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Physiology 1943

ANNUAL REVIEW OF PLANT PHYSIOLOGY

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ANNUAL REVIEW OF PLANT PHYSIOLOGY

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VOLUME 4

1953

PUBLISHED BY
ANNUAL REVIEWS, INC.

ANNUAL REVIEWS, INC.
STANFORD, CALIFORNIA, U.S.A.

FOREIGN AGENCIES

H. K. Lewis & Company, Limited
136 Gower Street
London, W.C. 1

Maruzen Company, Limited
6, Tori-Nichome Nihonbashi
Tokyo

PRINTED AND BOUND IN THE UNITED STATES OF AMERICA BY
GEORGE BANTA PUBLISHING COMPANY

PREFACE

Again, it is our pleasure to express gratitude to the reviewers whose efforts made this volume possible. We also wish to acknowledge with thanks the generous evaluations received from many colleagues about the function which the young *Review* is performing in advancing teaching and research in various fields of plant physiology.

The present volume, like its predecessors, does not aim at a complete coverage of the many phases of plant physiology. Our policy continues to be to review, at yearly intervals, only areas of great research activity and published output. Areas in which the volume of published contributions is more limited are reviewed at less frequent intervals. In this way, it is hoped that complete coverage of the current advances in plant physiology may be provided, not in a single year, but over a period of several years.

The rules governing the rotation of the Editorial Committee provide for the retirement of one member each year. To Professor K. V. Thimann, who is currently retiring, we wish to express our appreciation for the time and effort which he has generously given to the *Review* since it was first established.

We wish to acknowledge with thanks the loyal services of Beryl Daniel as editorial assistant, and the help given us by our printers, the George Banta Publishing Company.

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NITROGEN METABOLISM OF HIGHER PLANTS[1,2]

By J. G. Wood

Department of Botany, University of Adelaide, South Australia

This review deals with problems of nitrate reduction and of the metabolism of amino acids, amides, and proteins which are discussed in the light of work published since the recent reviews of Street (1), McKee (2) and Steward & Thompson (3).

Nitrate Reduction

Path of nitrate reduction.—Since the work of Meyer & Schulze (4) the opinion has been latent in the literature that reduction of nitrate in plant cells proceeds by successive steps through nitrate, hyponitrite and hydroxylamine to ammonia. More recently the hypothesis was given prominence by Chibnall (5) and Burström (6). It received support from the work of Virtanen and Arhimo (7) who reported that they had detected both nitrite and hydroxylamine in plants kept for 24 hr. in dilute nitrate solutions and concluded that only in the absence of α-keto acids does the reduction proceed to ammonia. In the presence of keto acids the hydroxylamine reacts with them to produce the corresponding oximino acids which are then reduced to amino acids. From these observations stems the so-called "alternative path" theory of amino acid formation, i.e. either by reduction of the corresponding α-oximino acid or by combination of ammonia with the corresponding α-keto acid.

A number of bacteria readily form nitrite from nitrate and the former accumulates in the culture medium [Street (1)]. Maeda (8) and Sakamura & Maeda (9) have reported accumulation of nitrite and of hydroxylamine in *Hansenula.*

Many observers have reported the occurrence of small amounts of nitrites in higher plants, though Steinberg (10) contends that reports of occurrence of nitrite and of hydroxylamine are caused by nitrohydroxamic acid which is relatively nontoxic to cells. Earlier reports of occurrence of nitrite in higher plants have been reviewed by Burström (6). Recently Steward & Street (11) have reported small amounts of nitrite in legumes and Kolesnikov (12) claims that chloroplast suspensions from barley leaves produce in light a substance, presumed to be nitrite, liberating iodine from potassium iodide; Rautenan (13) grew pea plants in nitrate solutions for periods of less than 24 hr. and could detect only traces of nitrite which did not accumulate though nitrate content increased rapidly. Jones *et al.* (14) used soybeans and

[1] The survey of the literature pertaining to this review was completed in July, 1952.

[2] The following abbreviations will be used: ATP, adenosinetriphosphate; DPN, diphosphopyridine nucleotide.

claim that nitrites accumulate in culture solutions in absence of air and of manganese. Robinson (15) found accumulation of nitrite in anaerobic cultures only when contaminated with nitrite-producing bacteria. The available evidence suggests that nitrite may occur in higher plants in extremely low concentration and reports of accumulation are probably due to bacterial contamination.

