose units in the middle :—



Some bacteria also produce mixed polysaccharides, the pneumococcus, for instance, giving products containing glucose and galactose, the tubercle bacillus glucose, arabinose and mannose, and the *Vibrios* glucose, galactose and arabinose.

The most important polysaccharides from a bacteriological point of view are the so-called "soluble specific substances" which are responsible for the serological behaviour of many organisms. They were first described in the case of the pneumococcus, but since then have been found to occur in a number of other bacteria of several genera. It was shown by Dochez and Avery that filtrates of cultures of pneumococcus contained a substance which gave specific reactions with antisera prepared against the same type of pneumococcus but not with antisera prepared against other types. Later Zinsser and Parker isolated "residual antigens" from alkaline extracts of the organisms; the residual antigens reacted with the homologous sera, but gave no reactions for proteins and were non-antigenic. Dochez and Avery's "soluble specific substance" was heat stable, was precipitated from aqueous solution by acetone or alcohol, was free from protein, and shown to be of a polysaccharide nature. It was accompanied by a nucleoprotein which was common to all the types of pneumococcus, that from any one type reacting with antisera prepared against any of the other types; it was antigenic and antisera prepared against it gave no reaction with the soluble specific polysaccharides.

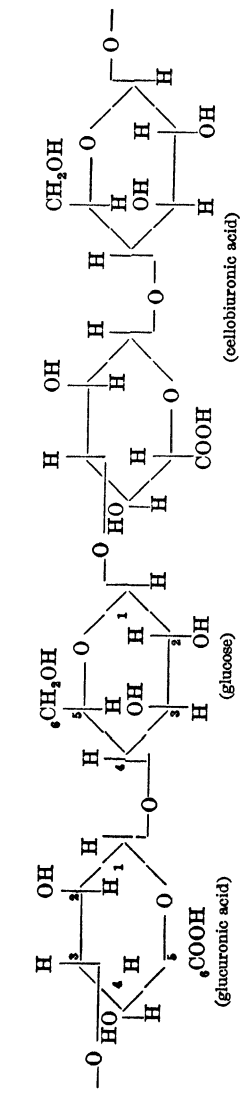
The specific polysaccharides from each type of pneumococcus are not only different in their serological behaviour but, also, have been shown to be chemically different, as may be seen from Table 20, where their main properties are summarised.

TABLE 20

	$[\alpha]_D$	Acid Equivalent	Total N	Amino-Nitrogen	Acetyl (Amino Group)	Products of Hydrolysis
			Per cent.	Per cent.	Per cent.	
Pneumococcus—						
Type I -	+280°	650	4·85	2·5	...	Amino-sugar, galacturonic acid.
„ II -	+ 55°	950	0·2	...	...	Glucose, aldobionic acid (glucose, glucuronic acid).
„ III -	− 33°	340	0·1	...	...	Glucuronic acid, glucose.
„ IV -	+ 30°	1,500	5·5	0·1	5·5	Acetic acid, amino-sugar, glucose.
„ VIII -	+125°	750	0·2	...	...	Glucose, aldobionic acid.
„ XIV -	+12·5°	...	2·7	2·7	9·5	Acetic acid, glucosamine, galactose.
Carbohydrate-C (species specific)	+61·3°	1,050	5·9	1·14	3·7	Acetic acid, phosphoric acid, amino-sugar.
Carbohydrate-F (Forsmann)	+68·9°	...	5·6	0·99	...	Acetic acid, glucosamine, reducing sugar, lipin, phosphoric acid.
Inactive carbohydrate Friedländer—	+ 10°	4,500	6·0	...	5·6	Acetic acid, glucosamine.
Type A - -	−100°	430	...	...	...	Glucose, aldobionic acid, 65 per cent. reducing sugar.
„ B - -	+100°	680	...	...	...	Glucose, aldobionic acid, 75 per cent. reducing sugar.
„ C - -	+100°	685	...	...	...	Glucose, aldobionic acid, 70 per cent. reducing sugar.

Although the Type I pneumococcus polysaccharide contained nitrogen it gave no protein reaction and, as it was first isolated, was not antigenic. Since then, however, by avoiding the use of alkali in its extraction, it has been obtained in an acetylated form, containing one acetyl group for each glucose unit; the acetyl polysaccharide is antigenic, and on removal of the acetyl group yields a non-antigenic polysaccharide identical with that originally isolated. Some doubt is cast on this finding by later work by Felton who could find no correlation between the acetyl content and antigenicity. Half the nitrogen of the Type I polysaccharide is in the form of amino-nitrogen since it is eliminated by treatment with nitrous acid, with production of reducing sugar and loss of serological activity. The polysaccharides of Types II and III pneumococci are not affected by treatment with nitrous acid. Type I polysaccharide is amphoteric, acting as a strong acid and a weak base; it has an iso-electric point at about *p*H 4.

The Type II polysaccharide is a weak acid; on acetylation it yields a serologically inactive product. Removal of the acetyl groups restores the original activity of the compound. The Type III polysaccharide is similar to that of Type II, but is lævorotatory. On hydrolysis it yields cellobiuronic acid, $[\alpha]_D + 10°$, an aldobionic acid having the structure 4-β-glucuronosidoglucose. In the polysaccharide these units are linked through the 3-carbon atom of the uronic acid to the reducing group of the glucose in the next unit :—


(glucuronic acid)
(glucose)
(cellobiuronic acid)

The polysaccharide from Type XIV pneumococcus resembles that of Type IV in not containing a uronic acid group. It is constituted of one molecular proportion of acetyl glucosamine and three molecular proportions of galactose. It closely resembles the Blood group A specific polysaccharide which occurs in group A red blood corpuscles and which can be isolated from saliva, gastric mucin, commercial pepsin and peptone. The two substances are not identical, since the blood group A substance contains nitrogen in addition to that as glucosamine, and the blood group A substance does not give a cross precipitin reaction with antisera to Type XIV pneumococci prepared in rabbits, although strong cross reactions are found when horse antisera are used. Horse antisera to Type XIV pneumococci agglutinate human red cells of all groups.

Pneumococci resemble *Shigella dysenteriæ* and *Salmonella schottmülleri* in containing the Forsmann hapten which is capable of provoking lysins for sheep's red blood corpuscles when injected into animals. In the case of the pneumococci it is a lipo-polysaccharide complex (carbohydrate F) associated with the bacterial bodies. It is probably made up of the species specific carbohydrate-C and a lipin fraction, chemically bound to it. The lipin, constituting 6·5 per cent. of the polysaccharide, is devoid of nitrogen and phosphorus, has m.p. 39–41°C. and an acid equivalent 372 ; it is possibly a C_{24} compound. Carbohydrate-C contains no lipin.

Enzymes have been found in various soil organisms, for example *Rhodobacillus palustris* and a *Myxococcus*, which hydrolyse the pneumococcal polysaccharides specifically. *Rh. palustris* attacks only Type VIII polysaccharide and not those of Types I, II or III. Another soil organism gives an enzyme attacking Type III polysaccharide only.

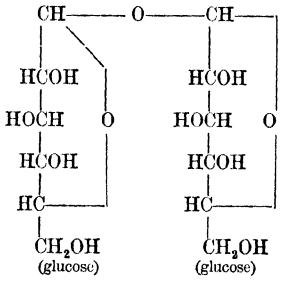
Autolytic enzymes isolated from pneumococci themselves have been shown to attack the corresponding polysaccharides and also those of *Str. salivarius*, of the bovine vitreous humour and of the umbilical cord, all of which contain an acetylglucosamine-glucuronic acid complex, hyaluronic acid.

The enzyme, hyaluronidase, is also found in many anaerobes, including *Cl. perfringens* and those of the gas gangrene group; it is identical with or very closely related with the so-called "spreading factor" of these organisms.

Similar specific polysaccharides were isolated from Friedländer's bacillus, *Klebsiella pneumoniæ*, Types A, B and C. Those from Types B and C, although very similar chemically (see Table 20), are quite distinct serologically.

The tubercle bacilli afford very complex mixtures of polysaccharides which have not yet been thoroughly worked out. A polysaccharide having a rotation of $[\alpha]_D +67°$ was isolated, which on hydrolysis yielded 30 per cent. of *d*-arabinose, together with glucose, galactose, mannose and a sugar acid. It was also found to be present in tuberculin. *Mycobacterium phlei* yields a polysaccharide, precipitated by basic lead acetate, which gives rise to arabinose, mannose and inositol on hydrolysis. The avian tubercle bacillus produces a polysaccharide which gives two molecules of mannose and one of inositol on hydrolysis. On a synthetic medium the human tubercle bacillus forms a polysaccharide, having a specific rotation $[\alpha]_D +32°$, which yields 19·7 per cent. of mannose and 10 per cent. of *d*-arabinose. The lipoid fractions contain glycerophosphoric acid, mannose, inositol, arabinose, glucose, fructose, glucosamine and other unidentified carbohydrates. The acetone soluble fat fraction of the

human tubercle bacillus and of *M. phlei* contains fatty acids linked to the non-reducing disaccharide, trehalose,



instead of to glycerol as in ordinary fats. The leprosy bacillus also contains trehalose together with a dextrorotatory polysaccharide yielding pentoses on hydrolysis. Heidelberger and Menzel have separated the polysaccharide from the human tubercle bacillus into three fractions having the properties outlined in Table 21 :—

TABLE 21

Type	Isolation	$[\alpha]_D$	Acid Equivalent	Nitrogen	Phosphorus	Products of Hydrolysis
				Per cent.	Per cent.	
A	Precipitated by $Ba(OH)_2$	+81°	1,500	0·7	1·8	...
B	Soluble in 75 per cent. methyl alcohol	+30°	2,200	0·7	1·0	61 per cent. reducing sugar, *d*-arabinose.
C	Insoluble in 75 per cent. methyl alcohol	+90°	6,700	0·1	0·2	87 per cent. reducing sugar, arabinose, mannose, magnesium palmitate.

The polysaccharide C is common to avian, bovine and human tubercle bacilli, whilst B, if present at all, occurs in only very small amount in the avian and bovine types. The removal of the magnesium palmitate from polysaccharide C does not affect its serological behaviour (compare the polysaccharide from *Salmonella typhimurium*, p. 361). An acetyl containing polysaccharide has been

obtained from human tubercle bacilli which gives precipitin reactions with the sera of tuberculous patients and with antisera to the organism. The complex mixture of polysaccharides from the bovine tubercle bacillus contains much more inactive carbohydrate than does that from human tubercle bacillus. An inactive carbohydrate, precipitated by 80 per cent. acetic acid, is common to both human and bovine bacilli ; another, which is soluble in 96 per cent. acetic acid, occurs in bovine strains only. The acetic acid soluble carbohydrate from human strains is serologically active. The inactive carbohydrates from bovine tubercle bacilli are strongly dextro-rotatory and contain phosphorus but little or no pentose. The serologically active polysaccharides contain *d*-arabinose. The wax fraction of the human tubercle bacillus (see p. 375) contains fatty acids esterified by a specific polysaccharide which gives precipitin reactions with anti-tubercle sera. On hydrolysis it yields mannose, *d*-arabinose and galactose, with small amounts of inositol and glucosamine. Two polysaccharides have been separated from the attenuated tubercle organism, Bacille de Calmette-Guerin (B.C.G.). Polysaccharide A, soluble in water, has $[\alpha]_D + 77{\cdot}4°$, and, on hydrolysis, gives 77 per cent. of reducing sugar containing mannose, arabinose and inositol, with 3 per cent. of an amino-sugar. The other polysaccharide, insoluble in water but soluble in acids, is a complex of about equal weights of a polysaccharide (giving 95 per cent. of reducing sugars on hydrolysis) and calcium phosphate.

Sal. typhimurium has been investigated by three methods which have given almost identical results. If smooth strains of the organism are extracted with dilute trichloracetic acid a polysacchride is removed which can be recovered by dialysis, concentration and precipitation by acetone. It is toxic to mice, is antigenic and reacts specifically with the corresponding antiserum. On treatment with hot dilute acetic acid it yields four components

(*a*) 69 per cent. of a soluble specific polysaccharide, (*b*) 16 per cent. of insoluble conjugated protein, (*c*) 3 to 4 per cent. of a benzene soluble lipin fraction and (*d*) 8 per cent. of an alcohol soluble acetyl polysaccharide. The conjugated protein is toxic but is neither antigenic nor a hapten. The lipin fraction, insoluble in acetone, appears to be a phosphatide. The main fraction, which can be precipitated by alcohol, is a hapten, reacting with antisera, but is neither antigenic nor toxic. It has $[\alpha]_D + 103°$, contains no nitrogen and on hydrolysis yields 93 per cent. of reducing sugar, of which 31 per cent. is glucose, 21·5 per cent. mannose and 19 per cent. galactose. It contains no ketose, pentose or uronic acid. The antigenic complex can be dissociated by precipitation from weakly alkaline solution to give a small amount of an amphoteric protein and a non-antigenic "undegraded" polysaccharide which reacts specifically with *Sal. typhi-murium* antisera. The complete antigen, possibly a calcium salt of a phosphatide-polysaccharide-protein complex, occurs only in the smooth organisms; the rough variants contain the "residual antigen," which is the complete antigen deprived of the phosphatide fraction. The rough variants apparently contain an enzyme which breaks down the complete antigen.

The second method of isolation depends on removal of the protein of the organism by digestion with trypsin and precipitation of the polysaccharide in the solution with alcohol. The complete antigen so obtained behaves in the same way as that extracted by trichloracetic acid. These are the so-called "F 68" polysaccharides since they are precipitated by 68 per cent. of alcohol. The third process is extraction of the dried organisms with diethylene glycol, which gives products which may not be degraded to such an extent as those obtained by the more drastic methods, although their properties are essentially the same.

The trichloracetic acid method has been applied to a

number of organisms, including *Esch. coli*, *Eberth. typhosa*, *Sal. paratyphi*, *Sal. schottmülleri*, *Shigella dysenteriæ*, *Proteus*, *Serratia marcescens*, *Ps. æruginosa*, *B. anthracis*, *Phytomonas tumefaciens* and *V. comma*, with similar results.

The polysaccharide antigens isolated by these methods are the somatic O-antigens of the smooth organisms.

The polysaccharides of a number of bacteria have been investigated. *Shigella dysenteriæ* in the smooth form produces a polysaccharide with a specific rotation $[\alpha]_D +98°$, containing 1·6 per cent. of nitrogen. It has a molecular weight about 5,100 and acid equivalent about 9,000. It contains no protein, no pentoses and no uronic acids. The nitrogen is present as an amino group, which, however, is masked by acetylation (the acetyl content is 5 per cent.). The composition and molecular weight correspond to four hexose units, probably glucose, and one acetamido-hexose unit, all repeated six times. This polysaccharide also appears to be responsible for the heterogenetic reaction between *Shigella dysenteriæ* antisera and sheep red blood cells. The polysaccharide-protein-phospholipin complex can be dissociated by treatment with formamide into the non-antigenic phospho-lipin and a polysaccharide-protein moiety which has the properties of the somatic antigen of the smooth organisms. Treatment of the complex with trypsin removes the protein and leaves the feebly antigenic phospholipin-polysaccharide. The free polysaccharide is a non-antigenic hapten which gives precipitin reactions with antisera. The polysaccharide-protein complex can be split by solution in 90 per cent. phenol and dialysis to give the polysaccharide hapten and the antigenic protein, which, however, has lost the somatic specificity of the complex. The complex can also be degraded by boiling with 1 per cent. acetic acid, yielding an almost non-antigenic protein which can be further dissociated by solution in phenol, when a prosthetic group is probably removed.

The polysaccharide and the protein can be recombined by solution in formamide and precipitation with alcohol. The conjugated protein gives rise to an antigen having the properties of the original somatic antigen of *Shigella dysenteriæ*, but the simple protein, when coupled with the polysaccharide, gives a non-antigenic complex, suggesting that the prosthetic group is essential for antigenicity.

A similar somatic antigen complex has been extracted from the O 901 strain of *Eberthella typhosa* by the trichloracetic acid, the trypsin and the diethylene glycol extraction methods. It can be dissociated by boiling 1 per cent. acetic acid to give an ether soluble phospholipin, a water soluble polysaccharide and an insoluble protein. The purified polysaccharide, which has $[\alpha]_{5461}+128°$, 1·2 per cent. of organic phosphorus and less than 0·1 per cent. of nitrogen, is non-antigenic and non-toxic to mice but gives a precipitin reaction with typhoid O-antisera. The purified protein, containing 11·5 per cent. of nitrogen, 0·47 per cent. of phosphorus and having $[\alpha]_{5461}-55°$, is soluble in alkali but not in acid and appears to be identical with that from Shiga's bacillus. The two proteins can replace one another in combination with the polysaccharide from either organism to give antigens having specificity which is determined by the polysaccharide. A similar protein has also been obtained from *Shigella paradysenteriæ* (Flexner 88), it contains 10·7 per cent. of nitrogen and 1·1 per cent. of phosphorus and has $[\alpha]_{5461}-50°$.

The virulent Vi strains of *E. typhosa* yield a similar polysaccharide complex which reacts specifically with antisera to Vi organisms.

Different polysaccharides corresponding to the rough (avirulent, Type B) and smooth (virulent, Type A) variants of *Staph. aureus* are known. They are acid to litmus, give no protein reactions but contain phosphorus

and nitrogen. Their chief properties are given in Table 22 :—

TABLE 22

	$[\alpha]_D$	Acid Equivalent	Nitrogen	Phosphorus	Reducing Sugar
			Per cent.	Per cent.	
Type A	+ 7°	776	4·1	6·3	25 per cent. glucose, mannose (?).
„ B	+69°	806	3·8	6·4	37 per cent. glucose.

The polysaccharide from the smooth organisms is precipitated by barium hydroxide, whilst that from the rough variant is not.

The streptococci afford both serologically active and inactive polysaccharides. The former, obtained from *Str. salivarius* by Lancefield, is a hapten, reacting with homologous antisera but being non-antigenic. The inactive polysaccharide, $[\alpha]_D$–73°, obtained from mucoid hæmolytic streptococci of Lancefield's Group A, contains no phosphorus, sulphur or amino-nitrogen. It contains 3·7 per cent. of nitrogen, 11 per cent. of acetyl group and 46 per cent. of uronic anhydrides ; it has an acid equivalent of 380. It is an acetyl-glucosamine-glucuronic acid complex, apparently identical with or closely related to the hyaluronic acid in bovine vitreous humour, since it is hydrolysed by the autolytic enzyme of the pneumococcus (see p. 358). A group specific polysaccharide has been extracted from the organisms with formamide and contains 1·72 per cent. of nitrogen, 0·7 per cent. of phosphorus and has $[\alpha]_D^{24}$–71·5°. It contains glucosamine and uronic acid residues. It reacts at a dilution of 1 in 2×10^6 with homologous antisera.

The *Vibrios* have been divided into six groups by Linton on the basis of their content of proteins and polysaccharides. Those derived from cholera patients, Group I, form a polysaccharide which on hydrolysis yields

galactose and an aldobionic acid composed of galactose and glucuronic acid. The water vibrios produce a polysaccharide containing arabinose linked with the same aldobionic acid. The Inaba variant of *V. comma*, Group VI, gives a polysaccharide having $[\alpha]_D + 58°$, and containing 2·6 per cent. of nitrogen, which yields 58 per cent. of reducing sugar on hydrolysis. The sugar is glucose only, no galactose or arabinose being present. The polysaccharides present in rough strains, and which also occur in the corresponding smooth strains, are stable to alkali, whilst the polysaccharides peculiar to the smooth variants are not stable to alkali.

The gonococcus and meningococcus contain non-antigenic and non-toxic polysaccharides which both react with antisera to Type III pneumococcus. That from the meningococcus is probably the sodium salt of an acid polysaccharide. The gonococcus gives two polysaccharides corresponding to two serological types.

Both capsulated and non-capsulated forms of *B. anthracis* yield a non-toxic, non-antigenic polysaccharide, containing 0·8 per cent. of nitrogen, which gives 60 per cent. of glucose on hydrolysis together with pentoses and a uronic acid. Another polysaccharide from virulent and avirulent *B. anthracis* has been described. It gives equimolecular proportions of galactose and of acetylated *d*-glucosamine on hydrolysis (corresponding to 68 per cent. of the pure polysaccharide) but no pentose and no uronic acid. This polysaccharide is antigenic. The capsules, unlike those of the pneumococcus, are not polysaccharide in nature but contain peptides (see p. 338).

Proteus yields two polysaccharides, one of which, stable to hot alkali, appears to be the common antigenic factor between *Proteus X*19 and *Rickettsia*.

Micrococcus lysodeikticus yields a polysaccharide of high molecular weight which is the specific substrate of the enzyme lysozyme, which splits it into an N-acetylaminohexose and a ketohexose. The polysaccharide can

be extracted by cold hypochlorite solution (antiformin), diethylene glycol or hot formamide, and precipitated with alcohol or acetone.

Hapten polysaccharides have also been isolated from *Brucella abortus* and other *Brucella* species, from *Cl. perfringens*, *C. diphtheriæ* and the diphtheroid bacilli, *H. pertussis*, *H. parapertussis*, from the capsules of *H. influenzæ*, from a number of *Salmonella*, from certain *Pasteurella* and from *Leptospira biflexa*.

Carbohydrate Synthesis.—We know practically nothing of the mechanism of the synthesis of this great variety of complex polysaccharides. Kluyver has suggested that their synthesis involves coupled oxidation-reduction processes, as do the syntheses of fats and proteins, and that it may follow similar lines to the resynthesis of glycogen from lactic acid in muscle during the recovery period :—

(1) $3CH_3CHOH.COOH + 3O \longrightarrow 3CH_3CO.COOH + 3H_2O.$

(2) $3CH_3CO.COOH \longrightarrow 3CH_3CHO + 3CO_2.$

(3) $3CH_3CHO + 3H_2O \longrightarrow 3CH_3C(OH)_2H$

(4) $3\,H(H)C(H)(OH)-C(H)(OH)H + 3O \longrightarrow 3CH_2(O)CHOH + 3H_2O$

(5) $3CH_2(O)CHOH \rightleftharpoons CH_2OH.CHO$ (glycolaldehyde).

(6) $3CH_2OH.CHO \longrightarrow C_6H_{12}O_6 \longrightarrow (C_6H_{10}O_5)_n$ (glycogen).

The lactic acid is oxidised to pyruvic acid, which is decarboxylated to give acetaldehyde and carbon dioxide. The acetaldehyde is oxidised *via* the hydrate to give

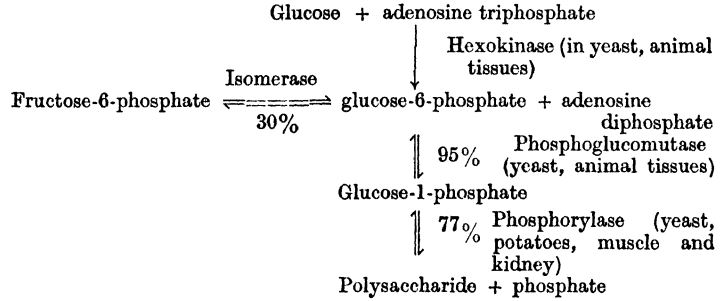
glycolaldehyde, which condenses to give glucose and finally glycogen. In summary, three molecules of lactic acid give one molecule of glucose and three molecules of carbon dioxide and water :—

$$3CH_3CHOH.COOH + 3O_2 \longrightarrow 3CO_2 + 3H_2O + C_6H_{12}O_6.$$

Or we may regard the process as being the oxidation of one-third of the lactic acid to provide the energy for the synthesis of the other two-thirds to glucose and glycogen :—

$$CH_3CHOH.COOH + 3O_2 \longrightarrow 3CO_2 + 3H_2O \text{ (energy).}$$
$$2CH_3CHOH.COOH \longrightarrow C_6H_{12}O_6 \text{ (synthesis).}$$

Hanes showed that starch was synthesised from glucose-1-phosphate under the influence of an enzyme, phosphorylase, present in potatoes, inorganic phosphate being liberated during the process. It has been suggested as a possible general mechanism that glucose is phosphorylated at the expense of adenosine triphosphate, as in yeast fermentation, and that some of the ester may be acted on by enzymes other than the normal fermentation enzymes to give rise to polysaccharides :—



The necessary energy for the process is held to be derived from the conversion of adenosine triphosphate to the di-phosphate. The latter is re-esterified to adenosine-triphosphate by the phosphate set free in the final stage

of synthesis. An alternative source of glucose-6-phosphate may be from phosphopyruvic acid by reversal of the reaction of alcoholic fermentation according to the Embden-Meyerhof scheme. Pyruvic acid is also formed in the fermentation of lactic acid and in most, if not all, bacterial fermentations. It will be remembered, however, that glucose-1-phosphate is not an intermediate in the conversion of sucrose to dextran by enzymes of *Leuconostoc mesenteroides*, and that potato phosphorylase is not effective in that synthesis (see p. 346).

The synthesis of carbohydrates and polysaccharides may also occur by way of the condensation of aldehydes with the intervention of adenosine phosphates as was suggested by Ruben for the autotrophic bacteria (see p. 78).

In the early stages of dissimilation of glucose by yeasts and by *Esch. coli* potassium and glucose disappear from the medium and a fermentable non-reducing polysaccharide is formed in equimolecular proportions. The disappearance of glucose is more rapid than is accounted for by the products of fermentation formed. It appears that the potassium is concerned in the synthesis, but the mechanism is unknown.

For further reading:—

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E. Mikulaszek, "Bakterielle Polysaccharide." *Ergebnisse der Hygiene* **17** (1935), 415.

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CHAPTER XX

THE LIPOIDS OF MICRO-ORGANISMS

THE term " lipoid " (lipide and lipin are synonymous) is employed as a general name for all the fat-like substances which are soluble in the " fat solvents " ether, alcohol, acetone, chloroform and light petroleum. Thus the fats, waxes, higher alcohols, sterols and phosphatides, together with certain of their degradation products, such as fatty acids and glycerol, are all lipoids.

Fats.—The fats are all glycerides, that is, esters of fatty acids with glycerol. When it is remembered that glycerol, $CH_2OH.CHOH.CH_2OH$, contains three hydroxyl groups capable of esterification and one, two or three of them may be involved, that the acids attached may be all the same or all different and may be chosen from a large number, both saturated and unsaturated, it is obvious that a great variety of fats is possible. Usually fats occur together as complex mixtures which are extremely difficult to separate, and their analysis is restricted to the identification of the fatty acids present and the determination of the ratio of saturated to unsaturated fatty acids. The fats of micro-organisms usually contain a high proportion of unsaturated fatty acids and are consequently liquid at ordinary temperatures or have low melting points. Palmitic, $C_{16}H_{32}O_2$, stearic, $C_{18}H_{36}O_2$, and oleic acids, $C_{18}H_{34}O_2$, are the chief acids found in the fats of micro-organisms as they are in animal fats, but butyric, $C_4H_8O_2$, caproic, $C_6H_{12}O_2$, lauric, $C_{12}H_{24}O_2$, dihydroxystearic, $C_{18}H_{36}O_4$, linoleic, $C_{18}H_{32}O_2$, linolenic, $C_{18}H_{30}O_2$, tuberculostearic, $C_{19}H_{38}O_2$, arachidic,

$C_{20}H_{40}O_2$, cerotic, $C_{26}H_{52}O_2$, phthioic, $C_{26}H_{52}O_2$, myristic, $C_{30}H_{60}O_2$, and isocetinic acids have also been reported as occurring in the fats of bacteria.

The acid-fast bacteria contain the following fatty acids in the acetone soluble fat fraction (Table 23) :—

TABLE 23

(*After R. J. Anderson*)

	Bacillus		
	Human Tubercle	Bovine Tubercle	Timothy Grass
Butyric acid, $C_4H_8O_2$ -	Trace	Trace	Trace
Palmitic acid, $C_{16}H_{32}O_2$	Large amount	Large amount	Large amount
Stearic acid, $C_{18}H_{36}O_2$	Small amount	None	None
Cerotic acid, $C_{26}H_{52}O_2$	Trace	Small amount	None
Linoleic acid, $C_{18}H_{32}O_2$	Small amount	Small amount	Small amount
Linolenic acid, $C_{18}H_{30}O_2$	Small amount	Small amount	Small amount
Tuberculostearic acid, $C_{19}H_{38}O_2$	Large amount	Large amount	Large amount
Phthioic acid, $C_{26}H_{52}O_2$	Large amount	Large amount	Large amount

Tuberculostearic acid is a liquid, saturated, fatty acid, very probably 10-methylstearic acid, $CH_3(CH_2)_7\overset{\displaystyle CH_3}{\overset{|}{C}}H.(CH_2)_8COOH$. It is optically inactive and has m.p. -10–11°C. Phthioic acid is also a liquid saturated fatty acid with a branched chain having m.p. 20°C. and $[\alpha]_D^{20}+12{\cdot}6°$. Phthioic acid has been shown, by a study of its degradation, X-ray diffraction and the areas of monomolecular films, to have the constitution 3 : 13 : 19-trimethyltricosanoic acid:

$$CH_3.(CH_2)_3.\overset{\displaystyle CH_3}{\overset{|}{C}}H.(CH_2)_5.\overset{\displaystyle CH_3}{\overset{|}{C}}H.(CH_2)_9.\overset{\displaystyle CH_3}{\overset{|}{C}}H.CH_2.COOH.$$

The structure has been confirmed by synthesis. A series of related acids has also been synthesised and tested for physiological activity (see p. 379).

The fats of the acid-fast bacilli are peculiar in that the fatty acids are linked to the disaccharide trehalose, in the case of the human tubercle bacillus and *M. leprœ*, and to an unidentified substance in the case of bovine and avian tubercle bacilli and not to glycerol.

The fat of the diphtheria bacillus consists mainly of free fatty acids, of which the solid saturated fatty acid, constituting about one-third, is exclusively palmitic acid ; the chief liquid unsaturated fatty acid is Δ^9-hexadecenoic acid, $CH_3(CH_2)_5CH=CH(CH_2)_7COOH$; 1 per cent. of the fatty acids consists of an unsaturated acid, $C_{14}H_{26}O_2$; a higher unsaturated fatty acid, diphtheric acid, $C_{35}H_{68}O_2$, m.p. 35°, was also isolated.

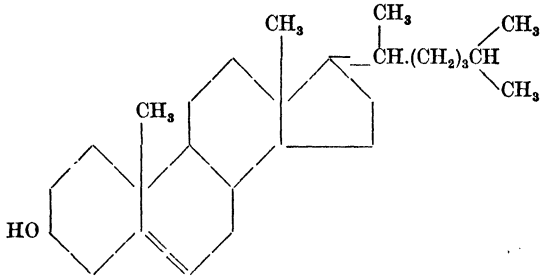
Lactobacillus acidophilus yields lauric, myristic, palmitic, stearic and oleic acids, together with a dihydroxystearic acid having an optical rotation $[\alpha]_D+7{\cdot}8°$.

In yeasts butyric, caproic, lauric, palmitic, stearic, oleic, linoleic, linolenic, arachidic, myristic and isocetinic acids have been found. The yeasts normally contain about 18 per cent. of fat, but *End. vernalis* and *Oöspora* (*Oidium*) *lactis* can produce up to 50 per cent., whilst *Torula lipofera* has been shown to give as much as 60 per cent. of its dry weight as fat. The yeast fats usually contain a high proportion of unsaturated fatty acids, oleic and linoleic acids, and are accordingly usually liquid, closely resembling olive oil.

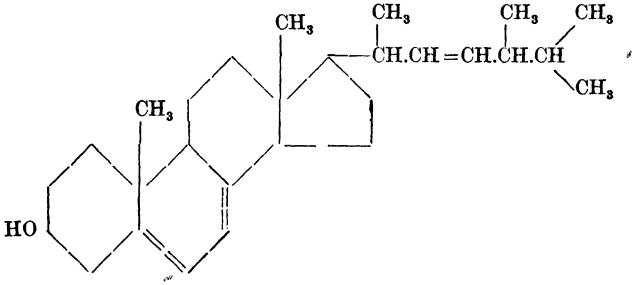
The mould fats contain palmitic, stearic, tetracosoic, $C_{24}H_{48}O_2$, oleic, linoleic and linolenic acids, in the proportion of approximately one-third saturated acids and two-thirds unsaturated acids.

Waxes, Sterols and Higher Alcohols.—The waxes are esters of fatty acids with higher monohydric alcohols instead of with glycerol as in the fats. The sterols are unsaturated alcohols having a condensed ring structure

of high molecular weight ; cholesterol has the structure :—



and ergosterol,



In view of their exceptionally high lipoid content the acid-fast bacteria have naturally been most closely examined in this respect. As early as 1898 a wax resembling beeswax had been isolated from the tubercle bacillus, and in 1904 a higher alcohol was isolated. In 1914 Tamura isolated an alcohol, $C_{29}H_{55}OH$, m.p. 66° C., which he named mykol. He showed that it stained Gram positive and had the property of acid-fastness ; he ascribed these properties of the tubercle bacillus to mykol. Other alcohols, including phytoglycol, $C_{26}H_{54}O_2$, have also been isolated from the tubercle bacillus, together with a wax shown to be an ester of mykol with lauric acid. R. J. Anderson and his co-workers have shown that most of the " waxes " of the acid-fast bacteria consist of

optically active hydroxy-acids esterified with carbohydrates together with smaller amounts of true waxes which are esters with higher secondary alcohols. Phthiocerol, a crystalline alcohol, m.p. 73°, $[\alpha]_D$–4·8° (in chloroform), $C_{35}H_{72}O_3$, containing two hydroxyl groups and one methoxy group, is found in all the wax fractions of human and bovine tubercle bacilli but not in those of other acid-fast bacteria. Avian tubercle bacilli, the Timothy grass bacillus and the leprosy bacillus contain the secondary alcohols *d*-2-eicosanol, $CH_3.(CH_2)_{17}.CHOH.CH_3$, m.p. 62°, $[\alpha]_D$+4·2°, and *d*-2-octadecanol, $CH_3.(CH_2)_{15}.CHOH.CH_3$, m.p. 56°, $[\alpha]_D$+5·7°. The wax of the human tubercle bacillus contains the specific polysaccharide, to which fatty acids are attached; avian tubercle bacilli, and the Timothy grass bacillus contain trehalose, whilst the leprosy bacillus gives only glycerol as the water soluble product of hydrolysis.