It is doubtful whether the occurrence of free hydroxylamine has ever been demonstrated unequivocably in plant tissues. Maeda (8) reported its presence in yeast; Lemoigne, Monguillon & Desvaux (16, 17), Virtanen and Arhimo (7), and Steward & Street (11) have reported hydroxylamine in fresh leaves. All these estimations of hydroxylamine were made using Blom's (18) method. Steward and Street (11) refer to difficulties involved in this method which depends on determination of nitrite present in deproteinized extracts before and after oxidation of hydroxylamine to nitrite. Csaky (19) has shown that nitrous acid, hyponitrous acid, and nitrohydroxamic acid destroy hydroxylamine under the normal conditions of Blom's method. Our own experience has shown the difficulty of completely extracting small quantities of nitrite from plant materials. Rautenan (13) could not detect free hydroxylamine in pea seedlings during growth for 24 hr. in nitrate solutions nor could Kolesnikov detect its presence in chloroplast suspensions exposed either to light or darkness (20). The writer of this review has developed a method specific for estimation of hydroxylamine based on condensation of hydroxylamine with nitrosobenzene in alkaline solution to form the diazotate which is coupled with α-naphthol. Accurate *in vitro*, its application to living tissue has not yet been possible since only a fraction of hydroxylamine added to ground plant tissue can be recovered in deproteinized extracts even after allowing for oxime formation. Readers who have tried to recover quantitatively nitrate added to plant material will appreciate the difficulties. To date we have not been able to detect free hydroxylamine in deproteinized extracts of fresh leaves or roots.

It is unlikely that an active and toxic substance such as hydroxylamine should accumulate in cells and the theory that hydroxylamine is an intermediary in nitrate reduction receives its main support from the reported occurrence in tissues of higher plants of oximes, evidence for the existence of which appear to be better substantiated than is the case for free hydroxylamine. Maeda (8) and Virtanen & Csaky (21) have reported formation of oximes in yeasts. Endres (22) isolated a carboxime from *Azotobacter* and Virtanen & Laine (23) reported isolation of oximinosuccinic acid among excretion products from legume roots nodulated with *Rhizobium* and fixing nitrogen. Lemoigne, Monguillon & Desvaux (17) found "combined hydroxylamine" in fresh leaves and Steward & Street (11) also record "combined hydroxylamine" in leaves of lucerne and peas. Kolesnikov (20) reports that in both light and dark the amount of nitrate is greatly decreased in the presence of glycolic and glyoxylic acids; under these circumstances no nitrite accumulated but, it is claimed, was converted to hydroxylamine; no hy-

droxylamine accumulated but formed oximes in the presence of glyoxylic acid.

Additional evidence that hydroxylamine is an intermediary in nitrate reduction comes from other sources. Cohen & Cohen-Bazire (24) report that *Clostridium saccharobutyricum* transforms hydroxylamine to ammonia in the presence of fumarate or oxalacetate probably by the path α-oximinosuccinic acid to aspartic acid to ammonia. Stumpf & Loomis (25) report the isolation of an enzyme from pumpkin seeds which catalyzes the formation of glutamohydroxamic acid from glutamine and hydroxylamine. Wood & Hone (26) have shown that oat plants utilize adequately both α-oximinosuccinic and α-oximinoglutaric acids as sole source of nitrogen under conditions in which bacterial contamination is minimized.

It is apparent that a body of evidence has accumulated which indicates production of oximes, especially oximinodicarboxylic acids, in tissues of higher plants. Whether these are formed as side reactions or whether they are key intermediaries in the formation of amino acids from nitrates still remains unsolved. Virtanen (27) has suggested that much of the nitrate absorbed by plants might be reduced to ammonia and that oximes are formed in relatively small amounts in a side reaction between hydroxylamine and keto acids. A critical experiment utilizing oximes labelled with N^{15} has not yet been performed with higher plants.

Mechanism of nitrate reduction.—Joklik (28), by fractional precipitation of extracts of ground *Escherichia coli*, obtained a cell-free nitrate reductase which reduced nitrate to nitrite under anaerobic conditions at pH 7.6 in the presence of methylene blue, or other reversibly reducible dyes, reduced with sodium hydrosulphite. Eckerson (29) reported the presence of a nitrate-reductase in expressed sap, in extracts of freshly ground plant tissue, and in alcohol-acetone precipitates from these extracts; she claimed that these preparations reduced nitrate to nitrite. In the writer's laboratory Robinson (15) repeated Eckerson's experiments and also applied Joklik's technique with extracts and with fractional precipitates of extracts from organs of grasses; in no case could nitrite be detected in less than 24 hr. After this time the amount of nitrite in the supernatant increased exponentially; in all such cases where nitrite accumulated plating from the medium revealed the presence of *Chromobacterium* and *Bacillus subtilis* which are both known to produce nitrite from nitrate. Rate of growth of these organisms paralleled production of nitrite in the medium. The organisms were present in the presence of toluene and occasionally spores survived treatment of tissues or extracts with acetone.

It is unlikely, therefore, that a simple nitrate reductase, similar to that found in bacteria, occurs in the tissues of higher plants: it is more likely that energy and hydrogen for reduction of nitrate are derived from the glycolytic and respiratory cycles. This is suggested by the high respiratory rate of nitrate-deficient plants supplied with nitrate (30, 31). Lewis (32) has reported that formate, succinate, lactate, citrate, and glucose all act as

hydrogen donators for nitrate reduction in the presence of washed microorganisms from sheep's rumen, though less efficiently than gaseous hydrogen.

The literature of the subject reveals that accumulation of nitrate occurs in deficiencies of several micronutrient elements. Stahler & Whitehead (33) have reported marked accumulation of nitrate in pastures sprayed with 2,4 dichlorophenoxyacetic acid (2,4D); sugar-beets so treated accumulated up to 8.8 per cent KNO_3 in their leaves on a dry weight basis. It has been suggested that ascorbic acid and also light energy play a role in nitrate reduction. Evidence for these claims is discussed below.

Micronutrients and nitrate reduction.—The role of micronutrient elements as components or activators of specific enzymes and the part which these might play in nitrate reduction have been the subject of many investigations. It is apparent that relations between nitrate assimilation and nutrient elements are complex and some sort of evaluation is called for.

Street (1) cites numerous authors whose work suggests that deficiencies of potassium, calcium, and phosphorus interrupt nitrogenous metabolism by their effect on nitrate absorption rather than on its reduction. However work cited below shows that deficiencies of sulphur, zinc, manganese, and molybdenum are associated with marked accumulation of nitrate in plant organs; on addition of the deficient element to the culture solution in which such plants are growing nitrate rapidly disappears from the tissues and organic nitrogen compounds appear.

Quite apart from the question as to whether specific nitrate and nitrite reductases are involved, the probability that nitrate reduction is connected with hydrogen transfer and with energy transfer links the process with dehydrogenases and with enzymes concerned with phosphate transfer. Indeed any block in the glycolytic and respiratory cycles could conceivably restrict nitrate reduction.

It is unlikely that elements, which when deficient cause nitrate accumulation, all affect the same enzyme; indeed the effects of any one element may be multiple so far as enzyme systems are concerned e.g., manganese and magnesium.

Any particular element may effect an enzyme in several ways: (*a*) by forming an integral part of the prosthetic group of the enzyme or by acting as a coenzyme; (*b*) by acting as a specific or nonspecific activator of the enzyme; (*c*) by affecting in any way any enzyme or process concerned in the long chain of reactions extending from nitrate reduction to protein synthesis and in this way restricting formation of the protein moiety of the enzyme; (*d*) by behaving as an inhibitor of enzyme activity; or (*e*) by combining with or removing inhibitors already present.

The effects of mineral deficiencies on a number of different enzymes have been described by comparing the activity of the enzyme in plant tissues grown in nutrient solutions deficient in one element with its activity in control plants of the same temporal age grown in complete nutrient solutions.

If enzyme activity is less in the deficient plant it has sometimes been assumed that the deficient element plays an essential specific role in the

particular enzyme system under examination. Comparisons of this kind, made between plants of different ontogenetic age and often with widely different protein contents, may be misleading and should be interpreted with caution. Recent work by Wood & Sibly (34) emphasizes this point. They grew uniform tomato plants in zinc-deficient culture solutions until deficiency symptoms appeared. Different concentrations of zinc were then added to the culture solutions; over intervals of a few days they measured the activity in the leaves of carbonic anhydrase, which in animals is known to contain zinc as a prosthetic group; at the same time they determined zinc and protein contents of the leaves. As the zinc content of the leaves increased so did carbonic anhydrase activity, but Wood & Sibly were able to show that zinc deficiency did not limit enzyme activity by providing insufficient zinc to fully saturate an apoenzyme. Rather, in zinc deficiency the protein moiety of the enzyme was not formed. In a case such as this where deficiency of an essential element restricts protein formation in general, it is probable that many enzymes might show decreased activity in a deficient plant. Little is known about the relative behaviour of enzyme-proteins and of cytoplasmic proteins during the life cycle of plants or under conditions involving synthesis or hydrolysis of proteins: the data of Wood & Sibly (34) indicate that carbonic anhydrase activity was different during ontogeny of zinc-deficient and of control plants and does not parallel total protein content; Axelrod & Jagendorf (35), however, report no change in activity of phosphatase, invertase, and peroxidase in tobacco leaves during a storage period of seven days after picking although soluble cytoplasmic proteins decreased by 45 per cent.

Observed relations between nutrient deficiencies, nitrate assimilation, and enzyme systems will be considered below with the above considerations in mind.