The properties of the various waxes are summarised in Table 24.

TABLE 24

WAX	PROPERTY	BACILLUS					
		Human Tubercle	Bovine Tubercle	Avian Tubercle		Timothy Grass	Leprosy
				(A) 54—55° C. +38·6°	(B) 53—55° C. +17·7°		
Purified Wax	m.p.	200—205° C.	47—54° C.	...	...	45° C.	50—51° C.
	$[\alpha]_D$	...	+15·5°	...	...	+15·1°	+ 4·0°
	P	0·41%	0·3%	...	...	0·29%	0
	N	0·77%	Trace	...	...	0·41%	0
	Products of hydrolysis	Specific polysaccharide	Carbohydrate 9%	Trehalose	12·3% 5·6%	Glycerol 3%	Glycerol 6%
		Mycolic acid 56%	Glycerol 1·3%	Opt. active hydroxy-acids of high mol. wt.	81·0% 84·8%	Carbohydrate 4·6% (cont. trehalose)	Fatty acids 93%
		Phthiocerol	Mycolic Acid 61·0%	Lower fatty acids	2·4% 6·8%	Fatty acids 68%	Unsaponified 7%
			Lower fatty acids 19·4%	*d*-2-Eicosanol, *d*-2-Octadecanol	9·9% 9·0%	Opt. act. hydroxy-acids, high mol. wt.	*d*-2-Eicosanol
			Palmitic acid			Dibasic acid, $C_{70}H_{138}O_4$, m.p. 56°	*d*-2-Octadecanol
			Branched chain tetracosanoic acid, m.p. 76—77°			*d*-2-Eicosanol	Myristic acid
			Branched chain stearic acid (opt. inact.) m.p. 29—30°			*d*-2-Octadecanol	Palmitic acid
			l-Rotatory acids, M. W. abt. 340				Stearic acid
			Phthiocerol 5·3%				Tricosanoic acid
							Tetracosanoic acid
							Leprosinic acid
Soft Wax from mother liquors from purified wax	Products of hydrolysis	Glycerol Mycolic acid Phthiocerol Lower fatty acids	...	...		...	...
Ether soluble wax	Products of hydrolysis	Glycerol Carbohydrate (mannose and inositol) *n*-Hexacosanoic acid Mycolic acid Phthiocerol Tuberculostearic acid Phthioic acid $C_{31}H_{62}O_2$ (*l*-rotatory)	...	...		...	...

Mycolic acid of human and bovine tubercle bacilli m.p. 54–56°, $[\alpha]_D + 1{\cdot}8°$, $C_{88}H_{176}O_4$, is a saturated acid containing one hydroxyl group, one methoxy group and one carboxyl group. On vacuum distillation at 280° C. it splits to give *n*-hexacosanoic acid, $C_{26}H_{52}O_2$, m.p. 87–88° and a colorless non-volatile residue. It is the acid-fast staining substance of both organisms. Avian tubercle bacilli give two mycolic acids α- and β-, both of which are acid fast. On pyrolysis at 210° α-mycolic acid, m.p. 69–70°C, $[\alpha]_D + 5{\cdot}6°$, mol. wt. 500, gives 25·4 per cent., of a branched chain crystalline pentacosanoic acid, m.p. 78–79°C. and β-mycolic acid, m.p. 60–61°C.c $[\alpha]_D + 5{\cdot}5°$, mol. wt. 1300, at 280° C., gives *n*-tetracosanoie acid, m.p. 83°C. Phleimycolic acid, from the wax of thd Timothy grass bacillus, is a mixture of a saturated aci_6, and an unsaturated, dibasic, hydroxy-acid, $C_{70}H_{138}Or$, which has m.p. 56–57° and $[\alpha]_D + 6{\cdot}1°$. Its methyl este on vacuum distillation, gives the volatile methyl ester o a branched chain tetracosanoic acid and a neutral non volatile residue. The optically active, dibasic, hydroxy- acid, leprosinic acid, from the leprosy bacillus has m.p 62–63°, $[\alpha]_D + 4°$ and has the formula $C_{88}H_{176}O_6$.

The specific polysaccharide of the human tubercle bacillus, which occurs in the purified wax and which gives precipitin reactions with homologous antiserum, contains nitrogen and phosphorus and, on acid hydrolysis, yields 2 per cent. of mannose, 36 per cent. of D-arabinose, 17·5 per cent. of galactose and traces of inositol and glucosamine. The carbohydrate from the bovine organisms, containing 2·2 per cent. of phosphorus and traces of nitrogen, gives mannose, inositol and inositol monophosphate on hydrolysis.

The acid-fast bacteria contain lipoids which can only be removed by extraction after treatment of the cells with 1 per cent. alcoholic hydrochloric acid. These

"firmly bound" lipoids can be precipitated from ether solution by alcohol or acetone. By filtration through a Chamberland candle the extracts from human and avian tubercle bacilli can be separated into filterable and unfilterable fractions. The extract from the leprosy bacillus is all filterable. Their composition is shown in Table 25:—

TABLE 25

(*After R. J. Anderson*)

	Unfilterable		Filterable		
	Human	Avian	Human	Avian	Leprosy
	Per cent.	Per cent.	Per cent.	Per cent.	Per cent.
Firmly bound lipoid -	4·7	2·68	7·5	8·16	19·5
Polysaccharide - -	50·5	31·3	25·5	15·0	40·5
Glycerol - - -	none	none	2·0	none	none
Hydroxy-acids - -	51·0	52·8	41·1	61·0	56·3
Lower fatty acids -	4·0	3·8	28·4	3·7	4·3
Neutral material -	none	10·6	0·8	8·2	5·5

The unfilterable fractions contain twice as much polysaccharide as the filterable fractions. The polysaccharides are similar to that of the human purified wax and give mannose, D-arabinose and galactose on hydrolysis (that from the leprosy bacillus contains no mannose and only 1 per cent. of galactose, being almost entirely composed of D-arabinose, with a small amount of an unidentified pentose). The polysaccharides all give about 50 per cent. of reducing sugar on hydrolysis. The non-reducing portion has not yet been identified. They give precipitin reactions with the homologous antisera.

The lower fatty acid fraction from the filterable fraction contained tuberculostearic acid but no phthioic acid. The hydroxy-acid from both human tubercle

fractions is mycolic acid; but that from the avian bacilli is different from the wax mycolic acids; it is called γ-mycolic acid and on vacuum distillation gave 18 per cent. of a branched chain tetracosanoic acid, $C_{24}H_{48}O_2$. The hydroxy-acid from the leprosy bacillus is leprosinic acid, which also gives a branched chain tetracosanoic acid on pyrolysis. The unsaponifiable material consists of *d*-2-eicosanol and *d*-2-octadecanol.

The chloroform soluble wax from the attenuated strain of tubercle bacillus, B.C.G. is a complex mixture giving a pentose polysaccharide,, palmitic and cerotic acids and an acid-fast wax, $C_{51}H_{102}.OH.COOH$, on hydrolysis. It has been claimed that the wax content of the ether extracts of the various acid-fast bacteria is characteristic of the different types; thus the lipoids of human and bovine tubercle bacillus contain 60 to 70 per cent. of wax, those of avian and cold-blooded tubercle bacilli, the leprosy bacillus and *M. phlei* contain 27 to 30 per cent., whilst the smegma bacillus and dung bacilli contain only 4 to 10 per cent. Waxes have also been obtained from the diphtheria bacillus, but have not been investigated chemically.

The evidence for the presence of sterols in bacteria is somewhat conflicting; it has been claimed that *Azotobacter chroococcum* contains ergosterol to the extent of 0·1 per cent. of the fat fraction, and that sterols occur in human and bovine tubercle bacilli, in the B.C.G. strain, in *M. phlei* and in *Esch. coli*. Cholesterol has been reported as occurring in *L. acidophilus*. The majority of reports, however, state that sterols are absent from bacteria. On the other hand, yeasts contain large amounts of ergosterol, up to 20 per cent. of the fat being quite common. Yeast is the chief commercial source of ergosterol, which is used in the manufacture of synthetic vitamin-D or calciferol. Ergosterol also appears to be

a common constituent of many moulds, having been found in *P. javanicum*, *P. puberulum*, *P. aurantio-brunneum*, *A. fischeri*, *A. oryzæ*, *A. sydowi*, *A. niger* and *Rhizopus japonicus* in amounts varying from 0·1 to 0·4 per cent. of the dry weight of the mycelium. It has been found as ergosteryl palmitate in *P. brevi-compactum*, *P. italicum* and *P. aurantio-griseum* in amounts between 0·02 and 0·5 per cent. of the dry mycelium.

The mycelium of *Aspergillus sydowi* contains fungus cerebrin, $\begin{matrix} C_{15}H_{31}.CHOH.CH.CHOH.CH_2.CH_2.OH \\ | \\ NH.CO.CHOH.C_{24}H_{49} \end{matrix}$, m.p. 143°C., $[\alpha]_D + 11{\cdot}9°$ (in pyridine), which is identical with that found in yeasts and in mushrooms.

Phosphatides.—The phosphatides yield fatty acids, glycerol or carbohydrates, phosphoric acid and choline or other nitrogenous bases on hydrolysis. According to the ratio of nitrogen to phosphorus in the molecule, they are classified as monoamino-monophosphatides (1 : 1), diamino-monophosphatides (2 : 1) and monoamino-diphosphatides (1 : 2). The phosphatides are soluble in ether and are precipitated from such solution by acetone, by which means they can be separated from the fats which are soluble in acetone. The commonest phosphatide is lecithin, having a nitrogen to phosphorus ratio of 1 : 1. It is very probably built up of glycerol esterified with one molecule each of stearic, oleic and phosphoric acids with a molecule of choline linked on to the phosphoric acid group, as represented in the formula :—

$$\begin{matrix} CH_2O.CO.C_{17}H_{35} \text{ (stearic acid)} \\ | \\ CHO.CO.C_{17}H_{33} \text{ (oleic acid)} \\ | \\ CH_2O.P.(OH).O.C_2H_4 \\ \quad \| \qquad\quad | \\ \quad O \qquad\quad N(CH_3)_3 \text{ (choline)} \\ \qquad\qquad\quad | \\ \qquad\qquad\quad OH \end{matrix}$$

The phosphatides are widely distributed in micro-organisms and are almost certainly present to more or less extent in all of them. The non-acid-fast organisms contain about 0·5 to 2 per cent., whilst the tubercle bacillus contains about 6·5 per cent. of phosphatide.

The phosphatides of the tubercle bacillus on hydrolysis yield palmitic and oleic acids and the two liquid saturated acids, tuberculostearic acid, $C_{19}H_{38}O_2$, and phthioic acid, $C_{26}H_{52}O_2$. The leprosy bacillus phosphatide contains an unsaturated C_{16} fatty acid. The B.C.G. strain yields also a phosphorylated polysaccharide, giving mannose on hydrolysis. The phosphatides of the acid-fast bacteria contain very little nitrogen (see Table 26) and no choline or aminoethanol. The nitrogen appears to be in the form of ammonia.

Phthioic acid appears to be responsible for the formation of the tubercles which are a characteristic of tuberculosis, since injection of the acid or of various fractions containing it gives rise to their production in experimental animals. Of a number of synthetic acids of similar constitution 3 : 12 : 15-trimethyldocosanoic acid and 4 : 13 : 16-trimethyltricosanoic acid were even more active than phthioic acid in producing tubercles. Tuberculostearic acid is irritant but does not cause tubercle formation.

The lipoids of the acid-fast bacteria contain, besides the phosphatides and waxes, substances akin to the cerebrosides which, on hydrolysis, yield glycerol, fatty acids and a carbohydrate. The carbohydrates have been identified as mannose, glucose and arabinose. The cyclic hexahydric alcohol, inositol, has also been found among the hydrolysis products of this fraction of the acid-fast bacilli. A comparison of the composition of the phosphatides of some acid-fast bacilli is given in Table 26 :—

TABLE 26

(*After R. J. Anderson*)

	Bacillus				
	Human	Avian	Bovine	Timothy Grass	Leprosy
Melting point	210° C.	210° C.	208° C.	190° C.	231° C.
	Per cent.	Per cent.	Per cent.	Per cent.	Per cent.
Phosphorus	2·30	2·18	1·87	2·80	1·75
Nitrogen	0·36	0·48	1·00	0·22	trace
Total ether-soluble fraction	66 to 67	55 to 56	57 to 58	60·0	62·2
Palmitic acid	30·5	18·4	27·0	20·0	18·6
Oleic acid	12·8	18·4	7·0	5·6	13·8
Liquid saturated fatty acids (tuberculostearic and phthioic)	20·9	14·1	16·0	18·0	13·5
Total fatty acids recovered	64·2	53·7	50·0	43·6	45·9
Water-soluble constituents	33 to 34	46 to 47	43 to 44	40·0	38·0
Mannose	9·2	13·3	6·7	9·5	5·2
Inositol	8·9	3·0	3·5	2·2	0·6
Other sugars	12·3	—	—	—	20·6
Glycerophosphoric acid	5·4	6·0	9·9	10·0	—

The distribution of the various lipoid fractions of the acid-fast bacteria is summarised in Table 27.

TABLE 27

(*After R. J. Anderson*)

	Bacillus			
	Human	Bovine	Avian	Timothy Grass
	Per cent.	Per cent.	Per cent.	Per cent.
Phosphatide	6·54	1·53	2·26	0·59
Acetone-soluble fat	6·20	3·34	2·19	2·75
Chloroform-soluble wax	11·03	8·52	10·79	4·98
Total lipoids	23·78	13·40	15·26	8·37
Polysaccharides	0·87	1·09	1·02	3·90
Bacterial residue	75·01	85·50	83·71	87·70

The phosphatides of the diphtheria bacillus, having the nitrogen-phosphorus ratio of a monoamino-monophosphatide, on hydrolysis yield aldohexoses, fatty acids, a compound with a high molecular weight and traces of a base. The solid saturated fatty acid was exclusively palmitic acid, and the liquid acids contained a substance, corynin, $C_{50}H_{100}O_4$, m.p. 70° to 71°, containing one carboxyl group and two hydroxyl groups.

Phytomonas tumefaciens contains about 2 per cent. of total lipoids when grown on a synthetic, glycerol-containing medium and about 6 per cent. when the glycerol is replaced by sucrose. About 70 per cent. of the acetone soluble fat consists of free fatty acids, mainly unsaturated. The phosphatides are lecithin and cephalin in approximately equal quantities. The fatty acids comprise normal saturated and unsaturated C_{16} and C_{18} acids as well as liquid saturated branched chain acids of high molecular weight, similar to those in the tubercle bacilli. Among the liquid, saturated fatty acids is crystalline phytomonic acid, $C_{20}H_{40}O_2$, m.p. 24° C. It is optically inactive and is probably a homologue of tuberculostearic acid. It constitutes about 14 per cent. of the total fatty acids. No chloroform soluble wax and no sterols could be isolated.

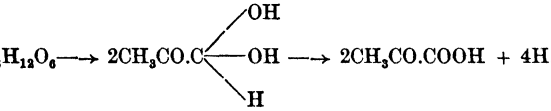
The phosphatide of *Lactobacillus acidophilus* yields glycerophosphoric acid, choline, palmitic, stearic and unsaturated fatty acids, together with a crystalline non-reducing polysaccharide which gives glucose, galactose and fructose on further hydrolysis.

The yeasts also contain a high proportion of phosphatides, but their constitution has not been worked out.

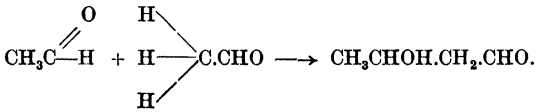
Fat Synthesis.—The mechanism of fat synthesis by micro-organisms has been most extensively studied in the case of the yeast, *Endomyces vernalis*, which was used as a source of fat in Germany during the 1914-1918 war.

Haehn and Kinntof, following a suggestion by Magnus Levy that acetaldehyde condensed to give aldol as an

intermediate, proposed the following scheme to account for the production of fats from sugar. Glucose breaks down, probably by the same mechanism as in alcoholic fermentation, to give methylglyoxal hydrate, which gives pyruvic acid and hydrogen :—



In view of more recent knowledge of alcoholic fermentation methylglyoxal hydrate probably should no longer be considered as an intermediate, the pyruvic acid being formed in accordance with the Meyerhof-Embden scheme of alcoholic fermentation. The pyruvic acid is decarboxylated to give acetaldehyde and carbon dioxide. Two molecules of acetaldehyde condense to give aldol :—



The latter loses water with formation of the unsaturated aldehyde, crotonaldehyde :—

$$CH_3CHOH.CH_2.CHO \longrightarrow CH_3CH{=}CH.CHO + H_2O.$$

The crotonaldehyde is reduced by hydrogen formed during the production of pyruvic acid to give butyraldehyde, $CH_3CH_2.CH_2.CHO$. The butyraldehyde condenses with another molecule of acetaldehyde to produce a homologue of aldol, β-hydroxycaproic aldehyde :—

$$CH_3CH_2.CH_2.CHO + CH_3.CHO \longrightarrow CH_3CH_2.CH_2.CHOH.CH_2.CHO.$$

Loss of water gives rise to $\alpha\beta$-hexylene aldehyde, $CH_3CH_2.CH_2.CH{=}CH.CHO$, which is reduced to caproic aldehyde, $CH_3(CH_2)_4CHO$. This process of condensation with acetaldehyde, dehydration and reduction continues until a chain of carbon atoms corresponding to oleic

or stearic acids is built up, when oxidation of the aldehyde group to a carboxyl group gives stearic acid, $CH_3(CH_2)_{16}COOH$, for instance. The fatty acids so produced esterify with glycerol, which is formed in the early stages of the process, as in alcoholic fermentation (see p. 277).

Very similar is the suggestion of Smedley and Lubrzynska that acetaldehyde and pyruvic acid undergo aldol condensation to give α-keto-γ-hydroxy-valeric acid :—

$$CH_3CHO + CH_3CO.COOH \longrightarrow CH_3.CHOH.CH_2.CO.COOH$$

On decarboxylation, followed by internal oxidation-reduction, butyric acid is formed :—

$$CH_3.CHOH.CH_2.CO.COOH \longrightarrow CH_3.CH_2.CH_2.COOH + CO_2$$

α-Keto-γ-hydroxyvaleric acid could also lose water to give the unsaturated acid, which on decarboxylation would give rise to crotonaldehyde :—

$$CH_3.CHOH.CH_2.CO.COOH \longrightarrow CH_3.CH=CH.CO.COOH$$
$$CH_3.CH=CH.CO.COOH \longrightarrow CH_3.CH=CH.CHO + CO_2$$

Crotonaldehyde then condenses with another molecule of pyruvic acid to give an aldol acid which in turn would give rise to caproic acid and also to the homologue of crotonaldehyde containing two extra carbon atoms :—

$$CH_3.CH=CH.CHO + CH_3.CO.COOH \longrightarrow CH_3.CH=CH.CH.OH.CH_2.CO.COOH$$
$$CH_3.CH=CH.CHOH.CH_2.CO.COOH \longrightarrow CH_3.CH=CH.CH_2.CH_2.COOH + CO_2$$
$$CH_3.CH=CH.CH_2.CH_2.COOH + 2H \longrightarrow CH_3.CH_2.CH_2.CH_2.CH_2.COOH$$
$$CH_3.CH=CH.CH=CH.CO.COOH \longrightarrow CH_3.CH=CH.CH=CH.CHO + CO_2$$

Continuation of such steps would give rise to the fatty acids starting with butyric acid and increasing in chain length two carbon atoms at a time. α-Keto-γ-hydroxy-valeric acid and α-keto-γ-hydroxy-valeraldehyde have

been found among the products of the action of enzymes from *Staph. albus* on glucose. That one of these views of the production of fats is probably correct is supported by the fact that fixation of acetaldehyde with sulphite or dimedon causes a lowering of the fat yield, and also that the fatty acids found in bacteria and yeasts all contain chains with an even number of carbon atoms (corresponding to the building up of the chain by addition of the two carbon atoms of acetaldehyde at a time); moreover, nearly all the shorter fatty acids from butyric up to arachidic acid are known to occur in micro-organisms. It is interesting, and perhaps significant, to note that this synthesis passes through the β-hydroxy-aldehydes, whilst the degradation of fats in the animal body proceeds through the β-hydroxy-acids, carbon atoms being split off two at a time.

The formation of fats from such substrates as alcohol probably also proceeds through acetaldehyde as intermediate, the aldehyde being produced by oxidation, or *via* a reserve carbohydrate.

For further reading :—

R. J. Anderson :

(*a*) " The Separation of Lipoid Fractions from Tubercle Bacilli." *J. Biol. Chem.*, **74** (1927), 525.

(*b*) " The Phosphatide Fraction of Tubercle Bacilli." *J. Biol. Chem.*, **74** (1927), 537

(*c*) " The Chemistry of the Lipoids of the Tubercle Bacilli." *Physiol. Reviews*, **12** (1932), 166.

(*d*) " Structural Peculiarities of Acid Fast Bacterial Lipides." *Chem. Rev.*, **29** (1941), 225.

R. E. Buchanan and E. I. Fulmer, " The Physiology and Biochemistry of Bacteria," Vol. I, Chapter III. Baillière, Tindall & Cox. London, 1928.

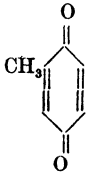
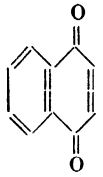
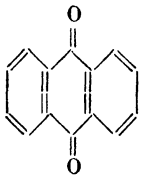
CHAPTER XXI

THE PIGMENTS OF MICRO-ORGANISMS

OUR present knowledge of the pigments of micro-organisms is in an unsatisfactory state. The constitution of comparatively few of them is known, and their classification is an arbitrary one depending on solubility relationships. However, there are three main chemical types into which they fall, namely :—

(*a*) **Carotenoid Pigments.**—These are red, orange or yellow pigments soluble in the fat solvents, ether, alcohol and chloroform. They are named after the type pigment carotene, an unsaturated hydrocarbon, $C_{56}H_{40}$, present as the red colouring matter of carrots. Hydroxyl derivatives of carotene, the xanthophylls, also belong to this group. Usually they occur together as more or less complex mixtures, which until recently were almost impossible to separate. Nowadays they are separated by means of chromatographic analysis, which depends on differential adsorption of the pigments on an appropriate adsorbent, such as calcium phosphate, alumina, kaolin and others, a principle originally developed by Tswett. A solution of the pigment in a suitable solvent is poured through a column of the adsorbant and the chromatogram "developed" by washing with the same or different solvents. The pigments separate into coloured bands at various depths in the column, which is then sliced between the bands and the separated pigments eluted. The carotenoids are usually characterised by the bands in their light absorption spectra. Many carotenoid pigments are readily bleached on exposure to atmospheric oxidation.

Most of them give the lipocyan reaction, an intense blue colour with concentrated sulphuric acid.

(*b*) **Quinone Pigments.**—Substituted toluquinones,  , naphthoquinones  and anthraquinones  are found quite frequently as pigments in bacteria and the lower fungi.

(*c*) **Melanins.**—The melanins are black or brown pigments which are very insoluble in nearly all solvents, even hot concentrated hydrochloric acid. They are soluble in warm concentrated sulphuric acid and are reprecipitated on dilution of the solution with water. They are formed as a result of the decomposition of proteins, either by boiling concentrated acids or by the action of the enzyme tyrosinase on the amino-acid tyrosine. Tryptophane and hydroxy-phenylethylamine are also sources of melanin pigments. They occur in the black *Torula* yeasts, in the *Dematiaceæ*, fungi with dark brown or black hyphæ, and in the black varieties of certain bacilli such as *B. mesentericus niger*.

The following is a classification of the pigments based on solubility characteristics :—

A. Cellular Pigments not colouring the medium.

I. Soluble in chloroform.

(*a*) Soluble in alcohol—

(1) Carotenoid—

(*α*) Red, e.g. pigments of *Rhodococcus*, sulphur bacteria, *Actinomyces*, *Torula*.

(*β*) Orange, e.g. pigments of *Staph. aureus*, *Sarcina aurantiaca*.

(*γ*) Yellow, e.g. pigments of *Staph. aureus*, *Staph. citreus*, *Sar. lutea*.

(2) Non-carotenoid—

(*a*) Colour change with acids and alkalies, e.g. prodigiosin, red in acid, yellow in alkali; bacterio-chlorin, the green pigment of sulphur bacteria; the red and yellow pigments of *Fusarium*, *Aspergillus*, *Penicillium*.

(*β*) No colour change with acids or alkalies—

(i) Fluorescent, e.g. pigments of some species of *Aspergillus*.

(ii) Non-fluorescent, e.g. *Flavobacterium brunneum*.

II. Insoluble in chloroform—

(*a*) Soluble in water, e.g. the anthocyanins of some species of *Fusarium*.

(*b*) Insoluble in water—

(1) Soluble in alcohol, e.g. violacein from *Chr. violaceum*; violet and purple pigments of similar species.

(2) Insoluble in alcohol—

(*a*) Soluble in alkali, e.g. the yellow pigments of some *Micrococci*; aspergillin from the spores of *A. niger*.

(β) Insoluble in alkali, e.g. the black pigment of *B. mesentericus niger*.

B. Extra-cellular Pigments colouring the medium.

I. Soluble in water—

(*a*) Soluble in chloroform, e.g. pyocyanin, the blue pigment of *Ps. æruginosa*.

(*b*) Insoluble in chloroform—

(1) Colour change with acid and alkali, e.g. the green fluorescent pigment of *Ps. æruginosa* (colourless in acid), red pigment of *Sacch. pulcherrimus* (colourless in alkali); methoxydihydroxytoluquinone from *P. spinulosum* (blue in alkali, purple when neutral, yellow in acid).

(2) No colour change with acid or alkali, e.g. the red pigment of some species of *Actinomyces*.

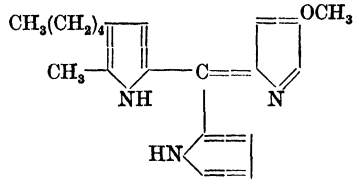
II. Insoluble in water—

(*a*) Soluble in other solvents, e.g. brown pigment of *Flavobacterium suaveolens*.

(*b*) Insoluble in other solvents, e.g. the black and brown pigments of *Actinomyces*, *Azotobacter chroococcum* ; the melanin from *Ps. œruginosa*.

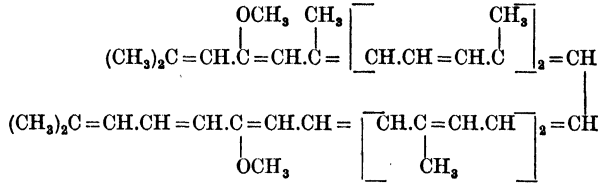
Certain organisms, notably of the genera *Pseudomonas* and *Acetobacter*, are capable of oxidising tyrosine, quinic acid and similar substances in the medium with formation of black or brown pigments.

The constitution of a few of these pigments has been established completely, and fragmentary knowledge is available about some others. Prodigiosin, $C_{20}H_{25}ON_3$, the red pigment of *Serratia marcescens* (*B. prodigiosus*), has been shown to have the structure :—



Violacein, $C_{42}H_{35}O_5N_5$ or $C_{50}H_{42}O_8N_6$, the violet pigment of *Chr. violaceum*, contains one or more pyrrole nuclei with hydrocarbon side chains, being similar in constitution to prodigiosin.

The purple bacteria of the genus *Rhodovibrio* contain complex mixtures of carotenoid pigments, including rhodopin, containing one hydroxyl group and two double bonds ; rhodovibrin, a polyene alcohol ; rhodoviolascene, $C_{42}H_{60}O_2$, containing two methoxyl groups and thirteen double bonds, probably having the structure :—



rhodopurpurin; flavorhodin, a hydrocarbon; and β-carotene.

Sarcina lutea produces bacterioxanthophyll; a crystalline xanthophyll pigment, sarcina-xanthine, m.p. 149° C., with absorption maxima at 480, 451 and 423 mμ in chloroform, has been isolated from *S. lutea*; *S. aurantiaca* gives β-carotene and zeaxanthin; *Staph. aureus* gives zeaxanthin as the only pigment; *M. phlei* gives lutein (a xanthophyll ester), kryptoxanthin and α-, β- and γ-carotenes; *M. leprœ* gives a pigment, leprotin, which is very like β-carotene; some strains of *Mycobacterium* have been shown to produce four carotenoid pigments when grown on media containing mineral oil as the sole carbon source. Two of the pigments had vitamin A activity and one was shown to be astacin; *Spirillum rubrum* gives the purple pigment, spirilloxanthin, $C_{48}H_{66}O_3$, containing one hydroxyl group and fifteen double bonds, and also other carotenoid pigments. *Bacterium cocovenenans* gives a yellow pigment, toxoflavin, $C_6H_6O_4N_2$, which is isomeric with methylxanthine. Anaerobic bacteria apparently do not produce carotenoid pigments.

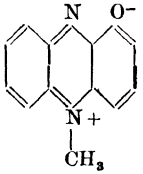
The purple sulphur bacteria give bacteriopurpurin, which is a mixture of the red pigment, bacterioerythrin, and the green pigment bacteriochlorin or bacteriochlorophyll, $C_{55}H_{72}O_6N_4Mg.H_2O$, which is very similar to plant chlorophyll; on removal of the magnesium it yields bacteriophæophytin. Bacteriochlorophyll is like chlorophyll-*a* but contains two more hydrogen atoms and has

an acetyl group instead of a vinyl group on one of the carbon atoms. Bacteriochlorophyll is probably carried on different proteins in the *Thiorhodaceæ*, *Athiorhodaceæ* and the green sulphur bacteria, since they give different absorption spectra. *C. diphtheriæ* produces porphyrins, possibly derived from cytochrome.

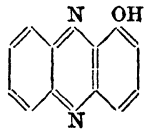
Azotobacter chroococcum and *Cl. welchii* produce black melanin pigments from tyrosine, whilst the latter also forms a pigment of the thio-amino type.

Actinomyces waksmanii is said to give an anthocyanin pigment, but the chemical properties of the pigment are not altogether those of an anthocyanin. *A. coelicolor* and *A. violaceus-ruber* give pigments which are blue in alkaline solution and red in acid solution, closely resembling azolitmin.

Several phenazine pigments are known. Pyocyanin, the blue, chloroform soluble pigment of *Pseudomonas æruginosa* (*B. pyocyaneus*) has the constitution

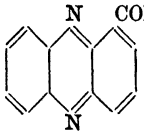


, or possibly a dimeric form of it. A second, yellow pigment, α-hydroxyphenazine,

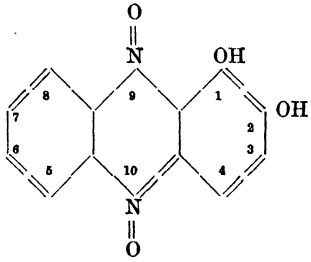


occurs in older cultures of *Ps. æruginosa*. Pyocyanin can act as a hydrogen carrier in the reduction of cytochrome, and can act as a hydrogen acceptor in the formation of phosphoglyceric acid from glucose by the action of the apozymase-cozymase system (see p. 202). Pyocyanin is bactericidal (see p. 177). *Pseudomonas chlororaphis* yields a green, crystalline pigment, chlororaphin, which, on exposure to air, changes to the crystalline, yellow pigment, oxychlororaphin, m.p. 241° C. The

latter is the amide of phenazine-1-carboxylic acid,

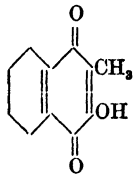


. On reduction with zinc dust in water it gives the orange-yellow, crystalline dihydrophenazine-1-carboxylamide, m.p. 192–4° C. Chlororaphin is composed of one molecule of oxychlororaphin and one molecule of dihydrophenazine-1-carboxylamide and can be produced synthetically by combining the components in acetic acid solution. *Chromobacterium iodinum* gives the pigment, iodinin, which is the N,N′ dioxide of a dihydroxyphenazine



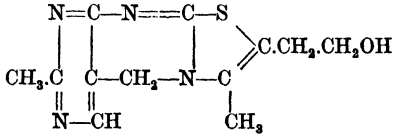
The positions of the two hydroxyl groups is not known but they are probably not at positions 2 : 3 or 2 : 5. Iodinin, like pyocyanin and chlororaphin, is inhibitory to bacteria, 2μg/ml. being sufficient to inhibit the growth of *Streptococcus pyogenes*. The effect can be reversed by the action of hydroxy-anthraquinones or by 2-methyl-1 : 4-naphthoquinone. It is possible that iodinin and the other phenazine derivatives interfere with the mechanism of hydrogen transfer which involves quinones, by reacting at the same enzyme centres, in virtue of their similarity in structure.

The human tubercle bacillus produces a yellow crystalline pigment, phthiocol, shown to have the constitution, 3-hydroxy-2-methyl-1:4-naphthoquinone,



. In alkaline solution it can undergo a two-stage oxidation-reduction reaction and may be concerned in the metabolism of the organism. It has some vitamin K (antihæmorrhagic) activity and raises the prothrombin content of the blood, on injection.