Sulphur.—Eaton (36) reported higher nitrate content in sulphur-deficient mustard plants than in fully manured controls; and Anderson & Spencer (37) found high nitrate accumulation associated with low protein content in sulphur-deficient plants of clover, oats, flax, and paspalum. In this case, where protein formation in general is restricted through failure of synthesis of essential amino-acids, it is possible that many enzymes, including those directly concerned in nitrate reduction, are present in lower concentration in deficient than in normal plants.

Zinc.—Little work on nitrogenous metabolism during zinc deficiency has been reported. Bean (38), cited also by Hoagland (39), states that nitrates accumulate in the leaves of zinc-deficient tomato plants, that continued proteolysis occurs, and that amino-acids and amides accumulate. He points out that a role in nitrate reduction is not the only function of zinc since severe deficiency symptoms appear also in plants supplied with ammonia-nitrogen. Wood & Sibly (34) found that proteolysis occurred in zinc-deficient tomato plants and that addition of zinc to these caused synthesis of protein in leaves. Relevant to these observations are others concerned with formation of β-indoleacetic acid. Skoog (40) found that stunting characteristic

of zinc deficiency was associated with low content of β-indoleacetic acid; Cheng Tsui (41) showed that zinc-deficiency in tomatoes did not inhibit conversion of tryptophan to β-indoleacetic acid but apparently inhibited formation of tryptophan since content of the latter was low in zinc-deficient plants; Nason, Kaplan & Colowick (42) report that the enzyme catalyzing synthesis of tryptophan from serine and indole is completely absent in zinc-deficient *Neurospora;* they also report that alcoholdehydrogenase activity was low in zinc-deficient plants. These results not only indicate that failure of protein formation occurs through absence of the essential amino acid tryptophan but also suggest a possible link between tryptophan formation and nitrate reduction. Umbreit, Wood & Gunsalus (43), and Fildes (44) found that synthesis of tryptophan from serine and indole required pyridoxal phosphate as coenzyme. Reed (45) using histochemical techniques reported accumulation of inorganic phosphate and referred to increased phosphatase activity in zinc-deficient plants. Sadisvan (46) presented evidence that the phosphatase of *Penicillium chrysogenum* is a zinc-containing enzyme but Robinson (47) in the author's laboratory has shown that zinc-deficient tomato plants possess high phosphatase activity towards glycerophosphate; addition of zinc to the culture medium caused marked inhibition of phosphatase activity.

It can be suggested, therefore, that failure of tryptophan formation, and consequently of protein formation in zinc deficiency could be attributed to destruction of pyridoxal phosphate. High phosphatase activity also results in hydrolysis of hexosephosphates and of ATP^2 with consequent reduced operation of glycolytic and respiratory cycles. This would be reflected in decreased synthesis of amino acids and in reduced nitrate reduction if the latter is linked with these cycles through hydrogen and energy transfer.

Quinlan-Watson (48) has reported that aldolase activity is considerably reduced in zinc-deficient plants compared with fully-manured controls. This may indicate a multiple role for zinc as an essential nutrient. Reduced aldolase activity implies less substrates for synthesis and less dehydrogenation for nitrate reduction. However, the evidence that zinc specifically affects only this enzyme is not necessarily conclusive; decreased aldolase activity in zinc-deficient plants can be accounted for in the same terms as the decreased carbonic anhydrase activity discussed above viz., decreased protein formation which may be accounted for by the observed increased phosphatase activity.

Molybdenum.—All investigators who have worked with molybdenum-deficient plants report a clear cut relationship between nitrate reduction and molybdenum. It is noteworthy that molybdenum is rapidly transported to leaves (49), and it is in leaves rather than in other organs that rapid disappearance of nitrate occurs in deficient plants supplied with molybdenum.

Accumulation of nitrate in leaves of molybdenum-deficient plants and its rapid disappearance following supply of the element to the culture solution have been reported for a variety of plants (50 to 54).

Vanselow & Datta (55) have reported that molybdenum is essential for citrus when ammonium salts and nitrate are present together. Recently Agarwala (55a) and Meagher (55b) have shown conclusively that molybdenum is essential for plants supplied exclusively with ammonium salts as source of nitrogen.

Anderson & Spencer (54) concluded from their data that molybdenum was essential for formation of proteins and Hewitt *et al.* (52) concluded that it was essential for formation of amino acids.

Nitrate reduction, formation of amino acids, amides, and of proteins have one feature in common—they all require expenditure of energy for synthesis which might be supplied by ATP or other energy-rich phosphates. It is of interest, therefore, that most of the work so far carried out on the effect of molybdenum on enzyme systems has indicated that phosphatase is concerned.

Courtois & Bossard (56) and Bossard (57) first demonstrated that addition of molybdenum to the phosphatase extracted from almond kernels inhibited the hydrolysis of glycerophosphate by the enzyme. Rothstein & Meier (58) reported that molybdate inhibited the surface phosphatases of yeast; molybdenum played a role in the medium and prevented utilization of glucose-phosphate by the intact cell. Recently, in the writer's laboratory Spencer (59) grew tomato plants in nutrient culture until molybdenum-deficiency symptoms appeared; molybdate was added to the culture solution and phosphatase activity of the leaves measured at short intervals thereafter. It was shown that the phosphatase of tomato leaves hydrolyzes glucose-6- phosphate, hexose-diphosphate, triose phosphate, ATP and glycerophosphate but not glucose-1-phosphate; hydrolysis of these substrates by the phosphatase was inhibited only by molybdenum added to living molybdenum-deficient plants and not by other heavy metals. He was able to show competitive inhibition for the enzyme between molybdate and ester-phosphate. We have also been able to show that in molybdenum-deficient plants inorganic phosphate is higher and the hydrolyzate 7 min.-phosphate and 30 min.-phosphate lower than in plants recciving molybdenum. It is suggested therefore that in molybdenum-deficient plants hexose-phosphates and ATP are present in low concentration owing to phosphatase activity and that consequently the glycolytic and respiratory cycles provide hydrogen for nitrate reduction and ATP for organic nitrogen synthesis at a reduced rate. If this view is correct molybdenum should also play a role in amino acid and in peptide bond synthesis and, when deficient, should restrict formation of amino acids and proteins even when plants are supplied with ammonia-nitrogen only as a source of nitrogen. Since the energy requirements for these syntheses are considerably less than for nitrate reduction, onset of deficiency symptoms might be expected later in the life cycle than is the case with plants receiving nitrate only.

We are led, therefore, to an essentially similar biochemical picture in molybdenum deficiency as in zinc deficiency viz., that general phosphatases appear to be affected by both elements though for different reasons. There

is, however, a difference in behaviour between the two elements which might determine the difference in visual symptoms of deficiency; molybdenum is translocated from deficient leaves as shown by senescence of older leaves and subsequent growth of usually only one or two new green leaves until the plant is many weeks old and Wood & Sibly (60) have shown that zinc is not translocated but is immobilized in leaves.

Hewitt *et al.* (61) have recently suggested a connection between molybdenum and ascorbic acid contents in relation to nitrate reduction which is discussed below.

Manganese.—Burström (6, 62) first stressed the importance of manganese, partially replaceable by iron, in nitrate reduction. He first considered that a specific manganese-protein catalyst was concerned and its location and operation restricted to roots. Several workers, reviewed by Mulder (63), have reported accumulation of nitrate in manganese-deficient plants and its disappearance on supplying manganese to the medium. Hewitt (64) has reviewed work dealing with relations between manganese content and the activity of different enzymes. Both manganese and magnesium behave as specific and nonspecific activators of various enzymes concerned with dehydrogenation and reversible carboxylations of acids of the respiratory cycle; in cases of nonspecific activation the roles of the two metals are usually interchangeable. Since the appearance of the above two reviews little significant work dealing with relations between nitrate reduction and manganese have appeared with the exception of certain aspects of recent work by Burström (65, 66).

Burström found that n-diamylacetic acid decreased nitrate assimilation of aseptic excised wheat roots but that increased supply of either manganese or iron restored assimilation, manganese being about three times as effective as iron. He concluded that these elements, which counteract the toxicity of n-diamylacetic acid, might function in a normal plant by behaving in a similar manner with naturally occurring inhibitors. Manganese and iron are no longer regarded as specific catalysts in nitrate reduction but as neutralizing effects of n-diamylacetate or some part of the system concerned with nitrate reduction.

Whether this is so or not, the function of manganese, as of other heavy metals, in enzyme systems leads us back to reactions capable of supplying hydrogen for nitrate reduction.

Ascorbic acid and nitrate reduction.—From time to time it has been suggested that ascorbic acid plays a role in nitrate reduction; these suggestions stem from the well-known rapid decomposition of nitrites by ascorbic acid.