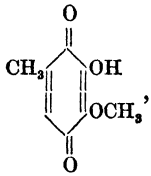
The red yeasts, such as *Torula rubra*, produce carotenoid pigments, including β-carotene, torulene, and a polycarboxylic acid pigment. The blue fluorescent pigment thiochrome, $C_{12}H_{14}ON_4S$,



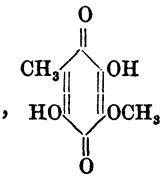
is derived from aneurin, or vitamin-B_1, which occurs in considerable amounts in yeasts.

The pigment aspergillin, from the black spores of *A. niger*, is a melanin type of pigment soluble in alkali.

Fumigatin, 3-hydroxy-4-methoxy-2:5-toluquinone,

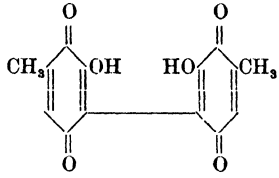


, maroon coloured crystals, m.p. 116° C., from *A. fumigatus* and spinulosin, 3 : 6-dihydroxy-4-methoxy-2 : 5-toluquinone,

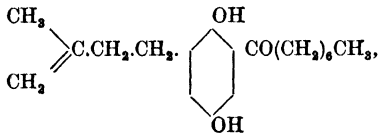


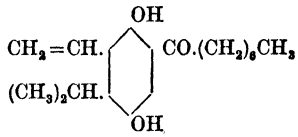
, purple plates, m.p.

201° C., from *P. spinulosum* and *A. fumigatus* have already been described as antibiotics (see pp. 163, 179). Phœnicin, 2 : 2′-dihydroxy-4 : 4′-di-methyldiquinone,

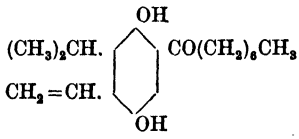


, yellow brown crystals, m.p. 230° C., produced by *P. phœniceum* and *P. rubrum* is a condensed toluquinone pigment. Under appropriate conditions of growth, fumigatin and phœnicin occur in the colourless reduced quinol form, in the culture media. As suggested on page 393 the quinones and quinols form an oxidation-reduction system and may serve as a hydrogen transfer mechanism in respiration. Flavoglaucin, $C_{19}H_{28}O_3$, lemon yellow needles, m.p. 105° C. and auroglaucin, $C_{19}H_{22}O_3$, orange crystals, m.p. 152° C., which occur in the mycelium of *A. glaucus* furnish examples of quinol pigments. Flavoglaucin has one of the structures :—





or



, and auroglaucin is the corresponding unsaturated analogue in which the side chain —CO.$(CH_2)_6CH_3$ is replaced by —CO$(CH{=}CH)_3$ CH_3.

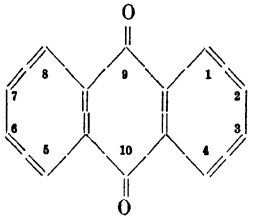
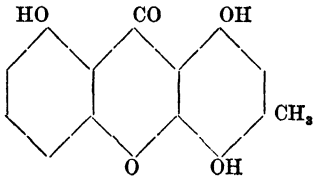
Pigments which are derivatives of 2-methyl anthraquinone,  , are responsible for the colours of many of the lower fungi, notably in the genus *Helminthosporium* and certain *Aspergillus* and *Penicillium* species. Some of them are listed in Table 28.

TABLE 28

PIGMENT	COLOUR	STRUCTURE	PRODUCED BY
Carviolacin	Light brown	Trihydroxy-methoxy-2-methyl-anthraquinone	*P. carmino-violaceum*
Carviolin	Chrome yellow	Trihydroxy-methoxy-2-methyl-anthraquinone	*P. carmino-violaceum*
Catenarin	Red	1 : 5 : 8-Trihydroxy-2-hydroxy-methyl-anthraquinone	*H. catenarium*, *H. gramineum*, *H. tritici-vulgaris*, *H. velutinum*
Cynodontin	Bronze	1 : 4 : 5 : 8-Tetrahydroxy-2-methyl-anthraquinone	*H. avenæ*, *H. cynodontis H. euchlænæ*
Emodic acid	Orange	4 : 5 : 7-Trihydroxy-anthraquinone-2-carboxylic acid	*P. citreo-roseum*, *P. cyclopium*
Erythroglaucin	Dark red	Trihydroxy-methoxy-methyl-anthraquinone	*A. glaucus* and related strains
Funiculosin	Deep red	Trihydroxy-methyl-anthraquinone	P. *funiculosum*
Helminthosporin	Maroon	4 : 5 : 8-Trihydroxy-2-methyl-anthraquinone	*H. catenarium*, *H. cynodontis*, *H. gramineum*, *H. tritici vulgaris*
ω-Hydroxy-emodin	Dull orange	4 : 5 : 7-Trihydroxy-2-hydroxy-methyl-anthraquinone	*P. citreo-roseum P. cyclopium*
Physcion, parietin, or emodin monomethyl ether	Reddish orange	4 : 5-Dihydroxy-7-methoxy-2-methyl-anthraquinone	*A. glaucus* and related strains and from lichens
Tritisporin	Red-brown	1 : 3 : 5 : 8-Tetrahydroxy-6 (or 7)-hydroxymethyl-anthraquinone	*H. tritici-vulgaris*

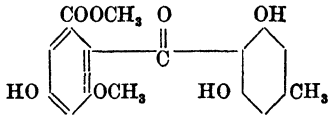
It is possible that the anthraquinones, like the toluquinones, may play the part of hydrogen carriers in mould respiration. Some species related to *A. glaucus*

were shown to contain the reduction products, 4 : 5-dihydroxy-7-methoxy-2-methyl-9-anthranol and the corresponding 10-anthranol in addition to the oxidised form, physcion. Instead of the usual polyhydroxy-anthraquinones, *H. leersii* gave two reduced compounds, luteoleersin, $C_{26}H_{38}O_7$, yellow rods, and colourless, alboleersin, $C_{26}H_{40}O_7$, which are regarded as the semi-quinone and quinol respectively and which can easily be converted into one another by oxidation-reduction processes.

Fairly closely related to the anthraquinone pigments are the yellow pigment ravenelin, 1 : 4 : 8-trihydroxy-3-methylxanthone, 

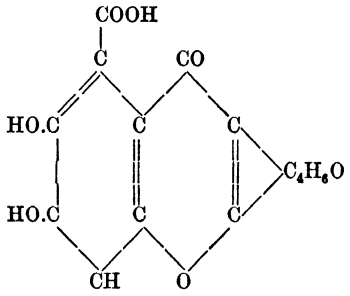
, which is produced by *H. ravenelii* and *H. turcicum*, and rubrofusarin, $C_{15}H_{12}O_5$, red crystals, which is a dihydroxy-methoxy-methylxanthone produced by *Fusarium culmorum*.

The yellow crystalline pigment of the mycelium of *Oospora sulphurea-ochracea*, sulochrin,

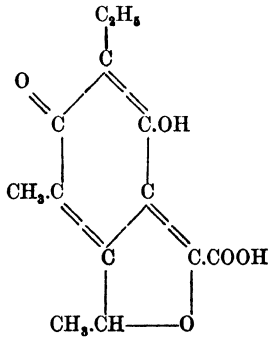


is a benzophenone which is related to ravenelin.

A number of other mould pigments are known but their constitution is unknown for the most part. The structure of citromycetin, the yellow pigment from *P citromyces-glabrum*, is partially known :—



whilst citrinin, a yellow pigment produced by *P. citrinum* and *A. terreus*, is :—



For further reading :—

R. E. Buchanan and E. I. Fulmer, "The Physiology and Biochemistry of Bacteria," Vol. I, Chapter III. Baillière, Tindall & Cox. London, 1928.

H. Raistrick and collaborators, Papers in *Biochemical Journal*, 1931 onwards.

CHAPTER XXII

ANTIGENS, HAPTENS, ANTIBODIES AND COMPLEMENT

IN this chapter it is proposed to describe some of the substances which enter into the reactions involved in immunological phenomena.

Antigens

An antigen is a substance which, when introduced parenterally into the animal body, calls forth the production of another substance, known as an antibody, capable of reacting specifically with the antigen. Antigens always react with their corresponding antibodies, but everything which reacts with an antibody is not necessarily an antigen. In order to be antigenic it appears that a substance must be (*a*) foreign to the animal into which it is injected, (*b*) colloidal and (*c*) introduced beyond the epithelial tissues of the animal. For our present purpose we can divide antigens into those which occur naturally and those which do not; the latter we may term artificial or synthetic antigens.

Natural Antigens.—The natural antigens fall into three classes, proteins, polysaccharides and lipoids.

Proteins.—The vast majority of antigens are proteins or contain a protein component. Almost all known proteins are antigenic provided that they are soluble. The notable exception is gelatin. It should be pointed out that gelatin, strictly speaking, is not a naturally occurring protein since it is produced by the hydrolysis

of collagen; it may therefore be degraded below the limits of colloidal dimensions necessary for antigenic power. It differs from the majority of proteins in not containing tyrosine or tryptophane among the amino-acids of which it is built up and in being devoid of carbohydrate, and it has been suggested that its lack of antigenic properties may be due to this deficiency. Insulin which is also non-antigenic lacks carbohydrate, but is rich in tyrosine.

If proteins are rendered insoluble, by heat denaturation or by treatment with alcohol, for instance, they are no longer antigenic. If the denaturation has not been carried too far and is reversible the regenerated undenatured protein regains its antigenic properties. Such proteins as casein which are not rendered insoluble by heating do not lose their antigenicity on such treatment.

The breakdown of a protein with loss of its colloidal properties is accompanied by a loss of antigenic properties. Thus a mixture of protein constituents obtained by hydrolysis is not antigenic. If, however, the fragments are re-united by enzyme action to form the colloidal plasteins, these are antigenic although they may have a specificity different from that of the original protein; the plasteins obtained by recombination of the amino-acids of different proteins usually give cross reactions, that is, they have a certain degree of common specificity.

Proteins from different sources differ from one another in the proportions and internal arrangement of their constituent amino-acids. Even such closely related proteins as the albumins of hens' and ducks' eggs can be distinguished by using anaphylactic shock in a sensitised animal as an indicator, although precipitin reactions are not sufficiently sensitive. It has been shown that these two albumins possess different amino-acids in the terminal positions of their molecules although their gross structure is the same. The fibrinogens and hæmoglobins of different species can be similarly distinguished. As a rule there is

a certain amount of cross-reaction between such heterologous antigens and antisera (that is, between the hæmoglobin, say, of one animal and an antiserum prepared against the hæmoglobin of another animal), especially if the antiserum is employed in high concentration and if the two species are closely related. However, homologous pairs of antigen and antiserum always react to a considerably higher titre than do heterologous pairs.

The non-structural proteins like globulin or albumin which circulate in the body are usually highly species specific. Structural proteins or depot proteins such as keratin, eye-lens protein, casein and the proteins of seeds are less specific and give wider cross reactions. The highly specialised proteins, for example insulin, which are common to many species are not only not specific in their immunological reactions but are not even antigenic.

Proteins of different chemical types, even if they are obtained from the same species, give distinct and specific reactions and show no cross-reactions. Thus the serum proteins, globulin and albumin, of the rabbit differ from one another immunologically as well as chemically, and they also differ from hæmoglobin and the protein of the lens of the eye and other proteins.

In view of the fact that most of the chemical methods, such as halogenation, nitration or the introduction of azo-compounds, by which proteins can be altered to give different immunological reactions, involve changes in the amino-acids, tyrosine, tryptophane or phenylalanine, which contain a benzene ring, it has been considered that these constituents of the protein play a particularly important rôle in determining antigenic properties. The fact that the non-antigenic protein, gelatin, contains none of these amino-acids lends a certain amount of support to the view. These amino-acids alone, however, cannot account for all the activity, since the amino, hydroxyl and carboxyl groups of the aliphatic amino-acids can also

be altered in various ways with corresponding changes in specificity. Acetylation, which involves the hydroxyl groups of tyrosine as well as amino groups, has a greater determining influence on specificity than does treatment with formaldehyde which acts primarily on amino groups. Acetylation usually eliminates species specificity but the action of formaldehyde does not. It may be mentioned here that the chemical alteration of a protein from a given animal may change it sufficiently to cause it to react as a protein foreign to that animal; thus rabbit serum treated with formaldehyde is sufficiently different from the original serum to elicit the production of antibodies when it is injected into the rabbit which supplied it.

Polysaccharides.—The majority of polysaccharides, bacterial and otherwise, which have been examined are not antigenic although they are haptens, that is, they can react with antisera prepared against a complete antigen of which they formed a part. It was shown by Zozaya, that starch, dextran, glycogen and the polysaccharides of several bacteria, including *B. anthracis*, the dysentery bacilli, streptococcus and pneumococcus, became antigenic if they were adsorbed on to collodion or aluminium hydroxide as a colloidal carrier. Since that time several polysaccharides have been suspected of being in themselves antigenic, without requiring any colloidal carrier, in spite of being free from proteins. The first of these was the acetyl polysaccharide isolated from the Type I pneumococcus by Goebel. This substance differs from the originally isolated soluble specific substance in the possession of one acetyl group, which is apparently sufficient to convert the hapten into a complete antigen. Some doubt has been cast on this finding by the work of Felton who could find no correlation between the acetyl content of various samples and their antigenicity.

The next supposedly antigenic polysaccharide to be

isolated was the "complete antigen" which Boivin and his co-workers isolated from smooth strains of *Salmonella typhimurium* by extraction with dilute trichloracetic acid. It was found to be toxic, capable of provoking antibody production and of immunising mice against subsequent injection of many times the fatal dose of the toxin or the living organisms. By the action of hot, dilute acetic acid the antigenic character and toxicity were rapidly lost and a polysaccharide, the "residual antigen," was obtained which was a hapten only, the hydrolysis having split off fatty acids and a phosphatide. It has since been shown that a protein fraction is also removed by hydrolysis and that the antigenicity of the "complete antigen" depends on its presence (see p 361). Rough strains of *Sal. typhimurium* contain the "residual antigen" only; they possess an enzyme which hydrolyses the "complete antigen." The same antigen was discovered almost simultaneously by Raistrick and Topley, who isolated it by treating the bacteria with trypsin in order to digest the bacterial protein and then precipitating the polysaccharide from solution with alcohol.

More recently, in a similar way, Raistrick and Topley have isolated a toxic, completely antigenic polysaccharide from the typhoid bacillus. It confers protective immunity on mice when injected in extremely small doses and elicits antibody formation in rabbits. On hydrolysis by very weak acid this too loses acid groups and yields a neutral polysaccharide which is non-toxic and non-antigenic (see p. 363).

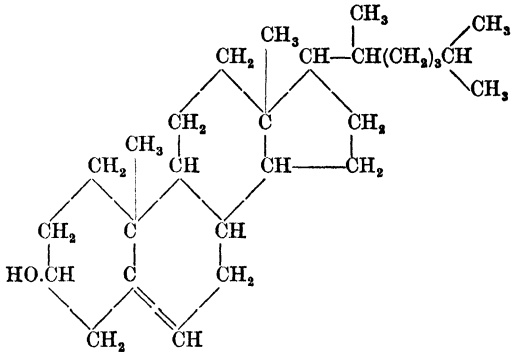
It is, therefore, becoming more certain that pure polysaccharides are not antigenic, but that when combined with a protein they act as the determinant groups of an antigenic complex.

Lipoids.—There is still considerable controversy as to the antigenic character of lipoids, although there is no doubt of the very important part which they play in

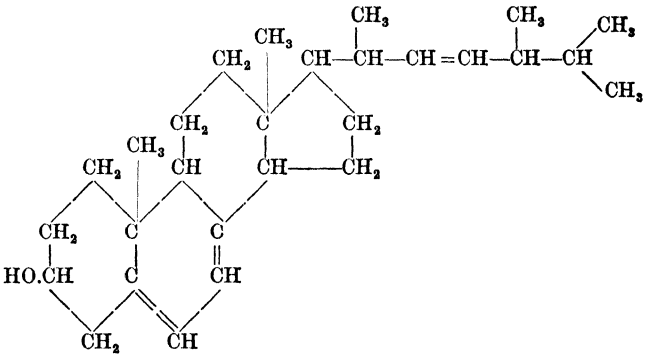
immunological reactions. Although they are not capable of such great variation in composition as the proteins and polysaccharides there is still a fairly wide range of possible lecithins and kephalins (containing different saturated and unsaturated acids) and sterols. It seems almost certainly established that they cannot act as antigens alone but that they may do so when mixed with serum ; that is, like most polysaccharides, they are haptens. Synthetic distearyl lecithin was shown to give complement fixation with an antiserum prepared against the compound mixed with pig serum ; cross-reactions with commercial lecithin preparations were also obtained. Synthetic and purified lecithins were found to be weaker antigens (when mixed with serum) than crude lecithins.

The Wassermann and flocculation reactions used in the diagnosis of syphilis employ lipoid " antigens " obtained by alcoholic extraction of heart muscle tissue. Their specific activity with syphilitic sera appears to depend on a substance, cardiolipin, isolated from the phosphatide fraction. It contains 4·11 per cent. of phosphorus but no nitrogen ; and is isolated as a sodium salt. On saponification it gives 62 per cent. of fatty acids, a non-reducing carbohydrate and phosphoric acid. Glycerol is absent, and the substance is analogous to the carbohydrate containing lipoids of the tubercle bacillus and *Lactobacillus acidophilus* (see p. 371). Lecithin and cholesterol are also necessary in the " antigen " for complement fixation to occur. Other lipoid fractions, fats and fatty acids, also afford reactions which, however, are not specific, occurring with normal as well as syphilitic sera. Bacterial lipoids, like those of plant and animal origin, behave in a similar way with sera, giving the same types of flocculation reaction.

The sterols, when mixed with pig serum, also seem to be antigenic and give antisera which show complement fixation but not flocculation reactions. Cholesterol,

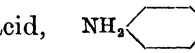


dihydrocholesterol, oxycholesterol (in which the double bond is saturated by hydrogen and oxygen respectively) and ergosterol,

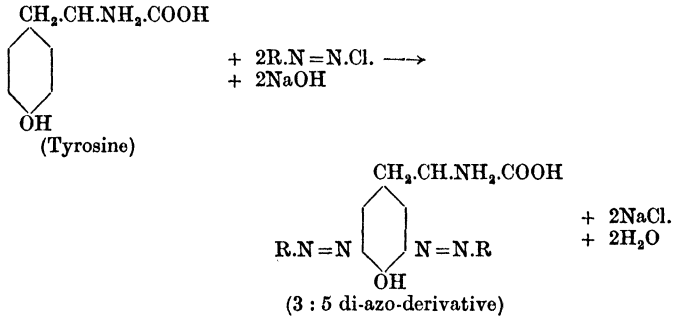


give cross-reactions, the reactions with homologous sera, however, being stronger than with heterologous sera. Cholestan (in which the hydroxyl group of cholesterol is replaced by a hydrogen atom), dibromocholesterol (in which the double bond is saturated by bromine) and cholesterol esters showed differences from one another, due to differences at the double bond or the hydroxyl group, but also gave cross-reactions due to the rest of the molecule.

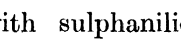
Artificial Antigens.—Our knowledge of artificial antigens has developed largely as a result of Landsteiner's investigations into the chemical basis of immunological specificity. He showed that if an antiserum is prepared by the injection of, say, horse serum into a rabbit that antiserum will react with horse serum but not with chicken serum or egg albumin. Similarly an antiserum to chicken serum will react only with the homologous serum and not with horse serum or egg albumin, which also gives a specific antiserum. Landsteiner made artificial antigens by introducing various known chemical groups into the proteins. For example, he diazotised the compound

atoxyl, *p*-aminophenyl-arsinic acid, 

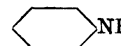
and coupled it with proteins, presumably through the tyrosine, histidine or tryptophane groups :—



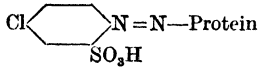
He used the resulting atoxyl-azo-proteins as antigens and found that any of the antigens reacted with any of the antisera irrespective of the protein (horse or chicken serum or egg albumin) which was present in the atoxyl derivative. In other words, the atoxyl group had abolished (or masked) the original specificity and conferred a new one determined by itself. If the proteins

were coupled with sulphanilic acid,  , instead of with atoxyl, again the original protein specificity disappeared, nor would sulphanilic acid-azo-proteins react with antisera to atoxyl-azo-proteins and *vice versa*.

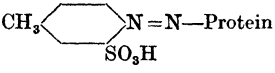
This specificity can be demonstrated in another way. The formation of the precipitate by the reaction of the antiserum with the atoxyl-azo-protein antigen, for instance, can be prevented by the previous addition of simple atoxyl derivatives to the antiserum. Atoxyl diazotised and coupled with tyrosine, or even atoxyl itself, can act in this way. These simple derivatives are not themselves antigenic nor do they give any visible reaction with the antiserum. They are named simple **haptens.** The inhibition of precipitin reactions by haptens is also specific ; atoxyl haptens inhibit the reactions of atoxyl antisera but not those of sulphanilic acid antisera, whilst the sulphanilic acid haptens inhibit only reactions between sulphanilic acid-azo-proteins and the corresponding antisera and not those between any other antigens and antibodies.

The groups, like atoxyl or sulphanilic acid, which modify the specificity of antigens in these ways are called determinant groups. Landsteiner studied a large number of aromatic amino-acids from this point of view and found that the specificity which they introduced depended partly on the substituent and partly on its position in the ring. Thus antisera to *o*-amino-benzene-sulphonic acid-azo-proteins gave reactions with both the *o*- and *m*-derivatives but not with the *p*-derivative, showing the effect of position. The same antisera also reacted with *o*-amino-benzoic acid,  , as hapten, or, when coupled with protein, as antigen, but they showed no reactions with the *m*- and *p*-amino-benzoic acids. This illustrates the combined effect of the substituent and its

position. The electric fields due to the polarity of the groupings (see p. 33) are sufficiently alike in the case of *o*-amino-benzoic acid and *o*-amino-benzene-sulphonic acid to allow of either of them reacting with the antiserum prepared against the other; but altering the position of the substituent to the *m*-position is sufficient to change the electric field so much that cross-reactions can no longer occur. The difference is, of course, even more enhanced when the *p*- and *o*-derivatives are compared. The addition of a second substituent is also sufficient to alter the charge distribution to a great enough extent to abolish the reactions; thus antisera to *o*-amino-benzene-sulphonic acid-azo-proteins will give no reaction with chloro-amino-benzene-sulphonic acid-azo-protein (1)

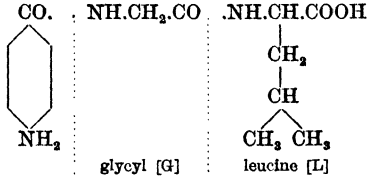


, nor with the corresponding methyl derivative (2),



, and *vice versa*. The fields of (1) and (2) are nearly enough alike, however, for antisera to (1) to react with (2), and *vice versa*.

Landsteiner also made some interesting experiments by coupling peptides to proteins. He diazotised *p*-amino-benzoyl-glycyl-leucine,



coupled it to proteins and made antisera in the usual way. He also prepared similar compounds with different arrangements of the amino-acids in the peptide, namely, LG, LL and GG, where L and G represent leucine and glycine respectively. The various combinations of

antigen and antiserum were then tested ; typical results are summarised in Table 29.

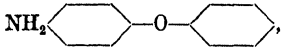
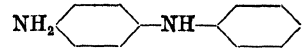
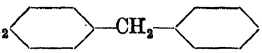
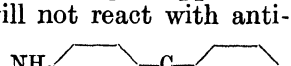
TABLE 29

Antigen	Antiserum	Hapten	Reaction
GLP	GLS	—	+ +
LLP	,,	—	+
GGP	,,	—	—
LGP	,,	—	—
GLP	,,	GL	—
,,	,,	LL	—
,,	,,	GG	+ +
,,	,,	LG	+ +

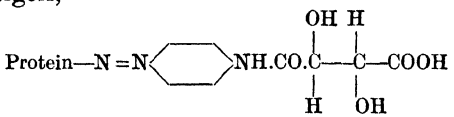
It was found that GL antiserum reacted strongly with GL antigen, less strongly with LL antigen and not at all with GG and LG antigen. The reaction between GL antiserum and GL antigen was inhibited by GL and LL as haptens but not by GG or LG haptens. Similar results were also found for the other antisera. It is seen that both the amino-acids in the peptide influence the specificity, but that the terminal amino-acid is the most powerful factor ; when the terminal amino-acid in the test antigen is different from that used in preparing the antiserum no reaction occurs, whilst a different amino-acid in the intermediate position merely weakens the reactions.

Landsteiner showed that the peptides having molecular weights between 600 and 1000, consisting of 8 to 12 amino-acid residues, obtained by hydrolysis of silk fibroin, were capable of inhibiting the reaction between silk fibroin and the antibody to it ; the inference is that the determinant groups in silk fibroin are not larger than these peptides. It is probable that the determinant groups of other proteins are of similar dimensions.

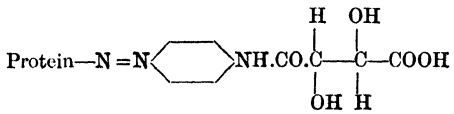
In cases where the determinant group has no very marked polarity the specificity is less sharply defined, and the actual nature of the particular groups involved has little

or no effect. Thus it has been shown that the determinant groups  ,  and  , linked in the usual way to proteins, are immunologically equivalent, that is, the antiserum to one will react equally well with any of them as antigen or hapten, the slight differences of field due to the comparatively inert groups —O—, —NH— and —CH_2— not being sufficient to influence the reactions. If, however, the strongly polar group,  , replaces the inert group, the equivalence is no longer apparent; antisera to the above antigens will not react with antigens or haptens containing  as the determinant group.

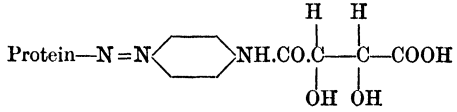
As would be expected from what was said about surface charges in Chapter III, spatial configuration plays an important part in the specificity induced by determinant groups. Landsteiner, for instance, coupled the amino-tartranilic acids, which exist in the optically active *d*- and *l*-forms and the optically inactive *meso*-form, with proteins and prepared the corresponding antisera. The antisera to the dextro-compound reacted strongly with the *d*-antigen,



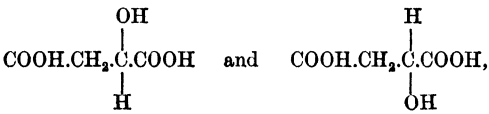
but only very slightly with the *l*-antigen,



whilst it gave weak reactions with the *meso*-antigen,

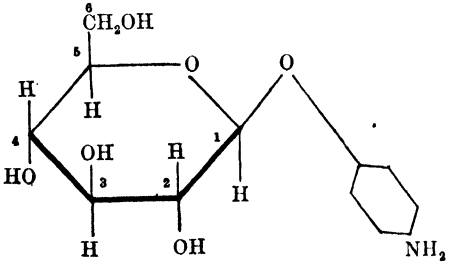


Similarly, the *l*-antiserum reacted strongly with the *l*-antigen, hardly at all with the *d*-antigen and weakly with the *meso*-antigen. The *meso*-antiserum reacted with the homologous antigen strongly and gave weak cross-reactions with the *d*- and *l*-antigens. The amino-tartranilic acids used as haptens gave the corresponding specific inhibition reactions. If, however, *d*- and *l*-malic acids,

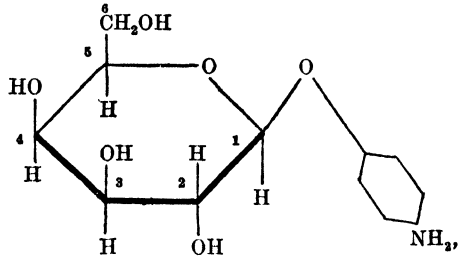


having only one optically active carbon atom instead of the two of the tartranilic acids, were used as haptens the inhibition reactions were much weaker, but took place with the antiserum to the corresponding isomer of tartranilic acid. Succinic acid, $COOH.CH_2.CH_2.COOH$, having no optically active carbon atom had no effect as a hapten.

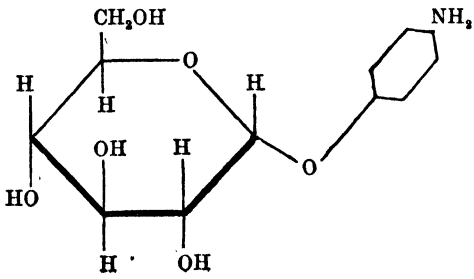
The effects of spatial distribution are also well illustrated in the case of the synthetic carbohydrate antigens studied by Avery and Goebel. They synthesised *p*-amino-phenol-*β*-glucoside,



and *p*-amino-phenol-β-galactoside,

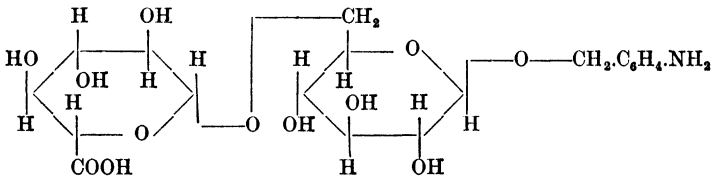


diazotised them and coupled them with albumin and globulin in the usual way to make antigens. It will be seen that the only difference between the glucose and galactose derivatives is in the arrangement of the hydrogen atom and hydroxyl group on carbon atom 4 of the sugar molecule. Yet this small difference is sufficient to make the new antigens specific and to obliterate the specificity of the proteins to which they are coupled ; thus the glucoside-globulin antigen gives no reaction with the galactoside-globulin antiserum but does react with the glucoside-albumin antiserum. The inhibition reactions by the homologous haptens were equally specific, the glucose-derivative hapten having no effect on the reactions between galactose-derivative antigens and antisera, and *vice versa*. When *p*-amino-phenol-α-glucoside,

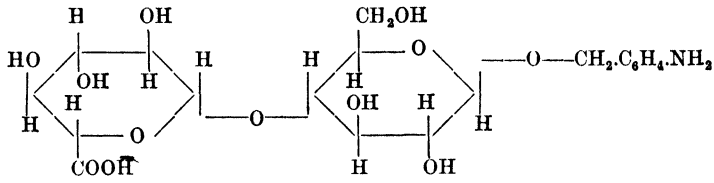


was diazotised and coupled with proteins, it also produced specific antigens in which the glucose molecule acted as the determinant group, but marked cross-reactions took place between the α- and β-glucoside antigen-antiserum pairs. It thus appears again that the terminal group, —CHOH, at position 4 in these cases, has a greater effect than groups situated within the molecule, such as the α- and β-glucoside links in these examples.

Artificial antigens containing glucuronic or galacturonic acids as determinants react with various anti-pneumococcal horse sera, but their injection into animals does not provoke antibodies which protect the animal against infection with pneumococci. If, however, an antigen made by coupling synthetic, diazotised *p*-amino-benzyl cellobiuronide (6-β-glucuronosidoglucose),



with horse serum globulin is injected, antibodies are formed which give precipitin reactions with the Type III polysaccharide, agglutinate Type III pneumococci and confer on mice passive immunity against virulent Types II, III and VIII pneumococci. The corresponding antigen containing gentiobiuronic acid (4-β-glucuronosido-glucose),

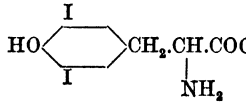
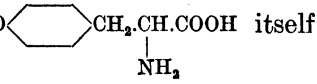
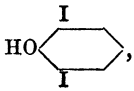


gives rise to antisera which have no protective effect in

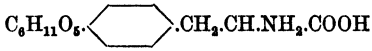
mice against Types III and VIII pneumococci, but which confer immunity against Type II organisms. Antigens containing cellobiose or gentiobiose in place of the aldobionic acids give antibodies which are devoid of protective effect against infection with pneumococci. Antigens made in the same way using the specific polysaccharides give antibodies with sharp type specificity whilst those containing aldobionic acids give antibodies with a wider specificity covering all types of pneumococci (for example Types II, III and VIII) which contain the same aldobionic acid in their specific polysaccharides (see p. 355).

Artificial antigens containing strychnine or various sulphonamides, have been prepared by coupling the diazotised substance with a protein. The resulting antibodies react specifically with the antigens. The antisera prepared against the strychnine antigen were too weak to protect mice against the toxic effects of strychnine. The specificity of the sulphonamide antigens is determined by the nature of the sulphonamide; thus sulphanilic acid, sulphanilamide and sulphacetamide azo-proteins give cross reactions with the antisera, whilst sulphathiazole and sulphapyridine are much more sharply specific, as would be expected from the difference in the structure of the substituents carried by the sulphonamide group.

All the artificial antigens which we have considered so far have been produced by coupling proteins with known simple chemical compounds by means of the diazo reaction. This coupling almost certainly occurs with those amino-acids in the protein, such as tyrosine, histidine and tryptophane, which contain aromatic groups. The aromatic nuclei can also be modified in other ways; for instance, by the introduction of halogens or nitro groups, with similar abolition of the original protein specificity and the formation of a new specificity depending

on the introduced determinant group. Thus iodo- and bromo-proteins were found to have lost their protein specificity and reacted only with antisera prepared against themselves, although there was strong cross-reaction between the iodo- and bromo-derivatives. The reactions between both iodo- and bromo-antigens and antisera were inhibited by di-iodotyrosine,  , which is apparently the corresponding hapten. Neither tyrosine,  itself, nor di-iodophenol,  , nor potassium iodide acted as haptens. Dibromotyrosine behaved as a hapten, but less strongly than the iodo-derivative.

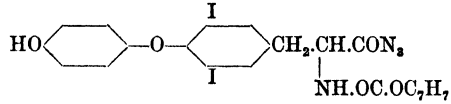
Harrington has made use of tyrosine for coupling various determinants to proteins. He prepared the 0-β-glucosidyl-tyrosyl-derivatives of gelatin and insulin by condensing glucosidyl-tyrosine,



with the free amino groups of lysine in the proteins. These normally non-antigenic proteins were thus converted into antigens which provoked specific antibodies when injected into rabbits, although rather poorly. This affords further evidence that gelatin and insulin are non-antigenic due to lack of tyrosine and carbohydrate and of carbohydrate respectively. Glucosidyl-tyrosine coupled to ordinarily antigenic proteins, such as globulin or albumin, gave very good antigens of a sharp specificity determined by the introduced groups.

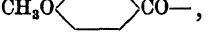
Harrington also made antigens containing thyroxine by

coupling the azide of N-carbobenzoxy-3 : 5-di-iodothyronine,



with proteins in alkaline solution and iodinating the product to convert the di-iodothyronine residues to thyroxine and to convert the tyrosine residues initially in the protein to di-iodotyrosine. Antisera prepared by the injection of these antigens were highly specific in their reactions and were able to prevent the metabolic activity of thyroglobulin or of thyroxine when these were injected into animals. Similar results were obtained by coupling aspirin to proteins ; passive immunisation of animals with antisera prepared against aspirin antigen prevented the ordinary pharmacological action of aspirin.

A method of affecting the specificity of proteins which does not necessarily affect the benzene nuclei is to substitute the hydroxyl and amino groups of the amino-acids by acyl groups. A certain amount of cross-reaction between different acyl proteins occurs ; for instance, acetyl-proteins react to some extent with antisera prepared against propionyl-proteins but not with those prepared against proteins containing longer chain substituents like butyryl, $CH_3(CH_2)_2CO$—, or valeryl, $CH_3(CH_2)_3CO$—. Proteins containing aromatic substituents like the anisoyl

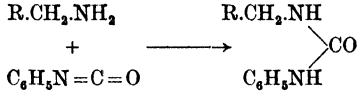




do not give cross-reactions with antisera to the aliphatic derivatives. Methyl or similar alkyl groups can be introduced into the hydroxyl or amino groups of the amino-acids with similar but weaker effects, a result which is to be expected in view of the less actively polar character of such groups. Methylated proteins react with the antisera to ethyl-proteins but not with those to untreated proteins

or to acyl- or nitro-proteins (which have strong polar groups).

The amino groups of proteins may be altered in other ways. Hopkins and Wormall treated proteins with phenyl isocyanate,  , which reacts with amino groups to give phenylureides or substituted ureas :—

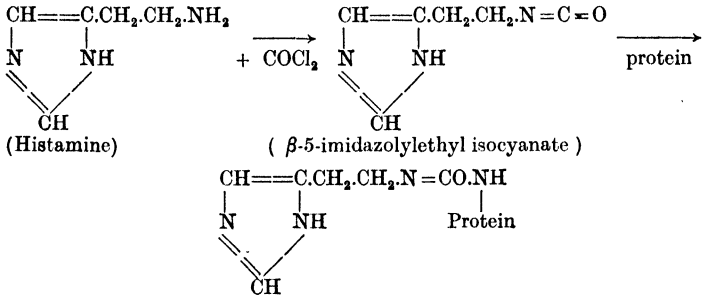


The protein-phenylureides were found to have lost the specificity of the original proteins and to have acquired a new one due to the phenylureide group. It was found that the precipitin reactions were inhibited by lysine phenylureide as hapten, indicating that the amino-acid lysine, $NH_2.CH_2.(CH_2)_3CH.COOH$, is involved in the

$$\begin{array}{c} \\ NH_2 \end{array}$$

formation of the protein phenylureide derivatives.

Isocyanate derivatives have been used for the introduction of other determinants into proteins. For instance the isocyanate derivative of histamine can be made by treating histamine with carbonyl chloride, and then coupled with proteins to give antigens :—



The coupling probably occurs through the free terminal

amino groups of lysine. The antiserum prepared against this antigen is specific for histamine and gives cross reactions with histamine-azoproteins. Guinea-pigs immunised by injection of the histamine antigen were protected against the physiological effects of histamine.

In contrast to the effect of isocyanates Landsteiner found that when the amino groups of proteins are condensed with formaldehyde with introduction of a methylene group,

$$R—NH_2 + HCHO \longrightarrow R—N=CH_2 + H_2O,$$

there is no apparent change in the specificity of the proteins, which behave immunologically exactly as the untreated proteins. This fact is made use of in preparing anti-toxins ; the toxin is converted to the toxoid by the action of formaldehyde. The toxoid, although non-toxic, is still capable of provoking the formation of antibodies which react specifically with the original toxin.

It was mentioned on p. 362 that the somatic antigens of *Shigella dysenteriæ* and *Eberthella typhosa* could be broken down by the action of 90 per cent. phenol into a polysaccharide hapten and an antigenic protein, and that the two components could be recombined by solution in formamide and precipitation of the complex by alcohol. The protein is also capable of being coupled with other polysaccharides in the same way to give antigenic complexes. Thus with agar, gum acacia, cherry gum, and the blood group A specific polysaccharide (isolated from commercial pepsin, peptone or gastric mucin) antigens are formed in which the specificity is determined by the polysaccharide moiety. Gum acacia gives no precipitin reaction with cherry gum antiserum, although cherry gum gives a weak reaction with gum acacia antiserum. Other polysaccharides such as kanten (a degradation product of agar), gum tragacanth, hyaluronic acid, the specific polysaccharides of Types I and II pneumococci

and the blood group A polysaccharide give no reaction with the two gum antisera.

Antibodies

Our knowledge of the chemistry of antibodies is at present rather vague. For the most part we only know antibodies by the reactions which they give, and we have but little insight into their chemical differentiation. As we have already stated, they are produced as a result of the injection of foreign colloidal substances into the animal system. It has been conjectured that they are not even new substances but merely an altered physical state of the normal serum proteins. Practically all the evidence, however, points to the fact that they are entities and capable of a separate existence ; they can be removed, either specifically by the corresponding antigen or by such non-specific agents as kaolin or alumina, from the antiserum and then recovered from the complex by suitable means.

Composition of Antibodies.—That antibodies are protein in nature is shown by the fact that the specific precipitates contain more protein than can be accounted for by the antigen. This is particularly striking when the antigen is a soluble specific polysaccharide, such as that of Type I pneumococci, which itself contains no protein. That the protein in these precipitates is not due to non-specific adsorption is shown by an experiment in which an antiserum was coupled with diazotised benzidine-R-salt to give a bright-red dye ; the dyed antiserum was used in specific and non-specific precipitin reactions, the red colour appearing only in those precipitates in which the antiserum was one of a homologous pair. Again, bacteria or red blood corpuscles sensitised with an antibody acquire a new isoelectric point and move in an electric field as though they were coated with globulin. Such systems, as well as toxin-antitoxin mixtures, containing, say, an antibody prepared in a horse, if injected

into a rabbit will produce antibodies corresponding to the protein (horse globulin) of the first antibody. Antibodies appear always to be associated with the globulin fraction of the serum proteins, but we do not know whether they are normal globulins modified in some way by the presence of the antigen or whether they are entirely new globulins.

The antibodies, generally speaking, are so closely related to the globulins chemically that it is almost impossible to distinguish between them except by serological reactions. They have the same distribution of amino-acids, the same nitrogen content and the same isoelectric point; it has been shown that the carbohydrate fractions of normal serum globulin, diphtheria antitoxin and the toxin-antitoxin floccules are identical, but different from that in albumin. Differences between the precipitability of ferric hydroxide sols by normal sera and antisera have been noted, but the results are not at all constant. The antibody globulin forms but a small portion of the total serum globulin, as the figures quoted on p. 427 show, so that it is not surprising that the antibodies cannot be distinguished chemically.

During immunisation the serum globulin content increases by about 5 per cent. and the albumin content falls slightly. The increase of globulin, however, bears no constant relationship to the antibody titre, and in any case only a very small proportion of it can be due to antibody globulin since precipitation results show, for example, that the unit of diphtheria antitoxin is associated with only 0·01 mg. of globulin.

The antibody globulins must, obviously, be different in some way from normal globulins, and it is generally thought that they carry active prosthetic groups which differ in stability from normal globulin. The group may survive treatment, considerably altering the globulin, such as the addition of iodo groups or acetylation, or it

may be eliminated by treatment not substantially affecting the globulin as a whole, depending on the point of attack.

Production of Antibodies.—We still do not know how antibodies are produced in response to the injection of antigens. It has been suggested that antibodies differ from normal serum globulins in that they incorporate the antigen in their structure and owe their specificity to that fact. This theory seems hardly tenable for several reasons ; an extremely small quantity of an antigen can induce the formation of almost limitless amounts of antibody. Animals repeatedly bled after injection of a single small dose of antigen go on manufacturing antibody in quantity which would exhaust the supply of antigen many times over, however little of it were incorporated. More positive evidence is afforded by experiments made with artificial antigens containing an easily detectable hapten group such as the atoyxl group or certain dye groups ; in no case has a trace of that group been detected in the antibodies resulting from the injection of such antigens. A suggestion, made by Breinl and Haurowitz and by Mudd, which seems more possible is that the antibodies are synthesised from amino-acids and peptides at the surface of the antigen which, due to its stereochemical spacing or distribution of surface charges, acts as a sort of template and impresses its specificity on the newly formed protein in that way. The antibody is supposed to differ from normal globulin in the order in which the amino-acids are incorporated into the protein chain, the new order being imposed by the presence of the antigen. The complex of antigen and antibody so formed is assumed to dissociate, the antibody being liberated into the blood-stream and the antigen being left free to influence the synthesis of further amounts of the antibody.

Work by Cannon and his collaborators has shown that the formation of antibodies is associated with the synthesis of globulins in the body, and depends on an adequate supply of amino-acids in the form of protein

or other dietary nitrogen compounds. It has also been shown by the use of amino-acids containing isotopic nitrogen as a " marker " or " label " that the proteins of the body, including globulin and albumin, are constantly being broken down and resynthesised and that the amino-acids themselves undergo similar changes. Pauling has suggested that antibody globulin molecules do not differ from normal globulin molecules in their chemical composition, and that they are synthesised in the normal way, but that they differ in the way that the peptide chains, particularly at the ends, are folded into a stable configuration. He regards the centre part of the chain of amino-acids to be held at the synthesising centres in the cell whilst the ends of the chain are more or less mobile, as pictured at (i) in Fig. 8. These free

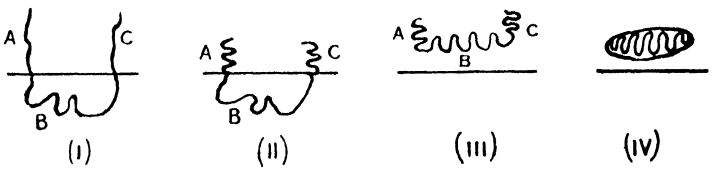


Fig. 8.—Synthesis of Normal Globulin.

ends fold into their natural stable configuration and remain so because held by hydrogen bonds and similar intramolecular weak bonds (at ii). In the course of time the molecule becomes liberated into the blood-stream (as at iii) where the whole of the peptide chain settles down to the stable configuration of normal globulin, represented at (iv). When globulin is synthesised in the presence of antigen, which is taken up by the cell at the site of synthesis, the polypeptide chain is built up as usual (i, Fig. 9), but the free ends now assume a configuration which is modified by the presence of the polar determinant groups on the antigen, which exert an attraction on groups in the polypeptide chain (see ii), and cause folding of the ends of the chain

in a pattern complementary to that of the particular part of the antigen in contact with them. The newly synthesised modified globulin chain becomes released from the cells as illustrated by (iii), and ultimately one end of the chain becomes dissociated from the antigen as at (iv), and the central part of the chain can then take up its normal globulin folding (v). In time the other end of the molecule is dissociated from the antigen and

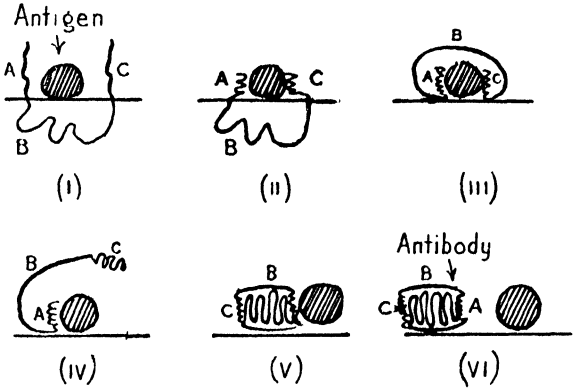


Fig. 9—Synthesis of Antibody Globulin.

the complete antibody globulin molecule is set free (vi) into the blood-stream, leaving the antigen molecule available to influence the folding of the ends of the peptide chains of freshly formed globulin molecules. The rate of antibody synthesis will depend on the strength of the attraction between the coiled ends of the globulin chain and the determinant groups on the antigen; the weaker the determinant groups the less time will occur before the attraction is overcome in the ordinary dynamic interchange which is constantly going on between molecules. Antibody molecules will continue to be produced until their concentration becomes so great that the antigen

molecules are almost constantly associated with already modified ends of antibody globulin chains and therefore not free to influence the production of further antibody molecules.

This theory accounts for the fact that more than one sort of antibody can be produced by a single antigen if that antigen carries more than one determinant group which can act as a template for the coiling of ends of the peptide chains. Thus it is known that the heat stable antigen and the heat labile antigen of the vaccinia virus are two parts of a single antigen molecule. That is, antigens may be monovalent or, more usually, since they are complex molecules, multivalent, in the sense of being able to combine with one or more antibodies. Thus if an antigen had two hapten groups, A and B, it would give rise to divalent antibodies carrying the specific end groups A′—A′, B′—B′ and A′—B′ as well as monovalent antibodies, A′ and B′. The fact that antibodies formed as a result of prolonged immunisation usually have a broader specificity and give wider equivalence zones of reaction with antigens (see Chapter XXIII) is also accounted for on this basis. The theory also accounts for the cross reactions which occur between the antisera to different antigens since several antigens may contain a common determinant group. The apparent paradox that antigens with powerful polar determinant groups are frequently poor antigens, giving only low concentrations of antibody although of sharp specificity, whilst antigens with weak polar groups, that is of low specificity, usually give high titre antisera is explained by the fact that the stronger the polar group the more firmly will it attract and hold the complementary antibody group and therefore the less frequently is the antigen molecule available to influence the formation of fresh antibody molecules.

On the basis of this theory Pauling predicted that if normal globulin were placed under mild denaturing

conditions, such as heating at 50° to 60° C, or solution in urea or alkali, which cause the protein chains to unfold, and the conditions then restored to normal in the presence of an antigen, the polypeptide chains would refold in a manner complementary to the antigen; in other words antibodies would be formed. He claims, on reasonable but not rigid evidence, to have produced *in vitro* in this way antibodies to 1 : 3-dihydroxybenzene-2 : 4 : 6-tri-*p*-azophenyl arsonic acid, methyl blue and the specific polysaccharide of Type III pneumococcus.

The Separation of Antibodies.—Many attempts have been made to separate antibodies from the inactive constituents of the antiserum. These depend on (1) non-specific methods and (2) specific methods :—

1 (*a*) *Alteration of the Salt Content.*—If serum is half-saturated with ammonium sulphate the globulin is precipitated, leaving albumin in solution; the albumin is precipitated on complete saturation of the serum with ammonium sulphate. Globulin can be further split into euglobulin which is insoluble in distilled water, and into pseudoglobulin which is soluble in distilled water. Dialysis of serum to remove all the salts, therefore, causes the precipitation of euglobulin. Dilution of serum with 9 or 10 volumes of distilled water and slight acidification, for example by passing carbon dioxide into the solution, precipitates most of the euglobulin fraction and a small proportion of the pseudoglobulin. Euglobulin, which can also be precipitated by one-third saturation with ammonium sulphate, comprises chiefly β- and γ-globulins which are the slower moving components in an electric field (see p. 425). The pseudo-globulin is composed mainly of the fast moving α-globulin fraction. It has been found that the antibodies are almost entirely associated with the globulin fraction, but there is little agreement as to the distribution between euglobulin and pseudo-globulin, even when dealing with the same antibody.

Depending on the source of the antibody, from horse or rabbit serum, for example, on the intensity and length of the course of immunisation, and on the exact method of fractionation, the antibody may be found to be associated with one or other of the globulins or divided between them. This is not surprising when it is remembered that the proportions of albumin, pseudoglobulin and euglobulin which can be separated even from normal serum are very variable.

(*b*) *Precipitation with Alcohol.*—By this method, also, the antibodies are separated with the globulin fractions. Denaturation of the proteins by alcohol is avoided by working at 4° C. or lower, or by bringing the alcohol concentration rapidly above 90 per cent. By precipitating the antibody from an anti-pneumococcus serum in the cold with 10 per cent. alcohol in the presence of N/200-sodium chloride at *p*H 6·7, Felton succeeded in removing about 90 per cent. of the inactive protein.

(*c*) *Adsorption Methods.*—Methods similar to those introduced by Willstätter for separating enzymes have also been found effective in separating antibodies. For instance the antibody to the typhoid bacillus can be adsorbed on alumina and then eluted with dilute (N/100) alkali. Diphtheria antitoxin, the flagellar typhoid antibody and the O-antibody of *Salmonella enteritidis* are adsorbed by kaolin from which they can be eluted with a solution of 2 per cent. glycine in 2 per cent. sodium chloride. The eluted typhoid antibody solution gave negative reactions for proteins but contained 0·6 mg. of nitrogen per ml.; it was not affected by proteolytic enzymes. Only 15 to 20 per cent. of the antibody could be recovered in this way, but that which was obtained was about six times as concentrated as the original serum.

(*d*) *Electrophoresis.*—Antibodies have an isoelectric point at about *p*H 5·5 and accordingly move in an

electric field with the globulins, which have about the same isoelectric point.

As a result of analysis of serum proteins by the use of Tiselius' apparatus for electrophoresis it is known that albumin shows greatest mobility in an electric field whilst normal globulin separates into three fractions, α-, β- and γ-, which move progressively more slowly. All these fractions contain cholesterol, phospholipoids and carbohydrates (glucosamine and mannose), the α- and β-globulins being richest in all of them. The antibodies formed as a result of the injection of polysaccharide-containing antigens, such as the soluble specific substances of the pneumococcus or the somatic O-antigens, are usually associated with the γ-globulin. Tetanus and diphtheria antitoxins are associated with a new component, T, not present in normal serum. The T component has a mobility of 2×10^{-5} cm. per second per volt per cm., which is midway between the mobilities of β- and γ-globulins. The antitoxins to *Cl. welchii*, *Cl. sordelli* and *Cl. œdemations* also have a T component. The antitoxins of hæmolytic streptococci, *Staphylococcus*, *Cl. botulinum*, *Cl. histolyticum* and *Cl. septicum* contain both T and γ-globulins in varying proportions, although the amount of γ-globulin is usually greater than in normal serum. So far all antitoxins which have been tested contain the T component. Diphtheria antitoxin shows a progressive increase of β-globulin as the course of immunisation proceeds, the γ-globulin increasing at first but soon reaching a steady value. The toxin-antitoxin floccules from γ-globulin antitoxin contain about twice as much nitrogen as do the floccules from β-globulin. The complexes can be expressed as having the composition $(TA_4)_n$ and $(TA_2)_n$ respectively. γ-Globulin antitoxin combines with toxin more rapidly than does β-globulin antitoxin, but the complex is less stable.

2. *Specific Methods.*—The specific methods depend on separating the antibody from the inactive part of the

antiserum by allowing it to react with the corresponding antigen and dissociating the antigen-antibody complex by an appropriate means. Hæmolysins have been eluted from sensitised red blood corpuscles with dilute acid or with glycine ; the eluates contained about 40 to 80×10^{-6} mg. of solid per hæmolytic unit. Agglutinins have been recovered from sensitised typhoid bacilli by extraction with dilute alkali, but with considerable loss ; no protein could be detected, but the nitrogen content was 0·4 mg. per 100 ml. Ramon dissociated the diphtheria toxin-antitoxin complex and obtained solutions containing 0·012 mg. of protein per unit of toxin. Northrop has obtained crystalline diphtheria antitoxin by digesting the toxin in the floccules with trypsin and crystallisation from ammonium sulphate solution. The purified antitoxin has molecular weight about 80,000, has an electrophoretic mobility of 4×10^{-6} cm. per second per volt per cm., at *p*H 7·3. It contains about one million antitoxin units per gram of protein nitrogen. Pneumococci have been agglutinated by the corresponding antiserum and the complex dissociated by extraction with dilute alkali ; the eluates were colloidal, did not give the ordinary protein reactions, and were not attacked by trypsin ; the antibody was not precipitated by 30 per cent. sodium chloride nor by dilution as would be globulins, but as the solutions contained only 0·00015 mg. of nitrogen per unit this lack of reactions is not surprising.

The most satisfactory results on these lines are those in which pneumococcus antisera have been precipitated by the protein-free soluble specific polysaccharides and the antibodies then separated from the precipitates. Felton decomposed the precipitates from Types I and II antisera with calcium or strontium hydroxide solutions in which the protein is soluble but which give insoluble precipitates with the polysaccharides. The antibody was then precipitated from solution by dialysis, behaving like euglobulin ; the activity was destroyed by proteolytic

enzymes and by denaturation; 90 to 95 per cent. of the protein (0·002 to 0·006 mg. of nitrogen per unit) in these preparations could be precipitated by the soluble specific polysaccharide. Heidelberger and Kendall dissociated similar precipitates with 15 per cent. sodium chloride and recovered the antibody by dialysis. The remaining undissociated precipitate was decomposed by barium hydroxide or barium chloride, which precipitate the Types I and III specific polysaccharides, leaving the antibody in solution; they succeeded in removing all the inactive protein in this way, 95 to 100 per cent. of the nitrogen in the final solutions being precipitated by the polysaccharides.

The Properties of Antibodies.—The size of the antibody molecule is about the same as that of normal serum globulins, as is shown by sedimentation rates in the ultra-centrifuge and by filtration through membranes of known pore size. Normal human serum globulin has a molecular weight of about 170,000, that of the rabbit about the same and that of the horse has two fractions, one with molecular weight about 170,000 and the other with molecular weight about 900,000. Antibodies to the pneumococcus in horse serum are associated with the heavy globulin fraction and have molecular weight about 900,000, and a diameter about 44 mμ. The rabbit antipneumococcus globulins have a diameter about 11 mμ and molecular weight about 170,000. This difference between the two antibodies is not restricted to size; the horse antibody is said to be associated with the pseudoglobulin and the rabbit antibody with the euglobulin. A further difference lies in their lipoid content; if the antisera are extracted with fat solvents, about 1·3 per cent. of lipoid is removed and they no longer give precipitin reactions, although their protective effect in mice is unaltered; the precipitin properties can be restored by the addition of lecithin to horse

antiserum and of kephalin to rabbit antiserum, but not *vice versa*.

Globulins are usually considered to have approximately spherical molecules but as a result of comparison of their behaviour in the ultracentrifuge, and studies of viscosity and electrophoresis it is becoming obvious that they must be elliptical or even rod shaped. Serum globulin in surface films opens up into fibre like molecules very readily—a state which is associated with denaturation. The normal ratio of length to breadth seems to lie between 7 to 1 and 10 to 1, although for horse anti-pneumococcus globulin the ratio is reported as 20 to 1. The molecules have a short axis 37 Å long whilst the long axis varies between 270 and 350 Å long.

Antibodies are comparatively labile substances ; they are much weakened by heating at 60° to 70° C., and are rapidly destroyed on boiling. They are more stable to dry heat (as are most proteins) and are not affected by cold. Prolonged heating at 57° C. precipitates antitoxins along with the globulins. The effect of heat runs parallel with denaturation and is independent of the particular serum in which the antibody is present. Antibodies are most resistant to heat at neutrality, the rate of destruction being increased by acid or alkali. If denaturation of the protein is hindered by the addition of sodium chloride (above 2N), glycerol or sucrose or by dilution of the serum, the destruction of the antibody is retarded. The resistance of antibodies to heat apparently varies from antibody to antibody ; thus the flagellar antibody of the hog cholera bacillus will withstand 90° C. for twenty minutes, whilst the somatic antibody is destroyed at 75° C., and loses half its activity at 65° C. in the same time. The differences are due, however, not to differences in heat stability but to the fact that the apparently more susceptible antibodies form complexes with non-specific nitrogen compounds, such as albumin, in the serum

more readily than do the others; these complexes combine with antigens, as shown by complement fixation, but do not flocculate, and thus antibody appears to have been destroyed by the heating. Antisera to rod shaped viruses, for example tobacco-mosaic virus or potato-X virus, behave like the flagellar, H-antibodies, whilst antisera to albumin and other more "globular" antigens behave like those to somatic, O-antigens. Some antibodies are destroyed at the earliest stages of denaturation, whilst others may withstand complete denaturation if coagulation is prevented by addition of urea or by dilution with physiological saline.

The deleterious effect of alcohol on antibodies also runs parallel with the denaturation of the proteins. Below the critical temperature of 4° C. there is no denaturation and no destruction, nor is there if the alcohol concentration is rapidly brought above 90 per cent. The addition of acid or alkali accelerates the effect of alcohol.

The Effect of Chemical Changes on Antibodies.—Antibody globulins, like normal globulins, are only slowly attacked by trypsin but are much more readily destroyed by pepsin. Brief treatment with pepsin at *p*H 4 and 37° C. causes the antibody globulin of diphtheria antitoxin, for example, to break into two parts, one of which does not carry antitoxic activity and is easily denatured and coagulated by heating at 60° C. for fifteen minutes, whilst the active part is much more resistant to heat and remains in solution. By precipitation of the active fraction by ammonium sulphate after removal of the easily coagulated fraction by heating, a considerable concentration and purification of the antibody can be achieved. During the digestion by pepsin the T component (see p. 426) disappears and is replaced by the slower moving γ-globulin. More prolonged digestion causes further breakdown into fragments which can no longer flocculate with antigen but can still react with it

as shown by the fact that such digested antibody inhibits normal toxin-antitoxin flocculation. The euglobulin of Types I and II antipneumococcus horse sera can be digested by pepsin to give smaller active molecules having a sedimentation constant of $5{\cdot}2 \times 10^{-13}$ cm. per second per dyne, corresponding to a molecular weight somewhat less than 100,000, and able to combine with twice as much specific polysaccharide per mg. of antibody nitrogen as the original antibody.

The introduction of azo-compounds or of iodo-groups or the action of formaldehyde lowers the titre of an antiserum, but is said to sharpen the specificity. Eagle and his co-workers have shown that coupling pneumococcus antiserum with diazotised atoxyl has a differential effect on the reactions of the antiserum; the power of agglutinating bacteria and of precipitating the specific polysaccharide may be lost, but not the power to protect mice against infection. The action of the azo-compound proceeds at different velocities for the different manifestations of the antibody, but a sufficiently long contact with the reagent causes a complete loss of all the antibody reactions. With the pneumococcus antiserum the precipitin reaction is lost before the agglutinating power, protective properties or ability to fix complement. In the case of diphtheria antitoxin the flocculating property is lost before the power to neutralise toxin. These effects are illustrated in Table 30. The addition of 1 part of formalin to a thousand parts of antiserum brings about similar effects. It is suggested that the addition of just enough formaldehyde to prevent aggregation increases the solubility of the antibody so that it no longer precipitates at the normal conditions of *p*H of serum.

In Type I pneumococcus antiserum replacement of a hydrogen atom in the amino group by acetyl, by the action of ketene, reduces its activity, whilst the action of formaldehyde causes loss of specificity. Esterification

of the carboxyl groups destroys the activity of the antibody very readily, indicating that the carboxyl group plays an important part in the reaction with the antigen. Substitution in the hydroxyl and amino groups has less effect.

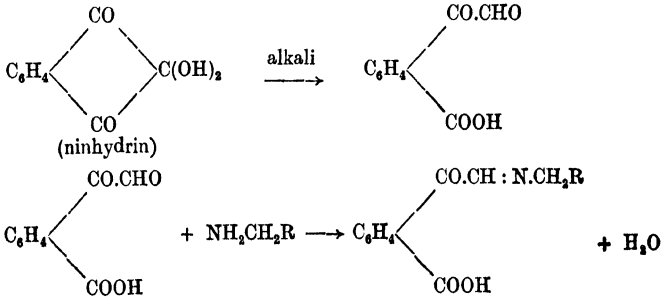
TABLE 30

(*After Eagle*)

Antibody	Reaction	Time for Loss
		Hours
Syphilis reagin	Wassermann and flocculation	0·01
Diphtheria antitoxin	Ramon flocculation	0·02
,, ,,	Protection	0·25
Horse pneumococcus antiserum	Polysaccharide precipitation	0·07 to 0·2
,, ,, ,,	Agglutination	0·24 to 1·0
,, ,, ,,	Mouse protection	0·5 to 2·0
Rabbit *v.* horse antiserum	Precipitation	0·25
Horse typhoid antiserum	Agglutination	24

The antitoxic power of tetanus antitoxin remains intact after the acetylation of 30 to 40 per cent. of the amino groups by ketene, but further acetylation causes a rapid loss of activity.

The action of ninhydrin is analogous to that of formaldehyde in that it reacts with the terminal amino groups of lysine residues :—



It has been shown that typhoid H-agglutinins in horse antisera can be concentrated by treatment with ninhydrin and precipitation by acid or salts. The O-agglutinins of typhoid and the Type I pneumococcus agglutinins in horse antisera are partially destroyed by ninhydrin. In rabbit antisera the apparent titre of typhoid H-agglutinins is increased 2 to fourfold by the action of ninhydrin, whilst the typhoid O-agglutinins and pneumococcus antibodies are not affected, unless the treatment is prolonged.

Complement

In the various lytic reactions, bacteriolysis and hæmolysis, for example, it has been established that two serum factors are involved—(1) the comparatively heat stable immune body which is increased in amount during immunisation and (2) the very labile complement, or alexin, which is present in normal as well as in immune sera and which is not increased in amount during immunisation.

In the case of hæmolysis, Ehrlich considered that the immune body, hæmolysin or amboceptor, had two affinities, one for the cell and the other for the haptophore or combining group of the complement. The complement then acted on the cell causing lysis in virtue of its active or ergophore group, as Ehrlich called it. Bordet regarded the immune body as a sensitiser with which the cell formed a complex which had an affinity for complement and adsorbed it ; the immune body itself has no visible effect on the cells ; it is the complement which brings about the lysis. It has been considered that in the lysis of cells the complement acts as a proteolytic enzyme, but it differs in one respect at least ; its effects are governed by the Law of Mass Action, that is, it appears not to act as a catalyst. Moreover, the cell walls of red blood corpuscles, for example, are not destroyed nor is the liberated hæmoglobin attacked, which appears to be in contradiction to

a proteolytic effect of complement. It seems much more probable that the lysis is due to an alteration of the physical state of the cell walls, making them permeable to the cell contents. This view is rendered the more likely since "artificial" complements, either colloidal silicic acid or one made by mixing sodium oleate, methyl alcohol and calcium chloride, have the same effect, which would appear to be due to a lowering of the surface tension.

Complement appears to be quite non-specific; the complement in the serum of any animal is effective in all reactions, but the activity of complement in the sera of different animals varies appreciably and also varies in any one animal depending on the system tested; the guinea-pig is a particularly good source of complement for hæmolytic systems.

Complement is very susceptible to heat, being destroyed by heating at 56° C. for fifteen minutes, resembling in this respect many enzymes. It is also very susceptible to physical and chemical changes, even mechanical shaking is sufficient to inactivate it. Complement is destroyed by acid and alkali and by ultra-violet light. If the inactivation has not been carried too far it is partially reversible and standing restores a part of the activity. This suggests that the inactivation is due to a change in the colloidal condition, probably to an increased aggregation of the particles which may spontaneously disperse again. Complement is almost certainly protein in nature since it is attacked by trypsin, which destroys the end-piece (see below) first. It is readily adsorbed by non-specific adsorbants, even by filtration through a Berkefeld candle.

The Structure of Complement.—The complex nature of complement was first demonstrated in 1907 by Ferrata, who showed that on removing the salts from serum it lost its complementary activity; the euglobulin was precipitated and the albumin remained in solution. Neither

of these fractions alone was active, but on mixing them the activity was restored. The two fractions were called mid-piece and end-piece respectively, because it was found that the albumin (end-piece) was not taken up by sensitised red blood corpuscles unless the globulin mid-piece had first been adsorbed. Although the mid-piece is adsorbed in the absence of the end-piece, it cannot induce lysis until the latter is also adsorbed. In Ehrlich's terminology the mid-piece carries the haptophore group and the end-piece the ergophore group.

Liefmann split complement into mid-piece and end-piece by diluting the serum ten times with distilled water and passing carbon dioxide through the chilled solution, whereupon the mid-piece (euglobulin, with some pseudo-globulin) was precipitated. He showed that the mid-piece was adsorbed in the Wasserman reaction but not the end-piece, which is left quantitatively in solution, and confirmed the finding that both mid-piece and end-piece are necessary for hæmolysis. Both mid-piece and end-piece are heat labile and can be adsorbed by kaolin.