Karrer & Bendas (67) showed that when ascorbic acid was boiled for an hour with potassium nitrate the latter could be recovered quantitatively; boiled with potassium nitrite at pH 6.0, 7.0, and 9.3 nitrous oxide was formed in neutral and acid but not in alkaline media. In alkaline solutions nitrous oxide was formed, however, if ammonium chloride was also present. It is worth recording here that some years ago the writer of this review (68) found rapid destruction of 0.0025 *M* nitrite by 0.0025 *M* ascorbic acid in the

presence of metaphosphoric acid at pH 2.0; but after 30 min. at pH 2.0 the amount of ascorbic acid was unchanged in the presence of nitrate, hydroxylamine, oximinosuccinic acid, and oximinoglutaric acid. Mikhlin (69) reported that ascorbic acid vigorously decomposed nitrite but that the reaction was greatly retarded in the presence of potato juice; he later claimed (70) that potato shoots and *Hortensia* leaves immersed in solutions containing potassium nitrite, formaldehyde, and ascorbic acid produced both hydroxylamine and ammonia. Kolesnikov (71) claimed that breakdown of chlorophyll in barley leaves is accelerated by addition of nitrate or nitrite to the leaves but that addition of ascorbic acid reduced the nitrite to hydroxylamine (no gas being evolved) and protected the chlorophyll from breakdown. In the purely chemical literature there is no record of formation hydroxylamine from nitrite and ascorbic acid, at least in acid media; the course of events in alkaline solutions, especially in the presence of keto-acids, is not known.

Virtanen (72) and Virtanen & Saubert v. Hausen (73) observed that there is considerable accumulation of nitrate in pea seedlings whether cotyledons are removed or left intact; they claim that addition of ascorbic acid to the medium prevents nitrate accumulation and strongly promotes growth in nitrate solutions but not in solutions of ammonium salts. They found that reductone, cysteine, and glutathione in suitable concentration also promoted growth in nitrate but was ineffective in solutions of ammonium salts. They suggest that ascorbic acid acts as a regulator of redox potential and that nitrate reduction does not occur unless the redox potential is reduced to a suitable level. On the other hand Prokoshev & Petrochenko (74) report when cut potato tubers are infiltrated with ascorbic acid then protein synthesis is independent of the ascorbic acid level.

Harmer & Sherman (75) have reported marked depletion of ascorbic acid in leaves of manganese-deficient plants. Hewitt *et al.* (61) observed a striking and significant reduction in ascorbic acid content in molybdenum-deficient plants compared with controls; a rise in ascorbic acid content occurred within 24 hr. following injection of molybdate into the petioles of deficient plants. They link changes in ascorbic acid content with nitrate reduction. Their report that potassium nitrate slowly decomposes ascorbic acid in pure solution is at variance with the data of Karrer & Bendas (67).

James & Cragg (76), James, Heard & James (77), Waygood (78, 79), Davison (80), and Mapson & Goddard (81) have all reported in a number of plants a transfer of hydrogen from hydroxyacids or triosephosphate through DPN^2, and a specific dehydrogenase, to ascorbic acid, ascorbic acid oxidase, and oxygen.

A reduced content in ascorbic acid might, therefore, be expected if there is disturbance of the normal glycolytic or respiratory cycles, as in manganese or molybdenum deficiency, whereby there is insufficient hydrogen transfer to keep the ascorbic acid in the reduced form. The data are not yet sufficient to decide whether ascorbic acid plays an essential role in nitrate reduction.

Light and nitrate reduction.—The role of light in reduction of nitrate has

had a long and controversial history (6) but interest in this topic has been revived following development of modern techniques.

Burström (6, 82) produced evidence that leaves of wheat reduced nitrate in the light by a photochemical reaction or by processes specifically linked with the photosynthetic reduction of carbon dioxide; he claimed that only negligible amounts of nitrate were reduced by leaves, though not by roots, in darkness. He suggested that nitrate assimilation was brought about by combination of intermediates of carbohydrate synthesis with a light-activated derivative of nitrate which might be nitrous acid. Stepka, Baron & Calvin (83) showed that radioactive alanine and aspartic acid were formed in leaves supplied with $C^{14}O_2$ during 30 sec. exposure to light and that during the first few minutes of exposure a major portion of the insoluble products was protein.

Exclusive light reduction of nitrate in leaves as claimed by Burström has not been substantiated. Delwiche (84), using excised tobacco leaves, found that labelled nitrogen from nitrate was incorporated in 24 hr. into organic-N fractions as readily in darkness as in light; Davis (85) has reported that in *Chlorella* direct nitrate reduction during photosynthesis does not occur but is coupled with oxidation of glucose and presence of phosphate. In a recent review Turner (86) has discussed evidence for compounds which might serve as hydrogen carriers between the Hill reaction and carbon-fixation in photosynthesis and also the role which these might play in nitrate reduction.

The evidence produced so far suggests that in light, as in darkness, nitrate reduction is closely connected with hydrogen transfer derived from dehydrogenations and with phosphate transfer. However, the pathway and details of the mechanism of nitrate reduction in green plants remains obscure.