In 1907 it had been shown that the complementary power of a serum was removed by treatment with yeast, and in 1914 Coca showed that the component adsorbed by yeast was heat stable and that adsorption by yeast leaves the mid-piece and end-piece in solution. Thus three factors are present in complement, two heat labile, the mid-piece and end-piece, and one, the "third component," heat stable. Ritz, in 1912, had also elicited the presence in complement of a heat stable third component which was inactivated by cobra venom. Whitehead, Gordon and Wormall in 1925 took the problem a step further when they showed that zymin (a preparation of acetone dried yeast) is more effective than fresh yeast for adsorbing the third component; by examining the effect of zymin on separated end-piece and mid-piece they showed that almost all the third component was associated

with the mid-piece, although a little was sometimes carried along with the end-piece. The active substance in zymin is an insoluble carbohydrate called zymosan. The same workers in 1926 showed that the addition of small amounts of ammonia to serum destroyed the complement action and established that it did so by acting on a "fourth component." The fourth component is heat stable, but is not identical with the third component since it is not adsorbed by zymin. The majority of the fourth component is associated with the end-piece. It is specifically removed by treatment with ammonia, methylamine, ethylamine, hydrazine or phenylhydrazine, other alkalies completely destroying all the factors, as does ammonia if it is allowed to react for too long. All these compounds can react with the potential aldehyde group of carbohydrates and it is suggested that the fourth component may be a carbohydrate carried by the pseudoglobulin end-piece. The claim of Takano that the loss of activity of complement on treatment with ether or chloroform or viper venom was due to the lipoid nature of the fourth component is inaccurate since treatment of dried complement with lipoid solvents has no effect on the fourth component, the loss of activity of liquid serum being due to denaturation of the proteins.

The symbols C′1, C′2, C′3 and C′4 have been suggested for midpiece, endpiece, third component and fourth component, respectively. Since the ′ serves no apparently useful purpose it is proposed to omit it here.

In hæmolytic reactions components C1, C2 and C4 combine with sensitised sheep red blood corpuscles but C3 does not. Although C1 combines with red cells in the absence of C4 it is hæmolytically inert unless C4 combines previous to, or simultaneously with, it. Although C3 is not fixed by antibody-red-cell aggregates it is essential for hæmolysis and acts on the sensitised cells after fixation of the other components. It appears to have catalytic activity.

In complement fixation reactions almost all the C2 and C4, 75 per cent. of the C1 and 25 per cent. of the C3 are removed. Elimination of C3 or C4 does not influence the fixation of the other components, but inactivation of C1 and C2 by heat inhibits the fixation of C3 and C4. The inactivation of complement by fixation is due mainly to the removal of C1, C2 and C4 from the serum. In the absence of C1, components C2 and C4 are not fixed, but C1 is fixed in the absence of all the other components.

Human complement has been fractionated in the same way and shown to contain components C1, C2, C3 and C4 which are almost, but not quite, identical with the corresponding fractions of guinea-pig complement.

The present state of our knowledge of the structure of complement can be summarised as follows (illustrated diagrammatically by Fig. 10) :—

End-piece.—Albumin and pseudoglobulin ; heat labile ; carries 70 to 100 per cent. of C4 and 20 to 40 per cent. of C3 ; constitutes 0·2 per cent. of total serum protein ; contains about 10 per cent. of carbohydrate ; very little adsorbed in complement fixation reactions, but necessary for lytic reactions.

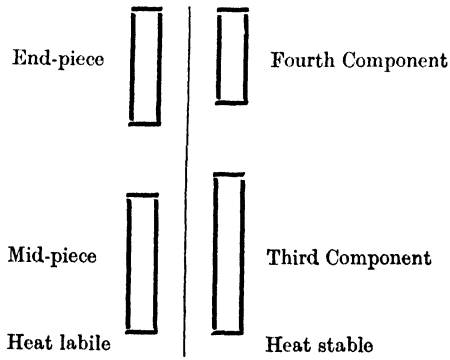


FIG. 10

Mid-piece.—Euglobulin; heat labile; carries 60 to 80 per cent. of C3 and 0 to 30 per cent. of C4; constitutes 0·6 per cent. of total serum protein; contains about 3 per cent. of carbohydrate; completely adsorbed in complement fixation and lytic reactions.

Third Component.—Mainly associated with mid-piece; heat stable; specifically adsorbed by zymin or zymosan.

Fourth Component.—Associated with end-piece; not adsorbed by zymin; specifically inactivated by ammonia; heat stable; necessary for complement fixation and lytic reactions but not for opsonin action; possibly a carbohydrate.

Complement, however, is probably even more complex than this summary suggests; the separation of these components is not always sharp and others may be present. For instance, complement can be reversibly inactivated by oxidation with iodine; reduction with ascorbic acid or glutathione restores the activity. The activity of oxidised complement can also be restored by the addition of complement from which the third and fourth components have been removed; the component inactivated by oxidation is therefore different from C3 and C4. The activity of complement is also lost on dialysis, but is restored by addition of a small quantity of the dialysate. Since the activity of complement devoid of C3, C4 and the oxidisable component is not restored by the dialysate whilst the activity of dialysed complement can be restored by the addition of dialysate from complement previously deprived of these components, the dialysable component must be different from them.

In view of this complexity it has been suggested that complement is not a definite substance but is really a particular state of the serum colloids, a state which can be very easily upset even by such means as mechanical

shaking. It has been maintained that complement is only active when the colloid particles are of a given size and when the correct concentrations of electrolytes are present; changes in the degree of dispersion or the proportion of the electrolytes disturb the balance with loss of activity. If the changes are small they may be reversible and activity spontaneously regained; large changes are irreversible. It has been pointed out that all changes which inactivate complement lower the surface tension, due to an alteration of the colloidal conditions which may be associated with the globulin, albumin, lipoid or electrolyte components of the system. This is borne out to some extent by the production of the artificial complement (p. 434) which is inactivated by heat and can be used in the Wassermann reaction like ordinary complement.

It has been claimed that complement may be a complex of ascorbic acid, proteins and lipoids, since guinea-pigs fed on a diet devoid of ascorbic acid (vitamin-C) produced no complement; the addition of ascorbic acid to the diet caused the almost immediate appearance of complement in the serum, only to disappear again if the vitamin were withdrawn.

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CHAPTER XXIII

THE MECHANISM OF ANTIGEN-ANTIBODY REACTIONS

WHEN an antigen and its corresponding antibody are brought into contact they react and the reaction manifests itself in a manner depending on the nature of the antigen and the conditions prevailing at the time of reaction. If the antigen is a soluble substance like a toxin or a serum protein precipitation may occur; if the antigen is carried by a bacterial cell or a red blood corpuscle agglutination may result, or the cell may be rendered sensitive to lysis or phagocytosis, which will take place if complement or leucocytes are also present. From the majority of evidence available it appears that antigen-antibody reactions occur in two stages. The first stage consists in the direct combination of the two reagents, the reaction being specific; this is followed by a non-specific stage, in the sense that one of several phenomena, such as flocculation, agglutination, complement fixation or lysis, may occur depending on the physical conditions operating at the time. Thus if bacteria are treated with the corresponding anti-serum in water instead of saline as the medium, no agglutination occurs, in spite of the fact that the two reagents have combined, as can be shown by separating the organisms by centrifugalisation, leaving a supernatant fluid devoid of antibody. If the sensitised deposit is suspended in saline agglutination immediately takes place.

In the first stage of the reaction between antigen and antibody the antigen can be replaced by the corresponding

hapten, following which, as a rule, the secondary reactions do not take place. There are certain haptens, however, notably the soluble specific substances and polyvalent haptens, sometimes called complex haptens, which lead to precipitin reactions, the products of their reaction with antibodies being insoluble. That reaction between hapten and antibody in the case of simple (non-precipitating) haptens also occurs is shown by the fact that an antibody so treated will no longer react with the complete antigen, its affinity for the active groups of the antigen having been satisfied by those of the hapten. Moreover it has been shown by direct means that the hapten combines with the antibody even when no visible reaction occurs. For example, phenyl-azo-*p*-benzene-arsonic acid was coupled with horse serum and an antiserum prepared against the complex. The antiserum was allowed to react with the hapten (phenyl-azo-*p*-benzene-arsonic acid) in a dialysis chamber and the concentration of free hapten which passed through the membrane measured. Most of the hapten was shown to be bound to the antibody. If an antiserum to the corresponding sulphonic acid was used instead of the homologous antiserum no combination occurred and the hapten passed through the membrane until equilibrium was established, showing that the reaction was specific. If the antibody was allowed to react with the antigen before introducing the hapten, no combination with the latter occurred.

Toxin-antitoxin Reactions.—Toxin and antitoxin combine in definite proportions to give a product which is non-toxic. Ehrlich defined the unit of antitoxin as that amount of it which would completely neutralise 100 minimal lethal doses (MLD) of toxin. The L_+ dose of toxin is that amount of toxin which when mixed with 1 unit of antitoxin will kill a 250 g. guinea-pig within ninety-six hours. The L_o dose is that amount of toxin which when mixed with 1 unit of antitoxin will just not

produce any reaction in the guinea-pig under standard conditions. It would be expected that $L_+ - L_o = 1$ MLD, but this is not the case, the difference being of the order of 20 to 50 MLD. Ehrlich explained this phenomenon as being due to the presence in toxin (T) of an epitoxoid (E) which is non-toxic and has a less affinity than, but the same combining power as toxin for antitoxin. If it is assumed, for purposes of illustration, that crude toxin contains equal parts of toxin and epitoxoid there will be nT+nE units of the mixture. If nA units of antitoxin were added to it, the non-toxic mixture nTA+nE would result. This corresponds to the L_o dose. The addition of 2nA units of antitoxin would yield the mixture nTA+nEA, also non-toxic. If more crude toxin, say 1 unit, is added, then the true toxin, having a greater affinity for antitoxin than the epitoxoid, would turn some of the latter out of combination :—

$$nTA + nEA + T + E \longrightarrow (n+1)TA + (n-1)EA + 2E.$$

The addition of more and more toxin will turn out more and more epitoxoid and combine with the resulting antitoxin until there is no epitoxoid-antitoxin complex left, the mixture remaining non-toxic ; after that free toxin can accumulate and the mixture becomes toxic, corresponding to the L_+ dose. Thus the L_+ dose will be bigger than the L_o dose according to the proportion of epitoxoid in the crude toxin preparation.

Another phenomenon first observed in connection with toxin-antitoxin reactions, and since found to occur with all antigen-antibody reactions, is the Danysz phenomenon. The amount of toxin neutralised by a given amount of antitoxin depends on the way in which the reagents are mixed. If equivalent amounts of toxin and antitoxin are mixed rapidly the product is non-toxic :—

$$nA + nT \longrightarrow nTA.$$

If, however, one-third of the toxin is added to the antitoxin, the mixture allowed to stand for some time, then another third of the toxin added, and after a further interval the last third of toxin added, the mixture is not non-toxic, as would be expected, but quite strongly toxic. Ehrlich explained this by assuming that crude toxin contained a non-toxic fraction, epitoxonoid, which combined only slowly with the antitoxin. If toxin and antitoxin were mixed rapidly all the antitoxin combined with the toxin and none with the epitoxonoid. When only part of the toxin was added and sufficient time allowed, part of the epitoxonoid combined with the excess of antitoxin until finally, after adding all the toxin, an excess of toxin remained corresponding to the quantity of antitoxin which had combined with epitoxonoid.

Other explanations than Ehrlich's of the toxin-antitoxin reactions have been proposed. Arrhenius and Madsen, for instance, claimed that the reactions followed the Law of Mass Action just as any ordinary chemical reaction. Assuming that when one molecule of toxin and one molecule of antitoxin combine two molecules of the toxin-antitoxin complex are formed,

$$A + T \rightleftharpoons 2at,$$

they carried out experiments in which the amount of free toxin was measured after the addition of various amounts of antitoxin. From the Law of Mass Action equation :—

$$[T]\,[A] = k\,[at]^2,$$

they calculated the dissociation constant, k, to be 0·0093, and used this value to calculate the amounts of free toxin which should be present for different concentrations of antitoxin. The agreement between the observed and calculated values was very close, as may be seen from Table 31 :—

TABLE 31

Antitoxin added	Amount of Free Toxin	
	Calculated.	Observed.
0·00 equivalent	100·0	100·0
0·25 ,,	75·0	75·0
0·50 ,,	50·5	48·0
0·75 ,,	27·0	26·0
1·00 ,,	8·8	9·6
1·25 ,,	3·1	3·1
1·50 ,,	1·7	1·6

Arrhenius and Madsen pointed out that there is a close similarity between this reaction and the purely chemical reaction between ammonia and boric acid. Ammonia is hæmolytic, and may be compared with the toxin; boric acid is non-hæmolytic and destroys the lytic action of ammonia by formation of ammonium borate, that is, it is analogous to antitoxin :—

$$3NH_4OH + H_3BO_3 \rightleftharpoons (NH_4)_3BO_3 + 3H_2O.$$

They carried out a similar experiment with these reagents, using the degree of hæmolysis as an indication of the amount of free ammonia, and again found a close agreement between the observed and calculated values (Table 32), although here the degree of dissociation is considerably higher than in the case of toxin and antitoxin.

TABLE 32

Amount of Boric Acid Added	Degree of Hæmolysis.	
	Calculated	Observed
0·00 equivalent	100·0	100·0
0·33 ,,	75·0	75·0
0·66 ,,	60·3	63·0
1·00 ,,	50·3	47·5
1·33 ,,	43·2	43·7
1·66 ,,	37·6	36·0
2·00 ,,	33·5	33·5

Ehrlich's phenomenon, that $L_+ - L_o = n$ MLD, is readily explained, according to Arrhenius and Madsen, as being due to the fact that sufficient toxin must be added to the toxin-antitoxin mixture to overcome the dissociation. This mechanism, however, does not afford a simple explanation of the Danysz phenomenon, although they quote a chemical reaction somewhat, though not completely, analogous. If sodium hydroxide is added to monochloracetic acid the sodium salt is formed :—

$$4CH_2Cl.COOH + 4NaOH \longrightarrow 4CH_2Cl.COONa + 4H_2O.$$

If half the sodium hydroxide is added at 70° C., however, a second reaction takes place, the chlorine atom being split off with formation of glycollic acid :—

$$4CH_2Cl.COOH + 2NaOH \xrightarrow{70^\circ C.} CH_2OH.COONa + NaCl + H_2O + 3CH_2Cl.COOH.$$

On adding the remaining half of the sodium hydroxide at the normal temperature, one molecule of monochloracetic acid will remain unneutralised (corresponding to an excess of toxin, as in the Danysz phenomenon).

This mechanism does not account, either, for the dilution effects observed in toxin-antitoxin reactions. If equivalent quantities of toxin and antitoxin are mixed in strong solution to give a non-toxic mixture, the solution remains neutral on dilution. If, however, the reagents are diluted before mixing the neutralisation is not complete, although if the Law of Mass Action applied, the result should be the same in each case.

Bordet regarded the toxin-antitoxin reactions as following the ordinary course of colloidal adsorption reactions, where the amount of adsorption depends on the relative concentration of the reagents. Thus according to Freundlich's adsorption isotherm, $x = maC^n$, where x = the amount adsorbed, C = the concentration of the remaining unadsorbed substance, m = the amount of adsorbant, and a and n are constants. According to this view there should not be any free toxin or antitoxin in

the mixture, but a complex containing more or less of the reagents according to the proportions in which they were mixed. This mechanism affords no explanation of the specificity of such reactions, nor does it account for the fact that on heating the toxin-antitoxin complex soon after its formation dissociation occurs, but heating an hour or more after the reaction has occurred no longer has any effect. It has been shown that the adsorption of toxin by colloidal ferric hydroxide and by antitoxin both follow the adsorption isotherm, but that the iron complex is just as toxic as the original toxin, whilst the toxin-antitoxin mixture is non-toxic ; in other words, adsorption alone is not adequate to explain the neutralisation. Bordet claimed that the neutralisation occurred as a secondary reaction after the adsorption was complete. The Danysz phenomenon is readily explained on the adsorption hypothesis as being due to the complete covering of the antitoxin by the toxin first added, toxin subsequently added not being completely adsorbed.

Precipitin Reactions.—When a soluble antigen, such as a protein, is allowed to react with its corresponding antibody precipitation follows. The precipitate consists of 70 to 90 or 95 per cent. of antibody globulin, most of the remainder being the antigen, although there is often a variable amount of lipoid material also present. The preponderance of antibody in the precipitate is not due to non-specific adsorption by the antigen-antibody complex, since added normal serum is not taken up and does not influence the proportion of antibody and antigen in the precipitate. Dean and Webb showed that the ratio of antigen to antibody which gave the quickest reaction (that is, showed precipitation first) was a constant for any given system. They showed that, in general, in such mixtures neither an excess of antigen nor of antibody could be detected in the supernatant solution by the addition of one or other of the reagents. It was also

shown that the optimum proportion of antigen and antibody, although giving the most rapid precipitation, did not, as a rule, give the greatest amount of precipitate, but that this usually occurred in the region of antigen excess.

In the presence of a considerable excess of either antigen or antibody no precipitation occurs, and a precipitate already formed may dissolve on adding an excess of either reagent. This is the so-called zone phenomenon; the precipitation only occurs over a limited range of antigen-antibody proportions. The variation of antigen-antibody proportions may be brought about in one of two ways: (*a*) the amount of antibody may be kept constant and the amount of antigen varied, as is the usual practice in precipitin reactions; or (*b*) the amount of antigen may be kept constant and the amount of antibody varied, as is the normal procedure in carrying out agglutination reactions and the toxin-antitoxin reactions. It has been found that the optimal proportions as determined by these two methods are not the same, but vary to an extent depending on the particular system being studied. For example, in the case of the Ramon flocculation reactions of diphtheria toxin and antitoxin, the optimum proportion is 1 : 8 as determined by the constant antibody method, and 1 : 64 as determined by the constant antigen method.

Since definite, chemically pure antigens have become available a considerable amount of accurate quantitative work on the composition of the precipitates has been possible. Dean and Webb estimated that horse serum as the antigen formed about 12 per cent. of the precipitate at the optimum proportion, and that it fell to about 6 per cent. in the region of antibody excess. Heidelberger and Kendall found that Type III pneumococcus polysaccharide (which contains no nitrogen) formed

about 2 per cent. of the precipitate at the optimum proportion and about 0·6 per cent. at the end of the zone of precipitation in the antibody excess region. The pseudoglobulin of horse serum, as antigen, forms about 20 per cent. of the precipitate at the optimum proportion ratio. When antigens which can be separately estimated in the precipitate and in the supernatant solution are employed it is possible to investigate the composition of the precipitate also in the region of antigen excess. Thus Heidelberger and Kendall, using R-salt-azo-diphenyl-azo-egg albumin, a red dye, as antigen and estimating its concentration colorimetrically, found that the precipitate contained 13 per cent. of antigen at the optimum proportion, 6·7 per cent. at the limit of antibody excess and 33 per cent. at the limit of antigen excess.

It will be seen that the precipitates do not have a constant composition, the amount of antigen present increasing with the proportion of antigen to antibody in the mixture. The range of composition varies with the particular antigen-antibody pair concerned. Marrack and Smith showed that, using diazotised atoxyl coupled to crystalline egg albumin or to horse pseudoglobulin, or using iodo-egg albumin or horse pseudoglobulin as antigens, the proportion of antigen to total protein in the precipitate increased with the amount of antigen added to a given amount of antibody. At the optimum proportion, addition of either antigen or antibody to the supernatant solution caused precipitation, showing the presence of small amounts of antigen and antibody in the solution, due to dissociation of the precipitate; at other ratios only antigen or antibody was to be found in solution. They showed that the change in composition of the precipitate was not due to non-specific adsorption of the antigen or the antibody since the introduction of normal serum or another antigen caused no change in the composition of the precipitate; for example, no azo-protein was carried down in a toxin-antitoxin flocculation.

nor with a pseudoglobulin-antipseudoglobulin precipitate. These authors suggested that the change in composition of the precipitate with the change in proportion of antigen and antibody in the reacting mixture might be due to the formation of a series of compounds of the general formula A_mG_n, due to the presence of several combining groups in the antigen and antibody.

Heidelberger and Kendall developed this idea further, and applying the Law of Mass Action were able to give a series of equations which could be used to predict the behaviour of antigen-antibody mixtures over a wide range of proportions. They used the nitrogen-free Type III pneumococcus specific polysaccharide as hapten and purified antibody preparations consisting of euglobulin as the antibody. They allowed varying proportions of the reagents to react, separated the precipitate by centrifugalisation and analysed it for nitrogen to determine the amount of antibody it contained. They showed that the addition of increasing amounts of the polysaccharide to a constant amount of antibody caused progressive removal of antibody from solution until the optimum proportion (or, as they name it, the equivalence point) was reached, when neither antibody (A) nor polysaccharide (S) could be detected in solution. On further addition of S it was taken up by the precipitate until the end of the zone of precipitation was reached. Actually the equivalence point was a zone due to a certain amount of dissociation of the precipitate, giving traces of A and S in solution. The extent of the zone depended on the particular specimen of antiserum which was used and on such factors as temperature, but it was fairly constant for any one system. The zone may be approached from either the hapten excess or the antibody excess side, giving limiting values. For the Type III polysaccharide-antibody system the values for the nitrogen : polysaccharide ratio were 13·5 for the antibody excess side and 8·6 for the hapten excess end of

the zone, with a mean value corresponding to an equivalence point of 10·8. The ratio of nitrogen to polysaccharide in the precipitates depended on the ratio of hapten and antibody reacting and not on the absolute concentrations. The range of antibody to hapten ratios in the precipitates was 40 : 1 at the antibody excess end and 5 : 1 at the hapten excess end of the zone. The soluble compound which was formed in the inhibition zone of hapten excess was shown to contain one more molecule of polysaccharide than the immediately preceding precipitate. They suggested that at the equivalence point a compound AS was formed. In the region of antibody excess compounds A_2S, A_3S, A_4S to A_mS were formed progressively depending on the relative excess of antibody. In the region of hapten excess AS_2 was formed as the insoluble precipitate, which yielded AS_3 as the soluble compound occurring in the inhibition zone. It was found, however, that this formulation would not fit the requirements of the Law of Mass Action, and later they suggested that the precipitation resulted from a series of bimolecular reactions. The first stage they considered to be the formation of the compound AS,

$$A + S \rightleftharpoons AS$$

having a composition corresponding to the equivalence point ratio of antibody to polysaccharide. Since both hapten and antibody are multivalent with respect to each other as a result of possessing a number of reactive groups, made up of repeated units of aldobionic acid and amino-acids respectively, the compound AS is capable of combining with more antibody or polysaccharide, whichever is in excess in the solution. In the region of antibody excess the following reactions can occur as a second step :—

$$AS + A \rightleftharpoons AS.A.$$
$$AS + AS \rightleftharpoons AS.AS.$$

Similarly these new compounds can take part in a third stage :—

$$\begin{aligned} AS.A + A &\rightleftharpoons AS.A.A. \\ AS.AS + A &\rightleftharpoons AS.AS.A. \\ AS.A + AS.A. &\rightleftharpoons AS.A.AS.A. \\ AS.A + AS.AS &\rightleftharpoons AS.A.AS.AS \\ AS.AS + AS.AS &\rightleftharpoons AS.AS.AS.AS. \end{aligned}$$

This process is supposed to continue on similar lines until insoluble aggregates are built up and precipitation occurs. When antibody and hapten are mixed in equivalent quantities the compound AS is believed to polymerise, by a similar mechanism, to give $(AS)_n$, which has the composition of the precipitate at the equivalence point. In the region of hapten excess analogous compounds are formed until the inhibition zone is reached. The latter only occurs when there is a considerable excess of hapten, when all the specific groups of the antibody tend to react with S rather than with AS or similar complexes, so that no aggregation to form an insoluble precipitate takes place. A similar explanation accounts for the non-precipitation with simple haptens which contain only one or two reactive groups, leading to the formation of soluble compounds of the type AH_x, which show no tendency to aggregation. If, as in complex haptens like the azo-dyes studied by Landsteiner, several reactive groups are present, compounds of the type AH.AH are formed, and aggregation followed by precipitation can occur.

Applying the Law of Mass Action to the above reactions, Heidelberger and Kendall deduced that the equation

$$\text{Mg. of antibody nitrogen precipitated} = 2RS - \frac{R^2S^2}{A} \quad . \quad (1)$$

held in the region of antibody excess, where R is the ratio of antibody to antigen at the equivalence point, S is the amount of polysaccharide added and A is the amount of antibody-nitrogen precipitated at the equivalence

point. This equation covers the range of precipitate given by mixtures with ratios of antibody to antigen from R to 2R. Similar but more complex equations were deduced for ratios up to 4R. For the region of antigen excess the equation

$$\text{Mg. of S precipitated} = 2R_1A - \frac{(R_1)^2A^2}{\text{Total S}} \quad . \quad . \quad . \quad (2)$$

results, where R_1 is the ratio of antigen to antibody at the equivalence point. Close agreement between the experimental values and the values calculated from the above equations was found, as the following examples for two antibody preparations show (Table 33) :—

TABLE 33

(After Heidelberger and Kendall)

	Antibody			
	1		2	
R	13·6		12·4	
A	4·08		1·86	
Mg. of S Added	Mg. of Nitrogen Precipitated			
	Calculated	Found	Calculated	Found
0·02	...	...	0·46	0·50
0·05	1·25	1·22	1·03	1·03
0·075	...	...	1·40	1·41
0·10	2·27	2·24	1·65	1·66
0·20	3·62	3·62	...	...
0·25	3·96	3·87	...	...

If the values for the ratio of nitrogen to polysaccharide in the precipitate at any two points in the region of antibody excess are known (by experiment), then the ratio (R) at the equivalence point can be calculated, since dividing equation (1) by S the equation

$$\frac{N}{S} = 2R - \frac{R^2S}{A} \quad . \quad . \quad . \quad . \quad . \quad . \quad (3)$$

results, in which all the values except R are known. Similarly the behaviour of the reagents and the composition of the precipitates in the region of polysaccharide excess can be calculated and the quantitative behaviour of the serum over the whole range can be predicted.

Kendall has derived the same equation (3) from a consideration of the number of combining groups available for combination and the proportion of them which are actually in combination for varying concentrations of antigen and antibody.

Heidelberger and Kendall showed that these equations hold not only for the Type III pneumococcus system (which is a hapten-antibody system) but also for R-salt-azo-diphenyl-azo-egg albumin and its antiserum, for crystalline egg albumin and its antiserum and for the Type I pneumococcus polysaccharide system. There was found to be a difference between horse and rabbit pneumococcus antisera, possibly due to the difference in molecular weights of the globulins, which are 500,000 for horse globulin and 150,000 for rabbit globulin. For rabbit antiserum to Type III pneumococcus the value of 2R is 13·5 and for the horse antiserum 32, giving ratios of 85 and 200, respectively, for antibody-protein to polysaccharide at the equivalence point. Using the above values for the molecular weights of the globulins, the molecular weight of the polysaccharide is thus 1,800 to 2,500, corresponding to 5 to 8 aldobionic acid units. In the case of the Type I pneumococcus system the values of 2R are 5·4 and 14·4 for rabbit and horse antisera respectively, giving values for the molecular weight of the Type I polysaccharide of 4,400 to 4,500. It can be calculated from these values that the composition of the precipitate in the equivalence zone of the Type III system with rabbit antisera would be from S_3A_2 to S_2A; at the

beginning of the inhibition zone it corresponds to S_4A, and the soluble complex is S_5A. With Type III horse antisera the compounds are S_3A to S_6A for the equivalence zone and $S_{10}A$ for the soluble complex in the inhibition zone.

The combination of antigen and antibody takes place as though the molecules behaved as fairly rigid bodies, the antigens being roughly spherical in shape and antibodies more or less ellipitical, with a ratio of length to breadth of about 7 to 10 (see page 429). Antigen molecules are probably multivalent in that they contain several reacting sites or determinant groups, even when the determinant groups are all the same. They will usually have a higher valency the larger the molecule. If Pauling's conception of antibody formation is correct antibody molecules are for the most part to be regarded as divalent, although some monovalent molecules are also formed (see page 423). If the antigen and antibody are of about the same size then at the equivalence point the ratio of the weight of antibody to the weight of antigen in the precipitate would be about $N/2$ where N represents the valence of the antigen and the limiting values would be 1 for excess of antigen and $N-1$ for excess of antibody. When the antigen is much larger than the antibody the ratio would be less than $N/2$ at the equivalence point. The value of N, the effective valence of an antigen, is determined by the number of antibody molecules which can be packed round the antigen molecule. If the two molecules are spheres of equal size, 12 antibody molecules can be fitted into place round one antigen molecule. If the antigen is larger than the antibody more molecules of the latter can come into contact with the antigen and N may be larger than 12; if the antigen is the smaller molecule then N is less than 12. These expectations are borne out by experiment, as the values of N in Table 34 show:—

TABLE 34

Antigen	Molecular Weight	N
Ovalbumin	40,000	6
Serum albumin	67,000	6—8
Thyroglobulin	700,000	30—40
Busycon hæmocyanin	7,000,000	74

Pauling has suggested that the forces which hold the complex of antigen and antibody together are a combination of the weak van der Waals forces, which need very close juxtaposition of the atoms concerned if they are to be effective; the weak forces exerted by the polarisation of one atom by the dipolar character of another; and the stronger electrostatic attraction between positively charged amino groups and negatively charged carboxyl groups, for example, and the even more powerful hydrogen bonds. The energy of electrostatic attractions may be quite considerable, of the order of 5 Calories per gram molecule, if the appropriately charged groups can come into close apposition. The hydrogen bond, which results from the attraction of a hydrogen nucleus from one electronegative atom by the unshared electron pair of another electronegative atom, depends for its strength on the degree of electronegativity of the two atoms, the most electronegative, oxygen and nitrogen, giving the strongest hydrogen bonds, with an energy of about 5 Calories per bond. Specificity is due to the complementary configuration and arrangement of the groups of atoms in the antigen and antibody molecules which could form hydrogen bonds, or give rise to electrostatic attraction, and this in turn depends on the size and dispositions of the areas on antigen and antibody molecules which could come into contact. If the arrangements of groups of atoms in the molecules are such that the molecules could only come into close contact at a few points, combination would be weak, whereas if the

contact was close over a considerable area then firm and specific combination would occur. The forces which hold the molecules together are not themselves specific ; the specificity depends entirely on the appropriate distribution of the atoms involved. The method by which such complementary structures might be built up in antibodies has been outlined on page 421.

The evidence points to the fact that the second stage of antigen-antibody reactions, especially in agglutination and precipitin reactions, is also specific. Thus agglutination carried out with mixtures of two bacteria and the corresponding antisera normally gives clumps which contain one type of organism only, and not the mixture of organisms which would be expected if the aggregation were non-specific. Similarly, precipitin reactions carried out in the presence of a heterologous antigen do not give precipitates containing the second antigen. It is reasonable to suppose, therefore, that the second stage, the actual formation of a precipitate, is a continuation of the first stage until aggregates of sufficient size to be insoluble are formed. This suggestion forms the basis of the " framework " or " lattice " theory put forward by Marrack and elaborated by Heidelberger and by Pauling and their collaborators.

The framework hypothesis requires that antibodies be at least divalent, otherwise antibody molecules could not form links between two or more antigen molecules to give the framework structure illustrated in Fig. 11 which represents the state at the equivalence point.

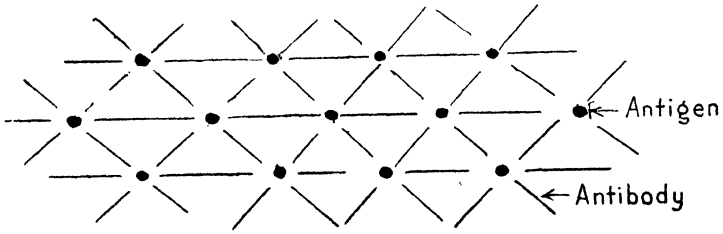


FIG. 11.

In the region of antibody excess the structure of the framework would be represented as in Fig. 12, in which only one valence of many of the antibody molecules

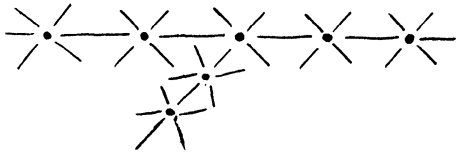
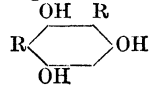
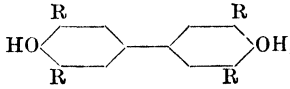
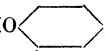
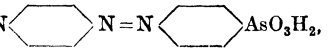


FIG. 12.

is involved and the precipitate has a higher proportion of antibody than it has in the equivalence zone. The proportion of antibody may be so high that the complex is soluble and give rise to the so-called "inhibition" or "pro-zone." The formation of precipitates by the interaction of multivalent haptens and antibodies is accounted for on the same basis, whilst monovalent haptens could not give rise to such a framework. Thus haptens such as  or  , where *R* represents diazotised arsanilic acid, —N=N  AsO_3H_2, give precipitates with antiserum prepared by injection into rabbits of diazotised arsanilic acid coupled to protein. Haptens like HO  R, containing only one determinant group, do not give precipitates although they combine with the antiserum.

Boyd suggests that antigen-antibody precipitates do not arise as the result of building up aggregates by the framework process but that they result from the prevention of the lyophilic polar groups of antibodies and antigens from exerting their normal "solubilising" function. The comparatively large antibody molecules are visualised as being held closely together by the antigen or hapten molecules in such a way that the uncombined polar groups of the antibody are "occluded"

and prevented from contact with solvent molecules, so that the complex composed of antigen or hapten and a few antibody molecules becomes lyophobic and insoluble. He accounts for the observed failure of some multivalent haptens to cause precipitation as being due to the too close proximity of the determinant polar groups so that there is not room for two or more antibody molecules to be brought into such positions that their polar groups are occluded, the complex, therefore, remaining soluble. If the hapten molecule is larger, for instance if *R* of the above formulæ is  , the polar groups are further apart, steric hindrance is less, more antibody molecules may be able to react with occlusion of a larger number of their polar groups and consequent precipitation.

The evidence for making a definite decision between the "framework" and "occlusion" hypotheses of precipitation is at present not adequate, but the balance seems in favour of the former.

Pauling and his collaborators have proposed equations to account quantitatively for the reactions between multivalent antigen molecules and divalent antibody molecules, assuming equilibrium between antigen-antibody soluble complexes of the types AB, A_2B and AB_2 and the precipitate ABp, where A represents antigen and B represents antibody. The resulting expression has been shown to hold for relatively simple systems, such as those composed of divalent antigen and divalent antibody.

Hershey has also proposed a rather elaborate series of equations which account fairly well for quantitative findings and also enable deductions to be made as to the results to be expected from alterations in the systems.

The Reacting Groups in Precipitin Reactions.—At present comparatively little is known of this subject, but some data are available. Chow and Goebel showed that

if the amino groups of a purified antibody to Type I pneumococcus were acetylated by the action of ketene, the antibody lost much of its power of reacting with the Type I polysaccharide. If the amino groups are treated with formaldehyde with introduction of methylene groups (see p. 417) the power of reacting is completely lost. Reconstitution of the amino group by treatment of the methylene derivative with dilute acid at *p*H 4·0 for several days at 0° C. restores the activity. It is, therefore, very probable that the amino groups of this antibody are involved in its reactions. It is also very probable that the strongly polar carboxyl group of the uronic acid or aldobionic acid of the hapten is involved, since esterification of the Type I polysaccharide with diazomethane causes complete loss of activity, although it must be remembered that one hydroxyl group and the amino group are also methylated by this procedure. Alkaline hydrolysis, however, removes the ester methyl group but not those attached to the hydroxyl and amino groups, with almost complete restoration of the hapten activity, which suggests that it is the carboxyl group which is largely responsible for the action, although the other two groups contribute to some extent.

It has been suggested that the prominent polar groups in antigens which determine their specificity actually fit into hollows or sockets in the antibody molecules which were modelled round the determinant groups either by the folding mechanism described by Pauling or by the actual selection of appropriately shaped amino-acid groups as in the theories put forward by Breinl and Haurowitz and by Mudd. It is considered that electronegative groups like the carboxyl ion on an antigen molecule would be matched by electropositive groups such as an amino group on the antibody molecule. This is in keeping with the fact that it is the terminal parts of determinant groups which have most influence on specificity, as with the peptide haptens mentioned on

page 408 and the carbohydrate determinants described on page 411.

Heidelberger and Kendall showed that partially hydrolysed Type III polysaccharide, giving products with molecular weights between 550 and 1,800, gave reactions with horse antisera but not with those from the rabbit; the aldobionic acid itself gave no reaction with either antiserum. This suggests that definite groups and not the molecule as a whole are concerned. Type III polysaccharide which has been methylated by dimethyl sulphate and sodium hydroxide (reagents which do not esterify the carboxyl group) reacts with the horse antiserum, precipitating about two-thirds of the nitrogen of the antibody. The remaining one-third of the nitrogen can only be precipitated by unmethylated polysaccharide in which the hydroxyl groups are free. This confirms that different groupings of a hapten may act independently in stimulating the production of antibodies and in reacting with them. That is, a single antigen may produce more than one antibody with specificities corresponding to different determinant groups in the antigen.

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APPENDIX 1

THE ISOLATION AND IDENTIFICATION OF METABOLIC PRODUCTS

IN carrying out investigations into the metabolic processes of micro-organisms it is obviously advantageous to start with substances of known composition only ; for this reason synthetic media should be employed whenever possible for the growth of the organisms, since the use of broth, meat extract and similar materials introduces mixtures of substances of unknown nature and amount. For ease in tracing the course of the metabolic changes a single source of carbon, such as a sugar or other suitable substance, and of nitrogen, such as an ammonium salt, should be used.

The fermented solution is usually an aqueous mixture which will contain the organism, possibly calcium carbonate (which may have been added to maintain neutrality) and insoluble organic calcium salts, volatile neutral and basic substances, volatile acids, non-volatile acids and non-volatile neutral or basic products. Gaseous products are also often produced, the usual gases encountered being carbon dioxide, hydrogen and methane ; these may be collected during the course of fermentation in the usual way over water after removal of the carbon dioxide by baryta or soda-lime.

The course of fermentation can be followed by observing the disappearance of the carbon source ; glucose, for instance, can be estimated by the optical rotatory power of the solution or by the reducing power as determined by the Shaffer-Hartmann method, for example, or by

Willstätter and Schüdel's alkaline iodine method. If more than one of these methods is employed any discrepancy between them may afford valuable information as to the products formed. Thus a low value for glucose by the optical method as compared with the reduction method would indicate the formation of a lævo-rotatory substance among the products, whilst a high value would suggest the presence of a dextro-rotatory product.

The fermentation mixture will consist of two parts, insoluble and soluble. The insoluble constituents are first removed by centrifugalisation or filtration. Difficult filtration may often be improved by shaking the metabolism solution with kieselguhr and filtering through a thin layer of kieselguhr on filter paper prepared by pouring a suspension of kieselguhr on to a wet paper in a large Buchner funnel (about 1 g. is adequate for a funnel 15 to 20 cm. in diameter). Filtration is usually effective for fungi, which form a more or less continuous felt of mycelium, less effective for yeasts and usually ineffective for bacteria for which centrifugalisation is usually employed.

The **insoluble residue** will contain the organism, such products as may be insoluble in water (usually only occurring in mould fermentations) and insoluble calcium salts and excess of calcium carbonate, if the latter has been employed. The organism is, of course, usually known but should be examined in order to detect any possible contamination with unwanted organisms. The metabolic products of bacteria and yeasts are almost invariably soluble in water unless the normal course of fermentation has been interfered with by addition of calcium carbonate or fixative agents such as sulphite, dimedon, or β-naphthylamine. In the latter cases the residue will naturally be examined for the presence of the expected products. If the fermentation has been carried out in the presence of calcium carbonate the production of calcium oxalate is possible, especially in

the case of mould action. It may be detected by solution of the residue in hydrochloric acid, filtration, addition of ammonia and acidification with acetic acid ; a precipitate usually indicates the presence of oxalic acid, although calcium fumarate or succinate are also possible. If the latter are suspected the solution of the salt in hydrochloric acid is extracted with ether in which fumaric and succinic acids are soluble. Precipitation of the calcium salt on boiling the aqueous solution usually denotes the presence of citrate.

The **solution** obtained by filtration or centrifugalisation is submitted to the following treatment :—

1. *Volatile Neutral Products.*—The solution is neutralised with sodium carbonate and a portion distilled off into a receiver cooled in ice. The distillate is tested for :

(*a*) Acetaldehyde, by addition of 2 : 4-dinitro-phenylhydrazine hydrochloride ; the yellow crystalline dinitrophenylhydrazone is recrystallised and its melting point, 162° C., determined.

(*b*) Acetone, by making the 2 : 4-dinitro-phenylhydrazone, recrystallising and determining the melting point, 125° C. ; by saturation of the solution with solid ammonium sulphate, addition of concentrated ammonia solution and two or three drops of a freshly prepared solution of sodium nitroprusside, when a purple colour indicates the presence of acetone ; the iodoform test may be carried out, using ammonia instead of sodium hydroxide.

(*c*) Ethyl alcohol, by the iodoform test ; or by oxidation to acetaldehyde ; 25 ml. of the distillate is treated with 0·1 g. of potassium dichromate and 0·5 ml. of 20 per cent. sulphuric acid and distilled slowly to collect about 5 ml. of distillate which is tested for acetaldehyde ; a green colour of the residue is confirmatory.

(*d*) Butyl and higher alcohols may be detected by their odour; the solution is redistilled and the distillate saturated with sodium chloride when the alcohols separate as oily drops. If present in sufficient quantity they can be submitted to fractional distillation and determination of the boiling points.

(*e*) Esters may be detected by their odour, and by fractional distillation and determination of the boiling points.

2. *Volatile Acids.*—The residue from the distillation of the volatile neutral products is made acid with sulphuric acid or, preferably, phosphoric acid (to prevent charring) and distillation continued. The distillate is tested for:

(*a*) Formic acid, by reduction of ammoniacal silver nitrate and the usual tests.

(*b*) Acetic acid, by the odour; by the production of the red-brown colour with ferric chloride.

(*c*) Propionic acid.

(*d*) Butyric acid. The latter two acids may be separated and determined by use of Duclaux's "distillation ratios" (see Bertrand and Thomas' "Manipulations de Chimie biologique"). Information as to the approximate composition of the mixture may be obtained by determining the acid equivalent by titration with sodium hydroxide using phenolphthalein as indicator, or by formation of the silver salts.

3. *Non-volatile Acids.*—If the fermentation has been carried out in the presence of calcium carbonate the non-volatile acids may be precipitated from the metabolism solution as the calcium salts by the addition of four volumes of alcohol.

(*a*) Bacterial products. The precipitate will probably be calcium succinate or lactate. The salt is acidified to Congo red with sulphuric acid and the solution extracted

with ether. Evaporation of the ether solution to dryness gives a crystalline residue of succinic acid or a liquid residue of lactic acid. Succinic acid may be identified by its melting point (183° C.) and acid equivalent. Lactic acid may be detected by evaporation to dryness, solution of the residue in alcohol, addition of concentrated sulphuric acid and a drop of dilute copper sulphate solution, heating on a boiling-water bath for a few minutes, cooling and addition of an alcoholic solution of thiophen ; a cherry-red colour indicates the presence of lactic acid. Lactic acid may also be isolated and identified as the crystalline zinc salt. Lactic acid may also be found among the volatile acid products since it is somewhat volatile in steam. It is not completely precipitated as the calcium salt by 80 per cent. alcohol, the salt being somewhat soluble.

The test for *pyruvic acid* may also be described here. With the sodium nitroprusside test, as carried out for acetone, a vivid blue colour is given by pyruvic acid. A second test is the addition of a few drops of an alcoholic solution of guaiacol followed by concentrated sulphuric acid to give a separate layer ; a carmine-coloured ring at the junction indicates the presence of pyruvic acid.

(*b*) Mould products. Oxalic, citric, gluconic, fumaric, succinic and malic acids are the most common acid products of fungi. The crystalline calcium salts may be obtained. Oxalic acid may be tested for as described above (p. 464). Gluconic acid may be readily characterised as its phenylhydrazide (m.p. 200° C.). The calcium salt of citric acid is less soluble in hot than in cold water, and is precipitated from fairly strong solutions on boiling. Citric acid may be identified by esterification with methyl-alcoholic hydrochloric acid solution when the trimethyl ester, m.p. 78·5° C., is formed, or by conversion into the amide, m.p. 207° C. Fumaric acid may be extracted with ether, crystallised and identified by its sublimation at about 200° C. and acid equivalent. Malic acid is best

identified by esterification and fractionation of the esters. Some acids, for example spiculisporic acid (see p. 286) from *P. spiculisporum*, are precipitated on acidification of the metabolism solution with hydrochloric acid.

4. *Non-volatile Neutral Products.*—Polysaccharides, if present, will, in general, have been precipitated with the calcium salts of the non-volatile acids on addition of four volumes of alcohol to the metabolism solution. They may be separated from the calcium salts by solution of the precipitate in water, acidification with hydrochloric acid and again precipitating with alcohol, the free acids being soluble in 80 per cent. alcohol. Neutralisation of the solution, freed from the polysaccharides, with calcium carbonate will then result in the precipitation of the calcium salts.

The residue remaining after the distillation of the volatile acids (see p. 465) is neutralised and the solution evaporated to dryness, preferably under reduced pressure, and the residue repeatedly extracted with hot absolute alcohol. The alcoholic solution is evaporated to dryness and the residue once more extracted with alcohol.

(*a*) Mannitol, if present, crystallises out on cooling as long needles, m.p. 168° C. It may be characterised by formation of the tribenzylidene derivative, m.p. 224° C.

(*b*) Addition of four or five volumes of ether to the alcoholic extract precipitates glycerol, trimethylene glycol or butylene glycol, which can be separated by distillation *in vacuo*. Glycerol and trimethylene glycol can be identified also as the benzoyl or phenylurethane derivatives, and butylene glycol as the phenylurethane derivative.

Acetoin may be detected in the metabolism solution by the Voges-Proskauer reaction or by O'Meara's modification (addition of creatine).

5. *Miscellaneous Products.*—The addition of ferric chloride to a metabolism solution (particularly useful in

the case of mould fermentations) may give rise to a range of colours :—

(*a*) Yellow colours (canary to orange yellow) usually indicate the presence of hydroxy-acids, such as gluconic, citric, malic or lactic acids.

(*b*) Blue or violet colours usually indicate phenolic acids.

(*c*) An intense blood-red colour is given by kojic acid (see p. 294).

(*d*) An intense green or black-green is given by citromycetin.

(*e*) A rusty brown-coloured precipitate soluble in excess of ferric chloride to give an iodine brown-coloured solution is given by citrinin (see p. 159).

The presence of unsaturated products may be detected by the addition of bromine water, which is decolorised in their presence.

APPENDIX II

SYNONYMS OF MICRO-ORGANISMS

THE official name according to Bergey "Manual of Determinative Bacteriology" 5th Edition is given in ordinary type; synonyms are given in italics :—

Acetobacter aceti	*Bacillus aceti, B. aceticus, Bacterium aceti, Mycoderma aceti.*
ascendens	*Bact. ascendens*
pasteurianum	*Bact. pasteurianum, Mycoderma pasteurianum*
suboxydans	
xylinum	*Bact. xylinum*
Achromobacter radiobacter	Alcaligenes radiobacter
Actinomyces antibioticus	
cœlicolor	*Act. violaceus, Act. waksmanii, Streptothrix cœlicolor*
lavendulæ	
violaceus	Act. cœlicolor
waksmanii	Act. cœlicolor
Aerobacter ærogenes	*B. ærogenes, B. lactis ærogenes, Bact. ærogenes, Bact. lactis, Bact. lactis ærogenes*
cloacæ	*B. cloacæ*
indologenes	Aer. cloacæ
levanicum	
Alcaligenes fæcalis	*B. alcaligenes, B. fœcalis alcaligenes, Bact. alcaligenes*
radiobacter	*Achromobacter radiobacter, B. radiobacter, Bact. radiobacter, Rhizobium radiobacter*
Azotobacter agile	*Azotobacter vinelandii*
chroococcum	*Azotobacter beijerinckii, B. azotobacter, B. chroococcus*
Bacillus *a*	Lactobacillus casei
aceti	Acetobacter aceti
aceticus	Acetobacter aceti
aceto-ethylicus	Probably identical with B. macerans
acidificans longissimus	Lactobacillus delbrückii

Bacillus *acidipropionici*	Propionibacterium pentosaceum
acidophilus	Lactobacillus acidophilus
ærogenes	Aerobacter ærogenes
ærtrycke	Salmonella typhimurium
alcaligenes	Alcaligenes fæcalis
aminovorans	
amylobacter	Clostridium butyricum
anthracis	
azotobacter	Azotobacter chroococcum
bulgaricus	Lactobacillus bulgaricus
butylicus	Clostridium butyricum
butyricus	Clostridium butyricum
casei α	Lactobacillus casei
casei γ	Lactobacillus brevis
cellulosæ dissolvens	Clostridium dissolvens
chlororaphis	Pseudomonas chlororaphis
chroococcus	Azotobacter chroococcum
cloacæ	Aerobacter cloacæ
coli	Escherichia coli
delbrückii	Lactobacillus delbrückii
diphtheriæ	Corynebacterium diphtheriæ
dysenteriæ Flexner	Shigella paradysenteriæ
dysenteriæ Shiga	Shigella dysenteriæ
enteritidis	Salmonella enteritidis
fluorescens liquefaciens	Pseudomonas fluorescens
friedländeri	Klebsiella pneumoniæ
granulobacter pectinovorum	Clostridium acetobutylicum
influenzæ	Hæmophilus influenzæ
lactis	
lactis acidi	Lactobacillus lactis
lactis ærogenes	Aerobacter ærogenes
lactis pituitosi	Bact. pituitosum
lepræ	Mycobacterium lepræ
macerans	
mesentericus	
methanicus	Methanomonas methanica
morgani	Proteus morgani
mycoides	
nitrobacter	Nitrobacter
oligocarbophilus	Carboxydomonas oligocarbophila
pantotrophus	Hydrogenomonas pantotropha
paratyphosus A	Salmonella paratyphi
paratyphosus B	Salmonella schottmülleri
pneumoniæ	Klebsiella pneumoniæ
polymyxa	
prodigiosus	Serratia marcescens
proteus	Proteus vulgaris
proteus vulgaris	Proteus vulgaris
pyocyaneus	Pseudomonas æruginosa
radicicola	Rhizobium leguminosarum

Bacillus	*radiobacter*	Alcaligenes radiobacter
	subtilis	
	tetani	Clostridium tetani
	thermocellulyticus	*Clostridium cellulyticum*
	tuberculosis	Mycobacterium tuberculosis hominis
	tumefaciens	Phytomonas tumefaciens
	typhosus	Eberthella typhosa
	violaceus	Chromobacterium violaceum
	vulgaris	Proteus vulgaris
	welchii	Clostridium perfringens
	xerosis	Corynebacterium xerose
Bacterium	*aceti*	Acetobacter aceti
	acidipropionici a	Propionibacterium freudenreichii
	ærogenes	Aerobacter ærogenes
	ærtrycke	Salmonella typhimurium
	æruginosum	Pseudomonas æruginosa
	alcaligenes	Alcaligenes fæcalis
	ascendens	Acetobacter ascendens
	coli	Escherichia coli
	dysenteriæ Flexner	Shigella paradysenteriæ
	dysenteriæ Shiga	Shigella dysenteriæ
	enteritidis	Salmonella enteritidis
	enteritidis Breslau	Salmonella typhimurium
	flexneri	Shigella paradysenteriæ
	fluorescens	Pseudomonas fluorescens
	friendländeri	Klebsiella pneumoniæ
	gluconicum	
	influenzæ	Hæmophilus influenzæ
	lactis	Aerobacter ærogenes
	lactis	Streptococcus lactis
	lactis ærogenes	Aerobacter ærogenes
	morgani	Proteus morgani
	nitrobacter	Nitrobacter
	nitrosomonas	Nitrosomonas
	paratyphosum A	Salmonella paratyphi
	paratyphosum B	Salmonella schottmülleri
	pasteurianum	Acetobacter pasteurianum
	pituitosum	*B. lactis pituitosi*
	pneumoniæ	Klebsiella pneumoniæ
	prodigiosum	Serratia marcescens
	pyocyaneum	Pseudomonas æruginosa
	radicicola	Rhizobium leguminosarum
	radiobacter	Alcaligenes radiobacter
	shigæ	Shigella dysenteriæ
	tumefaciens	Phytomonas tumefaciens
	typhosum	Eberthella typhosa
	violaceum	Chromobacterium violaceum
	vulgare	Proteus vulgaris
	xerosis	Corynebacterium xerose
	xylinoides	
	xylinum	Acetobacter xylinum

Beggiatoa	
Betabacterium breve	Lactobacillus brevis
Betacoccus arabinosaceus	Leuconostoc mesenteroides
bovis	Leuconostoc dextranicum
Borrelia recurrentis	*Spirochæta recurrentis* *Spironema recurrentis*
Brucella abortus	
Carboxydomonas oligocarbophila	*B. oligocarbophilus*
Cellfalcicula mucosa	*Cellulobacillus mucosus*
Cellulobacillus myxogenes	Pseudomonas myxogenes
Cellulomonas	
Chlamydothrix ferruginea	Gallionella ferruginea
ochracea	Leptothrix ochracea
Chromobacterium iodinum	
prodigiosum	Serratia marcescens
violaceum	*B. violaceus, Bact. violaceum*
Clostridium acetobutylicum	*B. granulobacter pectinovorum, Cl. acetonigenum*
botulinum	
butyricum	*B. amylobacter, B. butylicus, B. butyricus, Granulobacter butyricum, Gr. pectinovorum, Gr. saccharobutyricum, Plectridium pectinovorum*
cellulosolvens	
cellulyticum	B. thermocellulyticus
dissolvens	*B. cellulosæ dissolvens*
fossicularum	
methanigenes	
perfringens	*B. welchii, Cl. welchii*
sporogenes	
tetani	*B. tetani*
thermocellum	
welchii	Cl. perfringens
Corynebacterium diphtheriæ	*B. diphtheriæ*
xerose	*B. xerosis, Bact. xerosis*
Crenothrix polyspora	
Didymohelix ferruginea	Gallionella ferruginea
Diplococcus *gonorrhœæ*	Neisseria gonorrhœæ
pneumoniæ	*B. pneumoniæ, Bact. pneumoniæ, Str. pneumoniæ, Pneumococcus*
Eberthella typhosa	*B. typhosus, Bact. typhosum*
Enterococcus fœcalis	Streptococcus fæcalis
Escherichia coli	*B. coli, Bact. coli*
Flavobacterium brunneum	
suaveolens	
Flexner's bacillus	Shigella paradysenteriæ

Gallionella ferruginea	*Chlamydothrix ferruginea*
	Didymohelix ferruginea
Gärtner's bacillus	Salmonella enteritidis
Granulobacter butyricum	Clostridium butyricum
pectinovorum	Clostridium butyricum
saccharobutyricum	Clostridium butyricum
Hæmophilus *canis*	H. hæmoglobinophilus
ducreyii	
hæmoglobinophilus	*H. canis*
influenzæ	*B. influenzæ, Bact. influenzæ*
parainfluenzæ	
Hydrogenomonas pantotropha	*B. pantotrophus*
Klebsiella pneumoniæ	*B. friedländeri, B. pneumoniæ, Bact. friedländeri, Bact. pneumoniæ, Klebsiella friedländer, Friedländer's bacillus*
Lactobacillus acidophilus	*B. acidophilus*
arabinosus	Possibly identical with L. plantarum
brevis	*B. casei* γ, *Betabacterium breve.* Possibly identical with L. pentoaceticus
bulgaricus	*B. bulgaricus, Thermobacterium bulgaricum.*
casei	*Bacillus a, B. casei a, Streptobacterium casei*
delbrückii	*B. acidificans longissimus, B. delbrückii*
lactis	*B. lactis acidi, Thermobacterium lactis*
mesenteroides	
pentoaceticus	Possibly identical with L brevis
pentosus	Possibly identical with L. plantarum
plantarum	*Streptobacterium plantarum.* Possibly identical with L. arabinosus and L. pentosus
Lactococcus dextranicus	Leuconostoc dextranicum
Leptospira biflexa	*Spirochæta biflexa*
Leptothrix ochracea	*Chlamydothrix ochracea*
Leuconostoc citrovorum	*Streptococcus citrovorus*
dextranicum	*Betacoccus bovis, Lactococcus dextranicus, Streptococcus paracitrovorus*
mesenteroides	*Betacoccus arabinosaceus*
Methanobacterium aliphatica	
aliphatica-liquefaciens	
omelianski	
Methanomonas methanica	*B. methanicus*
Methanosarcina methanica	Sarcina methanica

Micrococcus *gonorrhœæ*	Neisseria gonorrhœæ
lysodeikticus	
nitrosus	Nitrosococcus
ureæ	
Microspira agar liquefaciens	Vibrio agar liquefaciens
Mycobacterium avium	*Avian tubercle bacillus*
lepræ	*B. lepræ, Leprosy bacillus*
paratuberculosis	*Johne's bacillus*
phlei	
tuberculosis bovis	*Bovine tubercle bacillus*
tuberculosis hominis	*B. tuberculosis. Human tubercle bacillus*
Mycoderma aceti	Acetobacter aceti
pasteurianum	A. pasteurianum
Myxococcus	
Neisseria gonorrhœæ	*Diplococcus gonorrhœæ, Micrococcus gonorrhœæ*
Nitrobacter	*B. nitrobacter, Bact. nitrobacter*
Nitrosococcus	*Micrococcus nitrosus*
Nitrosomonas	*Bact. nitrosomonas*
Pasteurella	
Phytomonas umefaciens	*B. tumefaciens, Bact. tumefaciens, Pseudomonas tumefaciens*
Plectridium ptectinovorum	Clostridium butyricum
Proactinomyces	
Propionibacterium arabinosum	
freuden-reichii	*Bact. acidi propionici* a
pentosaceum	*B. acidi propionici*
Proteus morganii	*B. morgani, Bact. morgani, Morgan's bacillus*
vulgaris	*B. proteus, B. proteus vulgaris, B. vulgaris, Bact. vulgare*
Pseudomonas æruginosa	*B. pyocyaneus, Bact. æruginosum, Bact. pyocyaneum, Ps. pyocyanea*
chlororaphis	*B. chlororaphis*
fluorescens	*B. fluorescens liquefaciens, Bact. fluorescens*
myxogenes	*Cellulobacillus myxogenes*
pyocyanea	Ps. æruginosa
tumefaciens	Phytomonas tumefaciens
Rhizobium leguminosarum	*B. radicicola, Bact. radicicola, Rhizobium radicicolum*
radiobacter	Alcaligenes radiobacter
Rhodobacillus palustris	
Rhodococcus capsulatus	Rhodorrhagus capsulatus
Rhodorrhagus capsulatus	*Rhodococcus capsulatus*
Rhodospirillum rubrum	*Spirillum rubrum*
Rhodovibrio parvus	

Sarcina aurantiaca - - -	
lutea - - - -	
methanica - - -	*Methanosarcina methanica, Zymosarcina methanica*
Salmonella enteritidis - -	*B. enteritidis, Bact. enteritidis, Gärtner's bacillus*
paratyphi - -	*B. paratyphosus* A, *Bact. paratyphosum* A
schottmülleri - -	*B. paratyphosus* B, *Bact. paratyphosum* B
typhimurium - -	*B. ærtrycke, Bact. ærtrycke, Bact. enteritidis* Breslau
Serratia marcescens - - -	*B. prodigiosus, Bact. prodigiosum, Chromobacterium prodigiosum*
Shiga's bacillus - - -	Shigella dysenteriæ
Shigella dysenteriæ - - -	*B. dysenteriæ* Shiga, *Bact. dysenteriæ* Shiga, *Shiga's bacillus*
paradysenteriæ - -	*B. dysenteriæ* Flexner, *Bact. dysenteriæ* Flexner, *Flexner's bacillus*
Spirillum rubrum - - -	Rhodospirillum rubrum
Spironema recurrentis - -	Borrelia recurrentis
Spirochæta biflexa - - -	Leptospira biflexa
recurrentis - -	Borrelia recurrentis
Staphylococcus albus - -	*Staph. pyogenes albus*
aureus - -	
citreus - -	
pyogenes albus -	Staph. albus
Streptobacterium casei - -	Lactobacillus casei
plantarum -	Lactobacillus plantarum
Streptococcus *acidi lactici* -	Str. lactis
citrovorus - -	Leuconostoc citrovorum
cremoris - -	*Str. hollandicus, Str. lactis* B
fæcalis - -	*Enterococcus fæcalis*
hæmolyticus -	Str. pyogenes
hollandicus -	Str. cremoris
lactis - - -	*Bact. lactis, Str. acidi lactici*
lactis B - -	Str. cremoris
paracitrovorus -	Leuconostoc dextranicum
pyogenes - -	*Str. hæmolyticus*
salivarius - -	*Str. viridans*
viridans - -	Str. salivarius
Streptothrix cœlicolor - -	Actinomyces cœlicolor
Sulphomonas - - - -	Thiobacillus
Thermobacterium bulgaricum -	Lactobacillus bulgaricus
lactis - -	Lactobacillus lactis
Thiobacillus denitrificans - -	*Sulphomonas denitrificans*
thio-oxidans - -	*Sulph. thio-oxidans, Thio-bacterium thio-oxidans*
thioparus - -	*Sulph. thioparus*
Thiocystis violacea - - -	
Thiothrix nivea - - -	

Vibrio agar liquefaciens - -	*Microspira agar liquefaciens*
amylocella - - -	
choleræ - - - -	V. comma
comma - - - -	*V. choleræ*
Zymosarcina methanica - -	Sarcina methanica

INDEX

ACETALDEHYDE, condensation, 278
— detection, 464
— fixation, 245
— formation by fungi, 302
— hydrogen acceptor, 202, 204
— intermediate in fat synthesis, 382, *et. seq.*
— — fermentation, 245, 309, 316
— reductase, 212
— — crystalline, 41
Acetarsol, 130
Acetic acid, 245
— — detection, 465
— — fermentation, 322 *et seq.*
— — formation, 247, 254
Aceto-acetic acid, 313, 316
Acetobacter aceti, water content, 58
— *ascendens*, 322
— *pasteurianum*, 322
— *suboxydans*, growth factors for, 101, 118
— — oxidation by, 241, 242
— *xylinum*, cellulose in, 61, 345
— — glyoxalase in, 246
— — oxidation by, 242, 243
— — vinegar fermentation, 322
Acetoin, detection, 467
— formation, 252
— reduction, 253, 278
— Voges-Proskauer reaction, 252
Acetone, 242
— detection, 464
— by fermentation, 312 *et seq.*
Acetonedicarboxylic acid, 284, 299
Acetylmethylcarbinol, see Acetoin
Acid, definition, 4
— production by bacteria, 245 *et seq.*
— — by fungi, 282 *et seq.*
— — theories of, 297
Acid-fast bacteria, fatty acids in, 370
— — phosphorus in ash of, 59
— waxes, 372
Aconitic acid, 285
cis-Aconitic acid, 302
Acridine dyes, drug resistance to, 155
Acriflavine, inhibition of hydrogen transportases, 135
— wound antiseptic, 131
Actinomyces albus, 157
— *antibioticus*, 157
— *cœlicolor*, 391
— *lavendulæ*, 182
— pigments in, 387, 388, 389
— *violaceus*, 157
— *violaceus-ruber*, 391
— *waksmanii*, 390
Actinomycetin, 157
Actinomycin, 157
— A-, 157
— B-, 157, 158
Acyl proteins, 415
Adenine, 333
— as growth factor, 131, 145
— reversal of sulphonamide activity, 145
Adenosine-3-triphosphate, in *Th. thiooxidans*, 72
— -5-triphosphate, 111
— — — in micro-organisms, 72
— — — as phosphate donor, 112, 257,
Adenylic acid, 109, 110, 275, 332
Adsorption, isotherm, 446
— polar groups and, 34, 35
Aeration, effect on lipoid content, 63
Aerobacter aerogenes, 174, 245, 252
— — action of sulphonamides on, 140
— — adaptive enzymes in, 91
— — aneurin in, 103
— — glyoxalase in, 321
— — growth factors in, 115
— *cloacæ*, 226
— *indologenes*, CO_2 fixation, 259
— *levanicum*, levansucrase in, 350

Aerobic oxidation, steps in, 206
Agar as determinant group, 417
Agglutinins, effect of ninhydrin on, 433
— separation, 427
Alanine, degradation, 230, 237
— synthesis, 339
— β-, 101, 110, 224
— — and drug resistant *C. diphtheriæ*, 153, 154
— — in bios, 100
— *d*-, as energy source, 218
Alboleersin, 396
Albucid, 133
Albumin, as antigen, 400
— in protein of hen and duck eggs, 399
— in protein of micro-organisms, 60, 330
— precipitation by ammonium sulphate, 60, 424
Alcaligenes fæcalis, activation as hydrogen donator, 195
— — anaerobic growth, 208
— — aneurin in, 103
— — growth factors in, 115
— *radiobacter*, nitrogen fixation, 221
Alcoholic fermentation, mechanism, 265, 269 *et seq.*
— — Meyerhof's theory of, 274
— — Neuberg's forms of, 272
— — products of, 262
Alcohols, oxidation by *Methanobacterium omelianski*, 76, 78
Aldehyde mutase, 314, 322
Aldobionic acids as determinant groups, 413
Aldol, 279, 313, 316, 382
Alexin, see Complement
Alkyl proteins, 415
Allicin, 185
Amides, utilisation of, 216
Amines, utilisation of, 215
Amino-acids, degradation, 227 *et seq.*
— — by bacteria, moulds and yeasts, 234
— — ease of attack, 216, 237
— — energy source of anaerobes, 218
— — estimation of, 238
Amino-acids in proteins of micro organisms, 60, 330
— requirements of heterotrophs, 81 *et seq.*
— synthesis, 339 *et seq.*
— toxicity to bacteria, 217
— utilisation of, 215
p-Aminobenzene sulphonamide, 131
Aminobenzene sulphonic acids, 406
Aminobenzoic acids, 406
p-Aminobenzoic acid, 99, 101
— — inhibition of sulphonamides, 101, 137
— — ionisation of, 141, 143
p-Aminobenzyl cellobiuronide, 412
— gentiobiuronide, 412
d-Amino-oxidase, prosthetic group, 41, 42
p-Aminophenol-β-galactoside, 411
— α-glucoside, 411
— β-glucoside, 410
ω-Aminovaleric acid, 228, 237
Ammonium borate, 445
— salts, utilisation of, 215
— — — by autotrophic bacteria, 68
— — — by heterotrophic bacteria, 81 *et seq.*
Amœbæ, chemotherapy of, 130
Amygdalin, 37, 51
d-Amyl alcohol, 280
iso-Amyl alcohol, 231, 280
Amylase, activation of, 48
— crystalline, 41
— effect of *p*H on, 48
Anaerobes, absence of catalase from, 199
— growth in oxygen, 207
Aneurin, 393
— diphosphate, 43, 150
— growth factor, 100, 102
— in yeast, 339
— pyrophosphate, as co-carboxylase, 103
Anisoyl proteins, 415
Anthocyanin pigments, 391
Anthranilic acid, in tryptophane synthesis, 343
Anthranols, 396
Anthraquinone pigments, 386, 395
Anti-amylase, 50