Metabolism of Organic Nitrogen Compounds

Amino acid composition of proteins.—Since appearance of the last review (3), several analyses of proteins and of free amino acids in tissues, chiefly by chromatographic methods, have been published. Steward *et al.* (87) and Thompson & Steward (88, 89) have described methods for the quantitative estimation of amino acids by filter paper chromatography.

Wildman & Bonner (90) showed that the bulk of leaf protein is electrophoretically homogeneous. Lugg & Weller (91) have described partial amino acid contents of whole protein from senescent leaves of *Trifolium subterraneum* and compared these with those from young leaves; changes in cysteine and methionine contents occurred during senescence, but it was shown that no definite progressive changes occurred. Fowden (92) has published an analysis of *Chlorella* protein showing similar amino acid composition to that of the proteins from grasses and legumes analysed by Lugg (91). Differences were found only in cystine content. Yemm (93) analysed tissue proteins (including cytoplasmic and chloroplastic proteins) from mature leaves and from seedlings of barley and found a high degree of uniformity in proportions of the constituent amino acids; appreciable dif-

ferences were found only in lysine content. Zelitch *et al.* (94) report the presence of 17 amino acids in the hydrolyzate from soya bean nodules with no unusual amino acid occurring in large amounts.

Some changes might be expected to occur during maturity and senescence. Little is known of changes in specific proteins brought about by changes in external conditions or during ontogeny. Camus *et al.* (95) report that protein synthesis in cells of tobacco leaves may be directed towards different paths by heat treatment; an inverse relationship exists between particulate proteins and soluble proteins, the latter being maximal in amount at low temperatures and the former at high temperatures. Wildman *et al.* (96) state that tobacco mosaic-virus protein increases at the expense of the homogeneous protein fraction. Specific proteins (e.g. enzymes) may not change during ontogeny at the same rate as other proteins; reference has been made previously to the observation of Wood & Sibly (34) that carbonic anhydrase activity was maintained at a time when total leaf protein was decreasing and to the report of Axelrod & Jagendorf (35) that during starvation of tobacco leaves in darkness no change in activities of phosphatase, invertase, and perioxidase could be detected although storage proteins decreased by 45 per cent. From analyses published so far it would seem that the effects of the changes described in this paragraph on composition of leaf protein are small and indicate relative uniformity of amino acid composition of most leaf proteins.

Folkes (97) has investigated the nature and extent of amino acid interconversions in the breakdown of seed storage-proteins and the synthesis of tissue proteins during germination of barley seeds. This was judged by comparing amino acid contents of the two sets of proteins; the results indicated synthesis or incorporation into tissue protein of aspartic acid, alanine, lysine, and arginine in quantity and of glycine, isoleucine, tryptophan, and threonine in smaller amounts. In these syntheses 90 per cent of the nitrogen involved was derived from amides, glutamic acid, and proline; small decreases occurred also in phenylalanine and in serine. The prominent role of glutamic acid as a source of nitrogen in amino acid synthesis, which is stressed by Folkes, has been described also by many workers and will be discussed later.

Free and bound amino acids in plant cells.—The amino acids which occur in the free state in tissues (i.e., not incorporated into protein) have been the subject of several investigations.

Dent *et al.* (98) and Steward *et al.* (99) extracted potato tuber tissue with 75 per cent alcohol and identified twenty-one amino acids in the extract by partition chromatography. Leucine and hydroxyproline were not detected though γ-aminobutyric acid, which is not a constituent of protein, was present in large amount. They found that under conditions of protein synthesis many of these amino acids decreased in amount to such an extent as to be no longer capable of detection in chromatograms; in contrast to these acids glutamic and aspartic acids and their respective amides were maintained at relatively high concentrations.

In lucerne leaves Steward *et al.* (87) found aspartic and glutamic acids, asparagine, glutamine, serine, and alanine present in large amounts in the free state; other amino acids, including leucine, were present in smaller amounts than in potato tuber tissue but no glycine, lysine, threonine, methionine, or proline could be detected in the free state.

Christiansen & Thimann (100, 101) investigated changes in content of free amino acids in sections of isolated pea internodes cultured in water and in auxin solutions over a 24 hr. period. Twenty-nine ninhydrin-reacting substances were found of which alanine and asparagine were present in greatest amount, and cystine was notable by its absence. During growth, increase in amounts of protein and of asparagine was accompanied by consumption of the free amino acids all of which decreased in amount, notably valine and arginine.

Synge (102) found glutamic acid, glutamine, alanine, serine, and γ-aminobutyric acid predominated among the free amino acids in rye grass; methionine and hydroxyproline were the only amino acids which were not seen in the chromatograms.