Antibiosis, definition, 156
Antibiotics, production by *Aspergillus*, 184
— production by *Penicillium*, 184
— use in selective media, 186
Antibodies, 418 *et seq.*
— antibacterial, 426
— effect of acetylation, 419, 431, 432, 460
— — azo-compounds, 431
— — esterification, 432
— — formaldehyde, 431, 460
— — iodo-groups, 419, 431
— — ninhydrin, 432
— — pepsin, 430
— H- and O- types, heat stability, 430
— molecular weight, 428, 454
— nature, 418
— production, 420 *et seq.*
— — *in vitro*, 424
— properties, 428
— resistance to heat, 429
— separation, 424 *et seq.*
— valency, 423, 455, 457
Anti-enzymes, 50
— immunological, 50
Antigen-antibody reactions, 441 *et seq.*
— — constant antibody method, 448
— — — antigen method, 448
— — forces involved, 456
— — inhibition zones, 448, 451, 452, 458
— — lattice theory of, 457
— — occlusion theory of, 458
— — optimal proportions, 447
— — pro-zones, 448, 451, 452, 458
— — specificity in, 456, 457
Antigens, molecular weight, 456
— multiple valency, 423, 451, 455
— natural, 398 *et seq.*
— reactive groups in, 460, 461
— synthetic, 405 *et seq.*
Anti-lecithinase, 50
Anti-pepsin, 50
Anti-ribonuclease, 50
Anti-trypsin, 50
Antisera, difference between horse and rabbit, 428, 433, 454, 461
Antitoxins, T component, 426
Anti-urease, 50
Apozymase, 269
Arabinose in phosphatides, 375
— in polysaccharides, 353
Arginine, 111
— synthesis, 343
Arsine derivatives from fungi, 305
Arsenoxides, action on SH groups, 152
Arsphenamine, 127
Ascorbic acid, 244, 288, 297
— — and complement, 439
— — in fungi, 65
— dehydrogenase, 41
Ash, see Mineral constituents
Asparagine, 111
— utilisation, 218
l-Aspartic acid, degradation, 230
— — energy source, 219
— — synthesis, 340
Aspergillic acid, 158
Aspergillin, 388, 393
Aspergillus candidus, 159
— — antibiotic from, 183
— *clavatus*, 160
— *elegans*, 303
— *fischeri*, 378
— *flavipes*, antibiotic from, 184
— *flavus*, 158, 161
— — kojic acid from, 295
— — penicillin formation by, 171
— *fumigatus*, 162, 163, 164, 167, 393, 394
— *giganteus*, 160, 163
— *glaucus*, 394, 395
— mannitol in, 62
— *melleus*, 308
— *nidulans*, 303
— *niger*, citric acid in, 292, 327
— — ergosterol, 378
— — gluconic acid, 292, 304
— — mannitol, 304
— — mycogalactan, 351
— — oxalic acid, 292
— — starch, 346
— — trehalose in, 62
— — utilisation of carbon source, 191
— *ochraceus*, 308
— *oryzæ*, 294, 302, 378
— *parasiticus*, 170

Aspergillus sydowi, 305, 378
— *terreus*, 306, 308, 399
— vitamin-C in, 65
Aspirin, antisera to, 415
— as determinant group, 415
Assimilation, 240
Astacin, 390
Atebrine, 128, 130
— inhibition of *Plasmodium* respiration, 151
Athiorhodaceæ, metabolism of, 74, 76
— pigments, 391
Atoxyl, 405
— azo-proteins, 405, 406, 449
— in chemotherapy, 127
— inhibition by *p*-aminobenzoic acid, 138
— — of lipase, 135
Atropine, inhibition of invertase, 135
Auroglaucin, 394
Autocatalysis, 331, 342
Autotrophic bacteria, 67 *et seq.*
— — chemosynthetic, 67, 78
— — classification, 69
— — photosynthetic, 67, 78
— — thermodynamic efficiency, 79
Avidin, inhibition of biotin by, 104
Azotobacter, fixation of nitrogen, 214
— *chroococcum*, fixation of nitrogen, 221 *et seq.*
— — gum of, 348
— — nucleic acids, 336
— — pigment, 389, 391
— — sterol in, 377
— utilisation of nitrate and nitrite, 215

Bacillus aceto-ethylicus, 245, 246, 312
— — fermentation of pentosans by, 312
— *acidificans longissimus*, 319
— aerogenes, see *Aerobacter aerogenes*
— *aminovorans*, 328
— *anthracis*, capsule of, 183, 338
— — carbohydrates of, 362, 365
— — *d*-glutamic acid in capsule, 183, 338
— — growth factors for, 106
— — — — — in, 116
— — inhibition of, 156
— — nucleic acids of, 336
Bacillus brevis, 165
— fæcalis alcaligenes, see *Alcaligenes fæcalis*
— *lactis*, 351
— — *pituitosi*, 351
— *macerans*, 312
— *mesentericus*, aneurin in, 116
— — antibiotic from, 183
— — capsule of, 183, 338
— — *d*-glutamic acid in capsule, 183, 338
— — levan, 349
— — *niger*, pigment, 386, 388
— *mycoides*, vitamin-B in, 65
— *polymyxa*, levansucrase in, 350
— prodigiosus, see *Serratia marcescens*
— pyocyaneus, see *Pseudomonas aeruginosa*
— *subtilis*, adenosine triphosphate in, 72
— — *d*-glutamic acid in capsule, 183, 338
— — levan, 349
— — proteolytic enzymes, 225
— — subtilin, 185
— — vitamin-B in, 65
— typhosus, see *Eberthella typhosa*
— *volutans*, 315
Bacitracin, 185
Bacterial gums, 326, 348
Bacterio-chlorin, 73, 387, 390, 391
— erythrin, 73, 390
— phæophytin, 390
— purpurin, 64, 69, 73, 390
— xanthophyll, 390
Bacterium cocovenenans, 390
— *gluconicum*, oxidation by, 243, 244
— *xylinoides*, oxidation by, 244
— — kojic acid from, 294
Bæyer-205, 126
Base, definition, 4
B.C.G., carbohydrates of, 360
— sterols in, 377
Beggiatoa, 69, 72
Benzaldehyde, reduction of, 278
Benzidine-R-salt, 418
Betabacterium vermiforme, dextran synthesis, 348
Biological reduction, 278

Bios, 99
— composition, 100
Biotics, 117
Biotin, 99, 100, 103, 110, 111. 305
Blood Group A ploysaccharide, 357
— — — — — as determinant group, 417
Botrytis cinerea, antibiotic from, 185
— — thio-urea from, 305
Bromo-proteins, 414
Brucella abortus, carbon dioxide requirement, 83, 255
— — growth factors for, 104, 118
Buffer solutions, 15 *et seq.*
— — amino-acids as, 18
— — composition, 18
— — dilution of, 16
— — effect of acids, 16, 17
— — — of alkalies, 16, 17
— — range, 18
— — relation to *p*K, 17
— — proteins as, 18
— — universal, 18
Butyl alcohol detection, 464
Butyl alcohol-acetone fermentation, 314 *et seq.*
— — — mechanism, 316
— — — products of, 316
1 : 3-Butylene-glycol, 279
2 : 3-Butylene-glycol, 253, 278
— detection, 467
Butyric acid, 245, 314, 369
— — detection, 465
Byssochlamic acid, 291
Byssochlamys fulva, 303

Calomel electrode, 11
Cannizzaro reaction, 248, 274, 303, 304
Carbasone, 130
Carbohydrates, content of bacteria, fungi and yeasts, 61
— "reserve," 61
— soluble specific substances, 353
— synthesis, 366
Carboligase, 253, 278
Carbon content of bacteria, fungi and yeasts, 59, 66
Carbon dioxide, fixation, 83
— — — autotrophic bacteria, 67, 68, 73, 76, 77, 78, 256
Carbon dioxide, fixation, distribution in acid products, 259
— — — heterotrophic bacteria, 250, 255
— — — phosphorylation, 257
— — — succinic acid formation, 251
— — — — — — by *A. niger* and *Rhizopus nigricans*, 294
— — requirement by heterotrophic bacteria, 87, 255
Carboxydomonas oligocarbophila, 75
Carboxylase, 43, 52, 212, 248, 272, 277
— absence from propionic acid bacteria, 254
— constitution, 43
— in amino-acid degradation, 230, 234
Carboxypeptidase, crystalline, 41
Cardiolipin, 403
Carlic acid, 287
Carlosic acid, 287
Carolic acid, 287
Carolinic acid, 287
Carotene, 385, 390
β-Carotene, 390, 393
Carotenoid pigments, 64, 385
Carviolacin, 395
Carviolin, 395
Casein, antigenicity, 399, 400
Catalase, 212
— crystalline, 41
— effect on notatin activity, 169
— hæm in, 41
— in bacterial respiration, 198
— in *Micrococcus lysodeikticus*, 93
— inhibition, 200
— prosthetic group, 41
Catalysts, 38
— mode of action, 53
Catenarin, 395
Cellobiase, 326
Cellobiose, 326
Cellobiuronic acid, 348, 355
Cellulase, 326
Cellulobacillus mucosus, 325, 326
— *myxogenes*, 325, 326
Cellulomonas, 325
Cellulose, degradation, 323 *et seq.*
— — thermophilic, 324

Cellulose, in *Acetobacter xylinum*, 61, 324, 345, 351
— in bacteira, 61
— staining by zinc chloriodide, 56
Cerebrin, 378
Cerotic acid, 370
Chemoreceptors, 135
Chemosynthetic bacteria, 67
Chemotherapeutic agents, modes of action, 134 *et seq.*
— index, 124
Chemotherapy, essential metabolite theory, 137
— of bacterial infections, 131
— of protozoal infections, 126
Cherry gum as determinant group, 417
Chinoform, 130
Chitin, in micro-organisms, 61
Chlorine metabolism of fungi, 306
Chlororaphin, 179, 391
Cholera red reaction, 210
Cholestan, 404
Cholesterol, 372, 377
— as antigen, 404
— growth factor, 121
Choline sulphate, 305
Chromatographic analysis, 385
Chromobacterium iodinum, 167, 392
— *violaceum*, 185, 388, 389
Chromoparous organisms, 64
Chromophoric organisms, 64
Chymotrypsin, crystalline, 41
— crystallisation of, 46
Chymotrypsinogen, crystallisation of, 45
Cinnamyl proteins, 415
Citric acid, 286, 292, 297, 327
— — detection, 466, 468
Citrinin, 159, 283, 397
— detection, 468
Citromycetin, 396
— detection, 468
Citrulline in arginine synthesis, 346
Clasterosporum, 303
Clavacin, 160
— inactivation by SH., 161
Clavatin, 160, 161
Claviformin, 160, 161
Clostridium acetobutylicum, butyl-alcohol-acetone fermentation, 314
Clostridium acetobutylicum, CO_2 fixation, 260
— — growth factors for, 101, 104, 118
— *botulinum*, antitoxin, 426
— — growth factors, 85, 114
— *butylicum*, growth factors, 104, 118
— *butyricum*, fermentation by, 314
— — fixation of nitrogen by, 220, 222
— — glycogen in, 345
— — granulose, 62
— — growth factors for, 101, 118
— — inhibition of, 156, 315
— *cellulolyticum*, 324
— *dissolvens*, 324
— *fossicularum*, 323
— *histolyticum*, antitoxin, 426
— *methanigenes*, 323
— *œdematiens*, antitoxin, 426
— *septicum*, antitoxin, 426
— *sordellii*, antitoxin, 426
— *sporogenes*, aerobic growth, 198, 211
— — growth factors, 114, 118
— *tetani*, growth factors for, 101, 113, 118
— *thermocellum*, 324
— *welchii*, antitoxin, 426
— — CO_2 fixation, 260
— — growth factors, 114, 118
— —hyaluronidase in, 358
— — pigment of, 391
— — α-toxin, 50
Cocaine, inhibition of invertase, 135
Co-decarboxylase, 112, 150, 259
Co-enzyme I, 49, 263, 267
— constitution, 268
— hydrogen carrier, 202, 203, 204, 268, 276, 320
— identity with V-factor, 110
— in acetic acid fermentation, 322
— — alcoholic fermentation, 263
— — lactic acid fermentation, 249, 320
— — propionic acid fermentation, 249
— magnesium in, 267, 269
— nicotinamide in, 109, 267
— occurrence, 268
Co-enzyme II, as growth factor, 110
— as hydrogen carrier, 204
— nicotinamide in, 109

Co-enzyme R, identity with biotin, 104
Colloids, 26 *et seq.*
— cadmium sulphide sol, 27
— coagulation, 28 *et seq.*
— — zone of, 29
— critical surface potential, 28, 29, 30, 33
— effect of electrolytes, 28 *et seq.*
— emulsions, 26
— ferric hydroxide sol, 28
— foams, 27
— fogs, 27
— gold sol, 27
— lyophilic, 27 *et seq.*
— lyophobic, 27 *et seq.*
— particle size, 26, 27
— polysaccharides, 27, 30, 33
— proteins, 27, 30, 33
— smokes, 27
— stability, 27, 28
— suspensions, 26
Comenic acid, 294
Comparator, Lovibond, 15
Complement, 433 *et seq.*
— artificial, 434, 439
— inactivation by dialysis, 438
— — by iodine, 438
— lability, 434
— role of components, 436, 437
— structure of, 434 *et seq.*
Continuous phase, 26
Copper protein enzymes, 52
Corynebacterium diphtheriæ, antitoxin, 419, 426, 431
— — — crystalline, 427
— — — effect of pepsin on, 430
— — carbohydrates, 61
— — drug resistant strains, 153, 154
— — fatty acids in, 371
— — growth factors for, 101, 108, 110, 111, 118
— — inhibition by pantoyltaurine, 149, 150
— — lipoids of, 62
— — metabolism, 80, 85, 216
— — nucleic acids of, 60, 336
— — phosphatides of, 381
— — porphyrins of, 391
— — waxes of, 377
Corynin, 381
Cozymase, see Co-enzyme
Crenothrix, 70
p-Cresol from tyrosine, 233
Cyclopium viridicatum, 176
Cynodontin, 395
Cystein, 207, 211
— de-amination, 237
Cytidilic acid, 333
Cytochrome, 105, 200
— distribution, 201, 202
— inhibition, 201
— in respiration, 201
— oxidase, 212
Cytophaga hutchinsoni, 324, 326
Cytosine, 333

Danysz phenomenon, 443, 446, 447
Deaminase, 212
Deamination, desaturative, 229
— hydrolytic, 229
— oxidative, 230
— reductive, 228
Decarboxylase, amino-acid, 111
Decarboxylation of amino-acids, 228
Dehydrase, 197
Dehydrogenase, 197
— inhibition, 49
— in respiration, 201
Desmolases, 53
Desoxyribonucleic acids, 336
— in Rough-Smooth conversion, 337
Desoxyribose, 333
Desthiobiotin, 104
Determinant groups, 406, 423
Dextran, 346, 348, 401
— constitution, 347
— serological reactions, 346
Diaphorase, 212
— and co-enzyme, 206
— prosthetic group, 41, 42
Diasone, 134
Diastase, 37
Diazotised arsenilic acid hapten, 458
Didymohelix, 70
— *ferruginea*, 74
Dibromo-cholesterol, 404
Dibromo-tyrosine, 414
Dihydro-cholesterol, 404

Dihydro-co-enzyme I, 203, 268, 276, 277, 321
Dihydro-erdin, 306
Dihydro-geodin, 306
Dihydro-penicillic acid, 171
Dihydro-penicillin I, 172
Dihydro-phenazine-1-carboxylamide, 392
Dihydroxyacetone from glycerol, 243
— phosphate, 274, 276, 320
(3:5-Dihydroxy-2-carboxybenzyl) methyl ketone, 290
(3:5-Dihydroxy-2-carboxybenzoyl) acetyl carbinol, 290
3:5-Dihydroxyphthalic acid, 291
Dihydroxy-stearic acid, 369
Di-iodophenol, 414
Di-iodotyrosine, 414
αγ-Diketo-adipic acid, 298
βγ-Diketo-adipic acid, 299
Dimedon, 77, 260, 272, 384
4:6-Dimethoxytoluquinone as bacteriostatic agent, 163
Dimethylpyruvic acid, 285
Dimethyl-selenium, 305
Dimethyl-tellurium, 305
Diphosphoglyceric acid, 276, 320
Diphosphopyridine nucleotide, 110, 202, 267
Diphtheria bacillus, see *Corynebacterium diphtheriæ*
Diphtheric acid, 371
Diplococcus pneumoniæ, antibodies to, 412, 427, 428, 431
— — antisera, cross reactions, 348, 412
— — autolytic enzymes in, 358, 364
— — carbohydrate F, 354, 357
— — carbohydrates of, 353, 401
— — growth factors for, 106
— — inhibition by pantoyltaurine, 149
— — Rough-Smooth conversion, 337
— — sulphonamide resistant strains, 152
— — synthetic antigens, 412, 413
Dismutation, 248
Disperse phase, 26
— — charge on particles, 27
— — size of particles, 26, 27
Dissimilation, 240
Dissociation constant, acetic acid, 10
— — acids, 5
— — bases, 6
— — phosphoric acid, 10
— — water, 6
Drug resistance, 151 *et seq.*
— — development of, 154

Eberthella typhosa, carbohydrates of, 362, 363, 402, 417
— — co-enzyme in, 109
— — exacting and non-exacting strains, 83
— — fermentation products of, 248
— — inhibition by indole-acrylic acid, 147
— — variants, 83
— — vitamins in, 114
Egg albumin-antiserum system, 454
Ehrlich's phenomenon, 443, 446
d-2-Eicosanol, 373, 374, 377
Electrode potentials, calomel, 11
— — hydrogen, 11, 12
— — quinhydrone, 13
Electrodes, calomel, 11
— glass, 12, 13
— hydrogen, 11, 12
— quinhydrone, 12
Electrophoresis, 425, 426
Elution, 45
Emetine, 125, 128
Emodic acid, 395
Emodin monomethyl ether, 395
Emulsin, 37
— specificity, 50
— synthesis by, 40
Endo-enzymes, 53
Endomyces vernalis, lipoid content, 63, 371, 381
— — resistance to pyrithiamine, 151
End-piece, 435, 437
Energy requirements of micro-organisms, 188
Energy liberated in oxidations, 191
Enolase, 212, 276, 320
Entamœba histolytica, 125, 128
Enterokinase, 49
Enzymes, 36 *et seq.*
— activators, 48

Enzymes activity, measurement of, 46
— adaptive, 89
— — speed of production, 96
— adsorption of, 45
— anti-enzymes, 50
— as catalysts, 38
— chemical nature of, 40 *et seq.*
— classification, 52
— colloidal carriers, 41
— — nature, 39
— constitutive, 89 *et seq.*
— crystalline, 41
— crystallisation of, 45
— effect of concentration, 46
— — of heat, 47
— — of *p*H, 48
— — of substrate concentration, 47
— — on equilibrium of reactions, 39
— — on velocity of reactions, 39
— endo-cellular, 53, 189
— exo-cellular, 53, 188
— inhibition by carbon monoxide, 49
— — by chloroform, 49
— — by cyanide, 49
— — by drugs, 135
— — by heavy metals, 49, 135
— — by substrate analogues, 135, 196
— — by sulphides, 49
— — by urethane, 49
— isolation of, 38
— nomenclature, 52
— physical properties, 43 *et seq.*
— prosthetic groups of, 41 *et seq.*
— purification, 45
— relation to cells, 38
— reversible effect of, 40
— separation, 44
— specificity, 50
— stability, 40
— synthesis by, 40
— theories of action, 53
— units, 46
— variability of content in micro-organisms, 95
— Warburg's yellow enzyme, 41, 42
Epitoxoid, 443
Epitoxonoid, 444
Equivalence point, 450
Erdin, 306
Ergosterol, 372
— as antigen, 404
— in fungi and yeasts, 63, 377
Ergosteryl palmitate, 63, 378
Erythroglaucin, 395
Escherichia coli, action of sulphonamides on, 139, 140
— — activation of hydrogen donators by, 194, 195, 209
— — adenine triphosphate in, 72
— — anaerobic growth, 208
— — aneurin in, 103, 115
— — carbohydrates of, 362
— — carbon dioxide fixation, 259
— — — — — and succinic acid formation, 260
— — — requirement, 82
— — co-enzyme in, 109
— — fermentation products of, 248, 250
— — growth in synthetic media, 82
— — inhibition by indole-acrylic acid, 147
— — — β-naphthyl-acrylic acid, 148
— — — pyrithiamine, 151
— — — sulphanilamide, 149
— — metabolism, 81
— — production of indole, 233
— — sterols in, 377
— — variants, 89, 96
— — vitamins in, 65, 115
— — water content, 58
Eserine, inhibition of esterase, 135
Ethyl acetate, 303
Ethyl alcohol, detection, 464
— — production by bacteria, 249, 251, 312
— — — by fungi, 302
— — — by yeasts, 262 *et seq.*
Ethyleneoxide-α-β-dicarboxylic acid, 284
Euglobulin, 424
— solubility, 60
Exacting strains, 83, 96
Exo-enzymes, 53

Fats, 369
— of yeast, 63, 371
— staining by dimethyl-amido-azobenzene, 62

Fats, staining by dimethyl-*p*-phenylene diamine, 62
— — α-naphthol, 62
— — osmic acid, 56
— — Sudan III, 56
— synthesis, 381 *et seq.*
Fatty acids, 62
— — of acid-fast bacteria, 371
— — of *C. diphtheriæ*, 371
— — of micro-organisms, 369
— — of moulds, 371
— — of yeasts, 371
Fermentation, nature of, 37, 192, 326
Fibrinogen as antigen, 399
Ficin, crystalline, 41
Flavacidin, 161
Flavicin, 161
Flavine adenine dinucleotide, 42, 248
Flavobacterium brunneum, 387
— *suaveolens*, 389
Flavoglaucin, 394
Flavoprotein enzymes, 41, 42, 52, 169, 212
— — and co-enzyme I, 203
Flavorhodin, 390
Fluoride, inhibition of alcoholic fermentation by, 275, 277
Folic acid, 104 *et seq.*
Formaldehyde, by reduction of carbon dioxide, 260
— effect on protein antigens, 401, 417
— fixation by dimedon, 77, 260
Formic acid, 245
— — breakdown, 203, 247, 248
— — detection, 465
— — formation, 247, 260
Formic dehydrogenase, 212
Forsmann antigen, 354, 357
Fourth component of complement, 436, 438
Friedländer's bacillus, polysaccharides, 354, 358
Fructosan, 348, 351
Fructose-1: 6-diphosphate, 266, 275
— — — in *Th. thio-oxidans*, 72
Fulvic acid, 291
Fumarase in *Micrococcus lysodeikticus*, 93
Fumaric acid, 202, 229, 258, 284, 293
— — detection, 464, 466
Fumaryl-*dl*-alanine, 286
Fumigacin, 162
Fumigatin, 163, 179, 393
Funiculosin, 395
Fusarium, 302
— *culmorum*, 396
— *javanicum*, antibiotic from, 185
— pigments of, 387
Fusel oil, 231, 279 *et seq.*

Galactan, 351
Galactose, fermentation by yeast, 89, 96
— in polysaccharides, 353
Galacturonic acid as determinant group, 412
Gallic acid, 289
Gallionella, see *Didymohelix*
Gas production by bacteria, 248, 251
Gas ratios, 251
Gelatin, 398
— conversion to antigen, 414
Gelatinase, formation by *Proteus vulgaris*, 96
Gentisic acid, 289, 307
Gentisyl alcohol, 307
Geodin, 306
Germanin, 126
Gigantic acid, 163
Glass electrode, 12, 13
Glaucic acid, 291
Glauconic acid, I, 291
— — II, 291
Gliocladium fimbriatum, 164
Gliotoxin, 164
Globulin, α-, 424, 426
— β-, 424, 426
— γ-, 424, 426
— T-, 426
— as antigen, 400
— carbohydrate in, 426
— in antibodies, 419
— in protein of micro-organisms, 60, 330
— precipitation by alcohol, 425
— — by ammonium sulphate, 60, 424
— shape of molecule, 428
Gluconic acid, 242, 285, 292, 300, 327
— — detection, 466, 468
Glucose monophosphate, 264

Glucose oxidase, 212, 293
— -1-phosphate, 72
— -6-phosphate, 72
O-β-Glucosidyl-tyrosine, as determinant group, 414
O-β-Glucosidyl-tyrosyl-gelatin, 414
— — insulin, 414
Glucosone, 295
Glucuronic acid, 285, 348
— — as determinant group, 412
— — intermediate in citric acid production, 300
Glutamic acid, 110, 111
— — as energy source, 219
d-Glutamic acid, 183, 338
l-Glutamic acid, degradation, 230
Glutamine as growth factor, 106
Glutathione, 136, 207
— as prosthetic group of glyoxalase, 208
— in bacterial respiration, 207
Glyceraldehyde, 270
3-Glyceraldehyde phosphate, 202, 270, 274, 276
Glycerol by fermentation, 310
— detection, 467
— formation in alcoholic fermentation, 269, 273, 277
— — by fungi, 302
— oxidation of, 243
Glycerophosphoric acid, 202
Glycine, 218
— degradation, 228, 232, 237
— methylamine from, 228
Glycogen as antigen, 301
— in *Aspergillus*, 345
— in bacteria, 345
— in yeast, 62, 345
— staining by iodine, 56
Glycollic acid, 284
— — intermediate in citric acid production, 300
Glyoxalase, 208, 212, 246, 321
Gonococcus, carbohydrates of, 365
— metabolism of, 81
— pyruvic oxidase in, 43
Gramicidin, 165
— action of formaldehyde on, 166
Gramicidin-S, 166
Gram stain, ribonucleic acid and, 337
Granulose, 62
Griseo-fulvin, 307
Growth factors, 82, 84, 98 *et seq.*
— — adenine, 101
— — β-alanine, 100, 101
— — *p*-aminobenzoic acid, 101
— — aneurin, 100, 102
— — assay of, 116
— — bios, 99
— — biotin, 100, 103
— — *Clostridium*, 85, 99, 103
— — *H. influenzæ*, 86, 107, 109, 110
— — inositol, 100, 107
— — *l*-leucine, 100, 107
— — nicotinamide, 108
— — nicotinic acid, 100, 108
— — pantothenic acid, 109
— — pyridoxine, 100, 111
— — requirement by heterotrophs, 82
— — sporogenes factor, 114
— — staphylococcus factor, aerobic, 113
— — — — anaerobic, 113
— — uracil, 113
— — *V*-factor, 86, 110
— — *X*-factor, 86, 107
Guaiacum, 37
Guanidine, effect on sulphonamide activity, 145
Guanine, 333
Guanylic acid, 332
l-Guluronic acid, 243
Gum acacia as determinant group, 417

Hæmatin as growth factor, 107, 110
Hæmocyanin in enzymes, 41
Hæmoglobin as antigen, 399
Hæmolysin, separation, 427
Hæmophilus canis, *V*- and *X*-factors, 86, 110
— — symbiosis, 110
— *influenzæ*, growth factors, 86, 107, 109, 110, 119
— — metabolism, 81, 216
— — *V*-factor, 86
— — *X*-factor, 86, 107
— *parainfluenzæ*, amino-acid degradation by, 230
— — growth factors, 110, 119
— — symbiosis, 111
— *pertussis*, growth factors, 108, 119

Haptens, 406
— combination with antibodies, 442, 452, 458
— complex, 442, 458
— simple, 442, 452
" Heavy " carbon, as tracer element, 247, 256
Helminthosporin, 64, 395
Helminthosporium avenæ, 395
— — antibiotic from, 185
— *catenarium*, 395
— *cynodontis*, 395
— *euchlanæ*, 395
— *geniculatum*, 303
— *gramineum*, 64, 395
— *leersii*, 396
— *ravenelli*, 395, 396
— *tritici-vulgaris*, 395
— *turcicum*, 395, 396
Helvolic acid, 162, 167
Hemipyocyanin, 178, 179
Heparin, 50
Heterobiotin, 104
Heterotrophic bacteria, 80 *et seq.*
Hexacosanoic acid, 374, 375
Hexadecenoic acid, 371
Hexose diphosphate, 320
Hexose monophosphate, 266
Hexose monophosphorylase, 212
Hexose phosphates, 264
Histamine, antisera to, 417
— as determinant group, 416
Histidine, 216, 405
— deamination, 230, 237
Histone, bacteriostatic action, 185
Holozymase, 269
Hyaluronic acid, 358
Hyaluronidase, 358
— in *Cl. welchii*, 95
Hydrogenase, 212
— in nitrogen fixation, 224
Hydrogen bacteria, 70, 74, 207
— donators, 194, 208
— — activation of, 194, 208
— — inhibition of, 196
— — mechanism, 195
— electrode, 11, 12
— ions, acidity due to, 7
— — concentration in acetic acid, 7
— — — in hydrochloric acid, 7
Hydrogen ions, concentration in water, 7
— — — measurement of, colorimetric, 13 *et seq.*
— — — — — electrometric, 11 *et seq.*
— — in water, 6
Hydrogenlyase, 94, 212, 260
Hydrogenomonas, 70
— metabolism of, 74
Hydrogen peroxide in bacterial respiration, 198 *et seq.*
— production from cellulose, 323
— — from formic acid, 249
— sulphide, activation of papain, 48
— —oxidation by sulphur bacteria, 72, 73
— transportase, 197
Hydrolases, 52, 188
p-Hydroxybenzoic acid, 232
Hydroxy-emodin, 395
Hydroxylamine in nitrogen metabolism, 223
Hydroxyl ions, alkalinity due to, 7
— — concentration in water, 7
— — in water, 6
2-Hydroxymethyl-5-carboxyfurane, 288
α-Hydroxyphenazine, 391
p-Hydroxyphenyl-lactic acid, 229
l-Hydroxyproline, 218
Hypoxanthine, 219, 336

Iminazole-acrylic acid, 230
Indicators of oxidation-reduction, 23, 24
— of *p*H, 13, 14
— — Congo-red, 14
— — litmus, 14
— — methyl orange, 13
— — phenolphthalein, 14
— — ranges of, 15
Indole-acrylic acid, antibacterial action, 147
Indole in tryptophane synthesis, 343
— from tryptophane, 209, 233
Indole-carboxylic acid, 232
Indole-lactic acid, 229
Indole-propionic acid, 228
Influenza bacillus, see *H. influenzæ*

Inositol as growth factor, 107, 110
— in bios, 100
— in phosphatides, 375, 379, 380
— relation to streptomycin, 182
Insulin, conversion to antigen, 414
— non-antigenicity, 399, 400
Invertase, 40
— inhibition, 49
— purification, 45
— specificity, 51
Iodinin, 168, 392
Iodo-proteins, 414
Ionic product, 6, 7
— — determination, 7
Ionisation, degree of 7
— of acids, 5, 7
— of bases, 6
— of water, 5, 7
Ions, activity, 5
Iron bacteria, 70
— — metabolism, 74
Isomerase, 212, 276, 320
Isopropanol, fermentation product, 318
— oxidation to acetone, 74, 242
Itaconic acid, 285

Johne's bacillus, nutrition of, 87
— — vitamins in, 114

Kala-azar, chemotherapy of, 129
Kanten, 417
Kephalin, 381, 403
— in antisera, 428
Keratin, as antigen, 400
α-Keto-acids, 230
— in protein synthesis, 340
β-Keto-acids, 232
2-Ketogluconic acid, 242, 244
5-Ketoglutaric acid, 242
α-Ketogluconic acid, 230
2-Keto-*l*-gulonic acid, 244
γ-Ketopentadecoic acid, 285
Klebsiella pneumoniæ, carbohydrates of, 354, 358
Kojic acid, 163, 171, 289, 294, 300
— — detection, 468
Kryptoxanthin, 390

L_4dose of toxin, 442
L_o dose of toxin, 442

Lactase, 39
Lactic acid, 245
— — as hydrogen donator, 194, 196, 208, 209
— — bacteria, 318
— — — growth factors for, 110, 113
— — breakdown to propionic acid, 254
— — detection, 466, 468
— — enzyme, 212, 321
— — enzymic breakdown, 135
— — fermentation, 249, 318 *et seq.*
— — — phosphorylation, 249, 320
— — formation, 208, 246
— — production by fungi, 247, 248, 251, 282, 284, 293
Lactic dehydrogenase, 212
Lactobacillus acidophilus, action of sulphonamides on, 140
— — cholesterol in, 377
— — fatty acids in, 371
— — phosphatides of, 381
— *arabinosus*, amino-acid requirements, 216
— — growth factors for, 104, 111, 113
— *bulgaricus*, 319
— *casei*, 146, 319
— — growth factors, for 104, 105, 106, 111, 112, 113
— *delbrückii*, 41, 43, 248, 319
— — glyoxalase in, 321
— — growth factors for, 105, 111, 112
— — pyruvic oxidase in, 43
— — "yellow enzyme" in, 41
— drug resistant strains, 153
— growth factors for, 105, 106, 111, 113, 116, 119
— *lactis*, growth factors for, 111
— use in assay of growth factors, 116
— *pentoaceticus*, 321
— — adaptive enzymes of, 93
— *pentosus*, growth factors for, 111
Lactoflavin-5-phosphoric acid, 42
Lauric acid, 369
Lecithin, 378, 381, 403
— in antisera, 428
Lecithinase, *Cl. welchii* α-toxin, 50
Leishmania, chemotherapy of, 130
Lentinus lepideus, 307
Leprosinic acid, 374, 375, 377
Leprotin, 390

Leptothrix, 70
— *crassa*, 74
— *ochracea*, 79
iso-Leucine, degradation, 280
— toxicity to bacteria, 218
l-Leucine, as energy source, 218
— as growth factor, 107
— degradation, 231, 280
— in bios, 100
— toxicity to bacteria, 217
Leucoflavoprotein as hydrogen carrier, 205
Leuconostoc citrovorum, 319
— *dextranicum*, 319, 348
— *mesenteroides*, adaptive enzymes in, 92
— — dextran in, 346
— — drug resistant strains, 153
— — glyoxalase in, 321
— — growth factors for, 111, 112, 113 119
Levan, 349
Levansucrase, 350
Linoleic acid, 369
Linolenic acid, 369
Lipase, nature of, 40
— specificity, 51
— synthesis by, 40
Lipocyan reaction, 386
Lipoids, 369
— as antigens, 402 *et seq.*
— content in bacteria, fungi and yeasts, 62
— "firmly bound," 376
— in complement, 436
— of acid-fast bacteria, 372 *et seq.*
Lovibond comparator, 15
Luteic acid, 351
Luteoleersin, 396
Lutein, 390
Luteose, 351
Lysine, 111
— phenylureide as hapten, 416
Lysozyme, 157, 365
— crystalline, 41
— substrate of, 157

Malic acid, 258, 284, 293, 300, 410
— — detection, 467, 468
Malonic acid, 284, 299
Maltase, adsorption of, 45
— specificity, 50
— synthesis by, 40
Mannitol, 300, 303 *et seq.*
— detection, 467
— in micro-organisms, 62
Mannocarolose, 352
Mannonic acid, 285, 297
Mannose in phosphatides, 375, 379, 380
— in polysaccharides, 352, 353
Mapharsen, 127
Marfanil, inhibition by *p*-aminobenzoic acid, 138
Melanin pigments, 64, 386
Melezitose, 349, 350
Mellein, 307
Meningococcus carbohydrates, 365
Mepacrine, 128
Mercuric chloride as antiseptic, 136
Metabolic products, separation, 462 *et seq.*
Metachromatic granules, nucleic acid of, 60
— — staining, 56
Methane bacteria, 75
— production, 323 *et seq.*
Methanobacterium omelianski, oxidation by, 76, 78
Methanol reduction, 76
Methanomonas aliphatica, 75
— *aliphatica-liquefaciens*, 75
— *methanica*, 75
Methionine, reversal of sulphonamide activity, 145
Methoxydihydroxytoluquinone, 179, 388
Methyl anisate, 307
— cinnamate, 307
Methylene blue as hydrogen acceptor, 202
— — technique, 193
Methylglyoxal, 208, 245, 272
— conversion to lactic acid, 246
— dismutation of, 273
— hydrate, 250, 271
Methylheptanone, 278
Methyl mercaptan, 305
— *p*-methoxycinnamate, 307
6-Methyl salicylic acid, 289, 308

γ-Methyltetronic acid, 286, 294
Micro-aerophilic organisms, absence of catalase, 199
Micrococcus lysodeikticus, carboxylase in, 259
— — polysaccharide in, 365
— — production of enzymes by, 93
— *ureæ*, metabolism, 190
Mid-piece, 435, 438
Mineral constituents in bacteria, fungi and yeasts, 58
— — phosphorus in, 59
— — potassium in, 59
Minioluteic acid, 286
Monilia, lactic acid from, 282
Monochloracetic acid, 446
Mono-iodoacetic acid, inhibition of alcoholic fermentation by, 275, 278
Mucoproteins, 61
Mucor, alcohol production, 302
— glycerol production, 302
— lactic acid production, 321
— *rammanianus*, growth factor for, 102
— *rouxii*, 282
Mutase, 193, 273
Mycobacterium leprœ, pigment of, 390
— —trehalose in, 359, 371
— *phlei*, extract, 114
— — nucleic acids of, 336
— — nutrition of, 86
— — pigment of, 390
— — soluble specific substances, 358
— — sterols of, 377
— — waxes of, 373, 377
— *tuberculosis*, carbohydrates of, 61, 353, 358
— — glycogen in, 345
— — lipoids of, 62, 373
— — nucleic acids of, 336
— — nucleoproteins of, 60
— — nutrition of, 85, 87
— — phosphatides of, 64, 379
— — soluble specific substances, 358
— — sterols in, 377
— — waxes in, 373, 374, 377
Mycodextran, 351
Mycogalactan, 351
Mycolic acid, 374, 375, 377
Mycolic acid, α-, 375
— — β-, 375
— — γ-, 377
Mycophenolic acid, 185, 291, 296
Mykol, 372
Myristic acid, 370
Myxococcus, 357

Naphthoquinones, 386
β-Naphthyl-acrylic acid, antibacterial action, 148
Neisseria gonorrhoæ, pyruvic oxidase in, 248
Neurospora crassa, growth factors for, 101, 117, 121
— — use in assay of amino-acids, 117
— — — — of growth factors, 116
— *sitophila*, growth factors for, 112, 121
— — use of mutants in amino-acid synthesis, 343
Niacin, 109
Nicotinamide, 108, 110
Nicotinic acid, 100, 108
— — in yeast, 339
Nitrate reduction, 209
— utilisation, 70, 82, 215
Nitrifying organisms, 70
— — metabolism of, 70
Nitrite reduction, 209
— utilisation, 70, 84, 215
Nitrobacter, 69, 70
Nitrobenzene, reduction of, 278
Nitrogen content of bacteria, fungi and yeasts, 59, 66
— estimation, 59
— fixation of, 214, 220 *et seq.*
— — mechanism, 223
— metabolism of heterotrophs, 81 *et seq.*
— requirements of micro-organisms, 214
Nitrosococcus, 69
Nitrosomonas, 69, 70, 215
— fixation of formaldehyde from, 71
Nomenclature of bacteria, 3
— of enzymes, 52
Non-exacting strains, 83
Norleucine, toxicity to bacteria, 218
Notatin, 168

Nucleic acids, constitution, 332
— — *Azotobacter chroococcum*, 336
— — *B. anthracis*, 336
— — *Cl. welchii*, 337
— — in *C. diphtheriæ*, 336
— — *Esch. coli*, 337
— — metachromatic granules, 60
— — *M. phlei*, 336
— — — *tuberculosis*, 336
— — pneumococci, 337
— — psittacosis virus, 336
— — *Sacch. cerevisiæ*, 60, 332, 337
— — *Staphylococcus*, 337
— — *Streptococcus*, 336
— — vaccinia virus, 336
Nuclein, 59, 332
Nucleoproteins, content of bacteria, 60, 332, 336
— constitution of, 332 *et seq.*
— hydrolysis of, 60, 332
— in *M. tuberculosis*, 60, 336
— in tobacco mosaic virus, 332
— in yeast, 60, 337
— properties of, 332
— staining by polychrome methylene blue, 56
Nucleosides, 332
Nucleotides, 333

Ochracin, 308
d-2-Octadecanol, 373, 374, 377
O-heterobiotin, 104
Oleic acid, 369, 378
Oospora lactis, fat in, 371
— — vitamin-B in, 65
— *sulphurea-ochracea*, 306, 396
Optoquin, 131
Ornithine, breakdown, 228
— in arginine synthesis, 343
Orotic acid, 105, 113
Oxalacetic acid, 223, 258, 259, 298, 302
Oxalic acid, detection, 464
— — production by fungi, 284, 292, 298
Oxidases, 193
— activation of, 49
— and cytochrome system, 201
— in bacterial respiration, 200
— inhibition, 49, 197, 200
— — by cyanide, 135, 200
Oxidation by oxygen, 197
— energy from, 191, 192
— mechanism, 192 *et seq.*
— of acetaldehyde, 193
— of carbon, 19
— of ethyl alcohol, 19
— of ferrous salts, 20
— of hydroquinone, 19, 192
— of methane, 19
— transfer of electrons, 20
— types of, 197
Oxidation-reduction potentials, 19 *et seq.*
— — — interpretation, 24
— — — measurement of, colorimetric, 23 *et seq.*
— — — — electrometric, 20 *et seq.*
— — use of, 19
Oxycellulose, 326
Oxychlororaphin, 391
Oxycholesterol, 404

Palitantin, 308
Palmitic acid, 369, 379
Paludrine, 128, 130
Pantothenic acid, 100, 101, 109, 148
— — antagonism by salicylate, 153
— — in bios, 100
— — in human blood, 150
— — in mouse blood, 150
— — in rat blood, 150
— — occurrence, 109
— — properties, 110
Pantoyltauramide, antibacterial action, 148
Pantoyltaurine, 125
— antibacterial action, 148
— — — inhibition of, 149
— resistant strains, 153
Papain, activation of, 48
— crystalline, 41
— synthesis by, 342
Parachromophoric organisms, 64
Parasiticin, 170
Parietin, 395
Pasteurella, growth factors for, 108
Patulin, 160, 161, 170
— effect on common cold, 161
Penaldic acid, 173, 174

Penatin, 168, 170
Penicillamine, 173, 174
Penicillic acid, 163, 170, 288, 296
Penicillin, 171, 283, 305
— -A, 168
— -B, 168, 176
— -F, 172
— -G, 172
— -X, 172
— -K, 172
— I, 172
— II, 172
— III, 172
— activity, 175
— drug resistance to, 155, 176
— in chemotherapy, 175
— purification, 172
— structure, 174
— unit, 174
Penicillinase, 174, 176
Penicillium aurantio-brunneum, 378
— *aurantio-griseum*, 378
— *aurantio-virens*, 176
— *brevicaule*, 305
— *brevi-compactum*, 185, 378
— *carminoviolaceum*, 395
— *charlesii*, 296, 352
— *chrysogenum*, 283, 303, 305
— — penicillin from, 171
— *citreoroseum*, 395
— *citrinum*, 159
— *citromyces*, 327, 397
— *citromyces-glabrum*, 396
— *claviforme*, 160
— *cyclopium*, 170, 395
— *digitatum*, 303
— *expansum*, 351
— *funiculosum*, 395
— *glaucum*, 346
— *griseo-fulvum*, 307, 308
— *italicum*, 378
— *javanicum*, 378
— *johannioli*, 176
— *luteum*, 293, 327, 351
— mannitol in, 62
— *notatum*, 168, 171, 283, 305
— *palitans*, 308
— *patulum*, 160, 307
— *phœniceum*, 394
— *puberulum*, 176, 296, 378
Penicillium resticulosum, antibiotic from, 184
— *rubrum*, 394
— *spiculisporum*, 286
— *spinulosum*, 179, 388, 394
— *stoloniferum*, 296
— *varians*, 352
— vitamin-C in, 65
Penicilloic acid, 173, 174
Penillamine, 173, 174
Penillic acid, 173, 174
iso-Penillic acid, 173, 174
Penillo-aldehydes, 173, 174
Pentacosanoic acid, 375
Pentamidine, 129
Pentosans, fermentation of, 312
Pentoses, in nucleoproteins, 62
Pepsin, 37
— crystalline, 41
— crystallisation, 45
— effect of *p*H on, 48
— specificity, 51
— synthesis by, 40
Pepsinogen, crystallisation of, 45
Peptidases, specificity of, 51, 330
Peptides as determinant groups, 407, 440
Peptone, action of papain, 48
— — of trypsin, 49
— in micro-organisms, 60
Peroxidase, 212
— crystalline, 41
— in bacterial respiration, 199
— inhibition, 200
— nature of, 40
— purification, 45
— test for, 200
*p*H, definition, 8
— effect on bacteria, 4
— — on enzymes, 4
— — on pepsin, 4
— — trypsin, 4
— — on *Vibrio comma*, 4
— of acids, 8, 9, 10
— of alkalies, 8, 9
— of blood, 8
— of water, 8
— determination of, colorimetric, 13 *et seq.*
— — electrometric, 11 *et seq.*

Phenazine pigments, 168, 177, 178, 179, 391
— — effect on quinones, 168
Phenol from tyrosine, 233
Phenylacetic acid, 231
Phenylalanine, absence from mould proteins, 60
— as energy source, 218
— deamination, 229
— in antigens, 400
Phenylarsenoxide, 127
— reaction with SH groups, 136
Phenyl-lactic acid, 229
Phenylureides, 416
Phenylureido-proteins, 416
Phleimycolic acid, 375
Phœnicin, 394
Phosphatase, 265
— production by propionic acid bacteria, 96
Phosphates, in fermentation, 249 *et seq.*, 263
— in metabolism of autotrophic bacteria, 72, 78
Phosphatides, constitution, 378
— content of bacteria and yeasts, 63, 378, 379
— of acid-fast bacteria, 379, 380
— of *C. diphtheriæ*, 381
— *of L. acidophilus*, 381
— *of M. tuberculosis*, 379
Phosphogluconic acid enzyme, 212
Phosphoglyceraldehyde, 320
— as hydrogen acceptor, 202
Phosphoglyceric acid, 249, 266, 275
— — breakdown inhibited by fluoride, 254, 275
— — in lactic acid fermentation, 321
— — in propionic acid fermentation, 254
Phosphoglycero-mutase, 212, 276
Phosphopyridine nucleotides, 110
Phosphopyruvic acid, 247, 248, 249, 250, 266, 275, 277, 321
Phosphorylase, potato, 367
Phosphorylation in citric acid fermentation, 298
Photobacterium fischeri ,158
Phthiocerol, 373, 37
Phthiocol, 392
— as growth factor, 114
Phthioic acid, 370, 374, 376, 379
Phycomyces blakesleeanus, 102
— — growth factors for, 121
Physcion, 395, 396
Phytochemical reduction, 279
Phytoglycol, 372
Phytomonas tumefaciens, 362, 381
— — polysaccharide, 348
Phytomonic acid, 381
Pigments, carotenoid, 385, 387
— classification, 387
— melanin, 386
— production by micro-organisms, 64
— quinone, 386
Pilocarpine, inhibition of invertase, 135
Pimelic acid, 111,
*p*K, definition of, 10
— of acetic acid, 10
— of phosphoric acids, 10
Plasmodia, chemotherapy of, 130
— inhibition of respiration, 151
Plasmoquin, 128, 130
— inhibition of *Plasmodium* respiration, 151
Plasteins, 399
Pneumococcus, see *Diplococcus pneumoniæ*
Polar groups, 30 *et seq.*
— — and adsorption, 34
— — and solubility, 33, 34
— — and specificity, 34
— — behaviour of, 33
— — distribution of, 34
— — effect in antigen-antibody reactions, 458
— — — of masking, 34
— — — on activation of hydrogen donators, 195
— — — on antibody production, 423
— — — on determinant groups, 407, 409
— — in polysaccharides, 33, 34
— — in proteins, 33, 34
— — strength of, 32
— — structure of, 31
Polypeptides in micro-organisms, 60
— utilisation of, 219

Polysaccharides, as antigens, 401, 417
— F68 fractions, 361
— isolation, 360 *et seq.*, 467
— of bacteria, fungi and yeasts, 61, 345 *et seq.*
— synthesis of, 257, 366
Polyuronides, 326
Porphyrin-protein enzymes, 52
Porphyrins, 391
Power alcohol, 311
— gas, 323 *et seq.*
— — from cellulose wastes, 325
— — — sewage, 325
— — mechanism of production, 326
Precipitin reactions, 447 *et seq.*
— — composition of precipitate, 448, 449, 454
— — reacting groups in, 456, 459
— — theories of, 450 *et seq.*
Proactinomycin, 176
Prodigiosin, 387, 389
Proflavine, as wound antiseptic, 131
— drug resistance to, 155
Proline, absence from mould proteins, 60
— as energy source for anaerobes, 218
— degradation, 225, 237
Promin, 134
Prontosil, 123, 131, 133
Propamidine, 129
— drug resistance to, 155
Propionibacterium pentosaceum, 258
— — fixation of CO_2, 258
— — formation of succinic acid, 258
Propionic acid, 245, 319
— — bacteria, 253
— — — fermentation of glucose, 254
— — — — of glycerol, 253, 259
— — — phosphorylation, 249, 254, 255
— — detection, 465
Proseptasine, 133
Prosthetic group in enzymes, 41 *et seq.*
— — growth factors and, 115
Protamine, action of trypsin, 49
— bacteriostatic action, 185
— in micro-organisms, 60
Proteinases, 342
Proteins, action of trypsin, 49
Proteins, amino-acids from, 329
— as antigens, 398
— conjugated, 60
— constitution, 328
— content of bacteria, fungi and yeasts, 59
— degradation, 224 *et seq.*
— hydrolysis, 329
— method of estimation, 59
— molecular weight, 328
— of tobacco mosaic virus, 330
— sparing action of carbohydrates, 226
— synthesis, 338 *et seq.*
— utilisation, 219
Proteolytic enzymes, 225, 227, 342
— — synthesis by, 342
Proteoses, utilisation of, 219
Proteus morganii, drug resistant strains, 153
— — growth factors for, 110, 119
— *vulgaris*, action of pyridine-3-sulphonic acid, 146
— — — of sulphonamides, 140
— — anaerobic growth, 208
— — aneurin in, 103 115
— — carbohydrates of, 365
— — carbon dioxide fixation, 259
— — growth factors for, 108, 110 119
— — proteolytic enzymes of, 225, 227
Protoplasm, 59
Pseudoglobulin, 424
— solubility, 60
Pseudomonas aeruginosa, action of sulphonamides on, 140
— — carbohydrates of, 362
— — pigment of, 64, 388, 389, 391
— — pyocyanase formation, 177
— *chlororaphis*, 179, 391
— *fluorescens*, 209, 228
— — adenosine triphosphate in, 72
Pseudopyridoxine, 112
Pteroic acid, 106
Pteroylglutamic acid, 106
Ptyalin, activation of, 48
Puberulic acid, 176, 290
Puberulonic acid, 177
Purines, from nucleoproteins, 60, 332
— utilisation of, 219
Putrefaction, nature of, 192
Pyocyanase, 177

Pyocyanase, lytic acition, 177
Pyocyanin, 177, 388, 391
— as hydrogen acceptor, 202, 391
Pyracin, 106
Pyridine-3-nitrile, 108
Pyridine-3-sulphonamide, 146, 149
Pyridine-3-sulphonic acid, 146
Pyridino-protein enzymes, 52
Pyridoxal, 112
Pyridoxamine, 112
Pyridoxine, 100, 111
— acetyl derivatives, 112
Pyrimidines, as growth factor, 102
— from nucleoproteins, 60, 332
— utilisation of, 219
Pyrithiamine, antibacterial activity, 151
Pyruvic acid, 245, 284
— — anaerobic dismutation, 248
— — breakdown, 247
— — carboxylation, 259
— — decarboxylation, 241, 271, 272, 273
— — detection, 466
— — fixation of, 246
Pyruvic oxidase, 212, 248
— — occurrence, 43
— — prosthetic group, 43

Quinhydrone electrode, 12
Quinic acid, 389
Quinine, 125, 128, 130
— inhibition of lipase, 135
— — of *Plasmodium* respiration, 151
Quinone pigments, 386
— — in oxidation-reduction systems, 393, 394

Racemiase, 321
Raffinose, 349, 350
Ravenellin, 306, 396
Reductase, 193
— acetaldehyde, crystalline, 41
Residual antigens, 353
Respiration, definition, 187
Rhizobium, fixation of nitrogen, 214, 223
— growth factors for, 104, 120
— *leguminosarum*, fixation of nitrogen, 223
Rhizobium, polysaccharide, 348
— — cross reaction with pneumococcus antisera, 348
Rhizopus chinensis, 282
— lactic acid production, 321
— *oryzæ*, lactic acid from, 282, 293
Rhodobacillus palustris, 357
Rhodococcus, pigment of, 387
Rhodopin, 389
Rhodopurpurin, 390
Rhodotorula ruber, growth factor for, 102, 120
Rhodovibrin, 389
Rhodovibrio, pigments of, 389
Rhodoviolascene, 389
Riboflavin adenine dinucleotide as hydrogen acceptor, 204 *et seq.*
— as growth factor, 99, 113
— in yeast, 339
— 5-phosphoric acid, in enzymes, 42
Ribonuclease, crystalline, 41
Ribonucleic acid, 334
— — in Gram staining, 337
Ribose-3-phosphate, 333
Robison's ester, 264
R-salt-azo-diphenyl-azo-egg albumin 449, 454
Rubrofusarin, 396

Saccharic acid, 285, 298, 299
Saccharomyces cerevisiæ, fixation nitrogen by, 215
— — Gram staining, 337
— — synthesis of proteins by, 33
— growth factors for, 101, 107, 111 112, 121
— *pulcherrimus*, pigment of, 388
Salicylate, antagonism to pantothenic acid, 153
Salmonella paratyphi, amino-acid degradation by, 232
— — carbohydrates of, 362
— *schottmülleri*, 232, 362
— *typhimurium*, 360, 402
— — action of sulphonamides on, 1
Salvarsan, 124, 127
Sarcina aurantiaca, 387, 390
— *lutea*, 387, 390
— *methanica*, 76
— xanthine, 390

Scatole from tryptophane, 233
Schizophyllum commune, 305
Serine, deamination, 237
— in tryptophane synthesis, 343
— toxicity to bacteria, 218
Serratia marcescens, anaerobic growth, 208
— — carbohydrates of, 362
— — pigments of, 64, 389
— — proteolytic enzymes of, 225
— — vitamins in, 116
Shigella dysenteriæ, carbohydrates of, 362, 401, 417
— — glycogen in, 345
— — growth factors for, 108, 109, 120
— — variants, 89
— *paradysenteriæ*, carbohydrates of, 363
Sideromonas, 70
Silk fibroin, constitution, 329
— — haptens from, 408
Soluble specific substances, 61, 353
— — — F68 fractions, 361
— — hydrolysis by enzymes, 357
— — — of *B. anthracis*, 362, 365
— — — of B.C.G., 360
— — — of *Brucella*, 366
— — — of *Cl. welchii*, 366
— — — of *C. diphtheriæ*, 366
— — — of *Diplococcus pneumoniæ*, 353
— — — — — — Type I, 354, 355, 401, 454, 460
— — — — — — Type II, 354, 355, 412
— — — — — — Type III, 354, 355, 356, 365, 412, 448, 450, 454, 461
— — — — — — Type IV, 354, 357
— — — — — — Type VIII, 354, 357, 412
— — — — — Type XIV, 354, 357
— — — of *E. typhosa*, 362, 402
— — — of Friedländer's bacillus, 354
— — — of gonococcus, 365
— — — of *H. influenzæ*, 366
— — — of *H. parapertussis*, 366
— — — of *H. pertussis*, 366
— — — of *Leptospira biflexa*, 366
— — — of meningococcus, 365
— — — of *M. phlei*, 358
Soluble specific substances of *M. tuberculosis*, 358, 359, 375
— — — of *Pasteurella*, 366
— — — of *Proteus*, 362, 365
— — — of *Sal. typhimurium*, 360, 402
— — — of *Shigella dysenteriæ*, 362
— — — of *Staph. aureus*, 363
— — — of *Strep. salivarius*, 364
— — — of *Vibrio comma*, 364
Soluseptasine, 133
Sorbitol oxidation of, 243, 244, 304
Sorbose bacillus, 242
Spiculisporic acid, 286, 467
Spinulosin, 179, 393
— dimethyl ether, 163
Spirilloxanthin, 390
Spirillum rubrum, 390
Spirochætes, chemotherapy of, 127, 130
Spores, water content of, 58
Sporogenes, factor, 114
Staphylococcus albus, phosphorylation enzymes in, 250
— antitoxin, 426
— *aureus*, action of pyridine-3-sulphonic acid on, 146
— — — of sulphonamides on, 140
— — adenosine triphosphate in, 72
— — carbohydrates of, 363
— — drug resistant strains, 155
— — growth factors for, 108, 113, 120
— — inhibition by pyridine-3-sulphonamide, 149
— — — by pyrithiamine, 151
— — pigments of, 387, 390
— *candidus*, CO_2 fixation, 259
— *citreus* 387,
— growth factors for, 104, 108, 109, 112, 113
Starch as antigen, 401
— in bacteria and fungi, 346
— staining by iodine, 56
— synthesis of, 367
Stearic acid, 369
Sterols, 371, 403
— in bacteria, 63, 377
— in yeast, 63
Stibacetin, 130
Stibenyl, 130
Stickland reaction, 218, 228, 230

Stilbamidine, 129
Stipitatic acid, 291
Streptamine, 181
Streptidine, 181
Streptobacterium plantarum, growth factor for, 111
Streptobiosaminide, 181
Streptococcus, antitoxin, 426
— *bovis*, dextran synthesis, 348
— *cremoris*, 319
— growth factors for, 106, 110, 112, 113
— Group N, antibiotic in, 185
— *hæmolyticus*, growth factors for, 109, 113, 120
— — pyruvic oxidase in, 43
— *lactis*, 105, 106, 112, 246, 259, 319, 321
— *paracitrovorus*, CO_2 fixation, 259
— *pyogenes*, drug resistant strains, 153
— — inhibition by iodinin, 392
— — — pantoyltaurine, 148
— — — sulphanilamide, 149
— respiration of, 198, 211
— *salivarius*, 358
— — carbohydrates of, 364
— — dextran synthesis, 348
— — levan synthesis, 351
Streptomyces griseus, 180
Streptomycin, 180
— inactivation, 180
— purification, 180
Streptose 181
Streptothricin, 180, 182
Strychnine, antisera to, 413
— as determinant group, 413
Substrate, definition of, 47
Subtilin, 185
Succinic acid, 245, 300, 410
— — detection, 464, 466
— — formation of, 250, 251, 284, 293
— — inhibition of hydrogen donators by, 196
— dehydrogenase, 212
— — inhibition, 135
Sulamyd, 133
Sulochrin, 306, 396
Sulphacetamide, 133
— activity, 144
— as determinant group, 413
— ionisation of, 143
Sulphadiazine, 132
— activity, 144
Sulphadiazine, inhibition by *p*-aminobenzoic acid, 139
— ionisation of, 141, 143
Sulphaguanidine, 133
— inhibition by *p*-aminobenzoic acid, 139
Sulphamerazine, 145
Sulphamethazine, 144
Sulphanilic acid, 406, 413
— — azo-proteins, 406
Sulphanilamide, 131
— activity, 144
— as determinant group, 413
— inhibition of action, 136, 137, 149
— — by *p*-aminobenzoic acid, 139, 140
— ionisation of, 141, 143
Sulphapyridine, 132, 146
— activity, 144
— as determinant group, 413
— inhibition by *p*-aminobenzoic acid, 138, 139
— ionisation of, 141, 143
Sulphasuxidine, 134
Sulphathiazole, 132
— activity, 144
— and decarboxylation, 147, 150
— as determinant group, 413
— inhibition by *p*-aminobenzoic acid, 138, 139, 140
— ionisation of, 141, 143
Sulphonamides, 132 *et seq.*
— and respiration, 147
— antibacterial index, 139
— antisera to, 413
— as determinant groups, 413
— inhibition by adenine, 145
— — by *p*-aminobenzoic acid, 138
— — by methionine, 145
— ionisation of, 141 *et seq.*
Sulphones, 134
Sulphur bacteria, 69, 70
— — bacterio-purpurin in, 64, 390
— — green, 391
— — metabolism of, 70 *et seq.*
— — pigments of, 387, 390, 391
— compounds in fungi, 305
— oxidation by sulphur bacteria, 71 *et seq.*

Sulphur reduction by yeast, 279
Sulphydryl groups, in enzymes, 136
— — reaction with phenylarsenoxide, 136
Symbiosis, 102, 109
Synthalin, 129
Synthetic media, 81, 462

Tartranilic acids as determinant groups, 409
Terrein, 308
Terrestric acid, 288
Tetracosanoic acid, 371, 374, 375
Tetranucleotides, 335
Tetrathionates, oxidation by sulphur bacteria, 71, 72
Tetronic acids, 171, 296
Thiamine, see Aneurin.
Thiazole as growth factor, 102
Thiobacillus, 69
— *denitrificans*, 69, 72
— *thio-oxidans*, 69, 71, 115
— — adenine triphosphate in, 72
— — aneurin in, 103
— — growth factors in, 115
— *thioparus*, 69, 70
Thiochrome, 393
Thiocystis, 69, 72
Thiorhodaceæ, pigment, 391
Thiosulphate, oxidation by sulphur bacteria, 71, 72
— reduction by yeast, 279
Thiothrix, 69, 72
Thio-urea, effect on sulphonamide activity, 145
— production by fungi, 305
Third component of complement, 435, 438
Threonine, toxicity to bacteria, 217
Thymine, 106, 333
Thymonucleic acids, 333
Thyroxine, antisera to, 415
— as determinant group, 415
Toluquinone derivatives as antibiotics, 163
— pigments, 386, 393, 394
Torula lipofera, 371
— — lipoid content of, 63, 371
— pigments in, 387
— *rosea*, vitamin-B in, 65
Torula rubra, 393
— *utilis*, protein synthesis by, 339
Torulene, 393
Toxin-antitoxin reactions, 442 *et seq.*
— — colloidal character, 446
Toxoflavin, 390
Toxoid, 417
Transaminase, 224
— in amino-acid synthesis, 224
Transamination, 341
Trehalose, in *Aspergillus niger*, 62
— in *M. phlei*, 359, 373
— — *tuberculosis*, 359, 371, 373
— in yeasts, 62
Tricarboxylic acid cycle, 302
Trichoderma viride, antibiotic from, 185
Tricosanoic acid, 374
Trimethyl arsine, 304
Trimethyleneglycol, 467
Triphosphopyridine nucleotide, 110, 268
Triose phosphate, dismutation, 204
Triose phosphorylase, 212, 320
Tritisporin, 395
Trypanosomes, arsenic resistant strains, 126, 152
— chemotherapy of 125 *et seq.*, 130
— growth factors for, 121
Trypan-red, 126
Tryparsamide, 127
Trypsin, 37
— activation of, 49
— crystalline, 41
— crystallisation of, 46
— effect of *p*H on, 48
— specificity, 51
Trypsinogen, 49
Tryptophane, 81, 84, 216, 399, 400
— degradation of, 209, 228, 229, 231, 232, 233
— in antigens, 399, 400, 405
— synthesis of, 343
Tryptophol, 231
Tubercle bacillus, see *Mycobacterium tuberculosis*
Tuberculostearic acid, 369, 370, 374, 376, 379
Tyrocidine, 182
— action on fungi, 183
— hæmolytic effect, 183

Tyrocidine hydrochloride, 166, 182
Tyrosinase, 52, 386
— copper in, 41
Tyrosine, 111
— absence from mould proteins, 51
— degradation of, 229, 231, 232, 233, 386, 389
— in antigens, 399, 400, 401, 405, 414
Tyrosol, 231
Tyrothricin, 165, 182

Uleron, 134
Uracil, 333
— as growth factor, 113
Urea, as energy source, 189, 211
— effect on sulphonamide activity, 145
Urease, crystalline, 41
— inhibition of, 49
— in *Micrococcus lysodeikticus*, 93
Uridylic acid, 333
Urocanic acid, 230

n-Valeric acid, from proline, 237
d-Valine as energy source, 218
Varianose, 352
Verticillium albo-atrum, 305
V-factor, 86, 110
Vibrio agar-liquefaciens, 325
— *amylocella*, 325
— *comma*, carbohydrates of, 353, 362, 364
— — co-enzyme in, 109
— — vitamins in, 116
Vinegar fermentation, 322
Violacein, 388, 389
— as antibiotic, 185
Viridin, 185
Virus, metabolism, 81
— of tobacco mosaic, 330 *et seq.*
Vitamin-A, absence from bacteria and yeasts, 65
Vitamin-B in bacteria, 65, 100
Vitamin-C, absence from bacteria and yeasts, 65
— production by fungi, 65
Vitamin-D, absence from bacteria and yeasts, 65
— from ergosterol, 63, 377
Vitamin-H, identity with biotin, 104
Vitamins, bacterial, 98, 116, 245
Voges-Proskauer reaction, 252
Volutin, staining of, 56

Warburg's yellow enzyme, crystalline, 41
— — — constitution, 42, 113
— — — riboflavin-5-phosphoric acid in, 42
Wassermann reaction, 403
Water content of bacteria, fungi, yeasts and spores, 58
— dissociation, 5
— hydrogen ions in, 6
— hydroxyl ions in, 6
Waxes, 371
— of acid-fast bacteria, 373, 374
— of *C. diphtheriæ*, 377
— of micro-organisms, 63

X-factor, 86, 107
Xanthine oxidase as bacteriostatic agent, 169
Xanthophylls, 385
Xanthopterin, 105
Xylonic acid, 293

Yeasts, amino-acid degradation by, 234
— carbohydrate content of, 61
— fatty acids in, 371
— fermentation of galactose by, 89, 96
— gums, 345
— fixation of nitrogen by, 215
— juice, 38
— — in fermentation, 263
— — zymase in, 53
— nucleic acids of, 332, 337
— nucleoproteins of, 60
— phosphatide content, 64
— proteins in, 338
— sterol content, 63
— *Torula*, pigments of, 64
— water content of, 58
Yellow enzyme (Warburg's), 113, 169
— — as hydrogen carrier, 206
— — constitution, 42, 113
— — crystalline, 41

Zeaxanthin, 390
Zephiran, lytic action, 177
Zinc chloriodide, 56
Zone phenomenon, 251
Zymase, 53, 268
Zymin, 263, 280, 435
Zymohexase, 212, 275, 320
Zymosan, 436