The presence of γ-aminobutyric acid among the free amino acids has been reported by Dent *et al.* (98) and by Steward *et al.* (99) in potato tubers and by Synge (102) in lucerne leaves. Hasse & Schumacher (103) reported that decarboxylation of glutamic acid was brought about by radish extracts. Beevers (104) found that aqueous extracts of barley roots contain an active L-glutamic acid decarboxylase; he suggests that the natural occurrence of γ-aminobutyric acid described above might be ascribed to the functioning of this enzyme *in vivo*.

The results reported in this section have certain features in common: (*a*) the presence in the free state at one time or another of all the amino acids present in tissue proteins; (*b*) the presence in quantity of glutamic and aspartic acids, glutamine, asparagine, alanine, and serine; and (*c*) decrease in amino acids during protein synthesis resulting sometimes in absence of certain amino acids in the free state.

Glutamic acid, aspartic acid and alanine.—It may be taken as established that the aminodicarboxylic acids are metabolites of great importance in plants both in relation to the formation of the amides, glutamine, and asparagine, and as amino group donors in enzymatic transamination.

The results described in the last section confirm earlier work of Rautenan (105) who found that, during intensive uptake of ammonia by pea seedlings over a 24 hr. period, the increase in organic nitrogen consisted almost exclusively of the aminodicarboxylic acids, their amides, and alanine. MacVicar & Burris (106) found that glutamine had a higher concentration of isotopic nitrogen than other amino acids in tomato plants supplied with $(N^{15}H_4)_2SO_4$. Roine (107) and Virtanen *et al.* (108) showed similar accumulations of glutamic and aspartic acids and of alanine in yeasts within 15 min. after supply of ammonium salts.

Further information concerning the synthesis of glutamic acid comes from two sources. Davison (109) has described a DPN^2-linked reaction

between formate oxidation and reductive amination of α-ketoglutarate in extracts of bean seeds. Vishniac & Ochoa (110) state that through the photochemical reduction of DPN, chloroplast grana can mediate the reductive amination of α-ketoglutarate in the presence of glutamic dehydrogenase. Whether the latter mechanism is operative in the entire leaf remains undecided since Calvin (111) states that although aspartic acid, alanine, and serine containing radio-carbon accumulate after short periods of photosynthesis in $C^{14}O_2$, radio-glutamic acid does not accumulate under such conditions but only after a period in darkness. Calvin has suggested that pyruvic and oxalacetic acids are produced by carboxylation during photosynthesis but that in the light, respiration (which supplies α-ketoglutaric acid), is suppressed. This concept has been the subject of controversy (cf. 86, 112).

Data concerning transamination in green plants remain scanty. Kritzman (113) found that her enzyme preparations from pea seedlings transaminated aspartic acid more rapidly than glutamic acid with pyruvic acid. Virtanen & Laine (114), Rautenan (105), and Kretovich & Bundel (115) using pea and lupin seedlings and infiltration methods have each shown that glutamic acid was transaminated more readily than aspartic acid with pyruvic acid. Virtanen & Laine state that transamination could be demonstrated with the following combinations: glutamic plus oxalacetic acids; glutamic plus pyruvic acids; glutamic plus α-ketoisocaproic acids; aspartic plus pyruvic acids. In addition Rautenan claims that transamination of valine occurs to a small extent with α-ketoglutaric acid but not with oxalacetic acid. Transamination could not be demonstrated in the following combinations involving aromatic amino acids: glutamic plus phenylpyruvic acids; α-ketoglutaric acid plus tyrosine; aspartic plus phenylpyruvic acids; and oxalacetic acid plus tyrosine.

It is apparent that the formation of alanine and of aspartic acid occurs through transamination. There has been no further work on the origin of other amino acids in green plants though investigations with *Neurospora* have thrown considerable light on their synthesis in that fungus. A summary of reactions involved in the biogensis of the principal amino acids has been given by Bonner (116).

Amides.—From time to time the existence of a third amino acid amide in plant tissues has been suggested and the possibility as often denied (cf. 3). Recently Done & Fowden (117) claim to have isolated a third amino acid amide from peanut plants which they consider may be the amide of either α-aminoadipic acid or γ-methylglutamic acid.

Yemm (118) has investigated further the occurrence of amides in barley leaves and concluded that asparagine and glutamine were the only amides concerned during starvation; secondary synthesis of both amides took place under these conditions though asparagine accumulated in much greater amounts than glutamine. Proteolysis could account for only 10 to 20 per cent of the total amide and the amount of asparagine present was 4 to 5 times greater than could be attributed to that released by protein

hydrolysis; he concluded that both aspartic acid and asparagine were formed by transamination from other amino acids. McKee (119) found a significant linear correlation between amounts of glutamine and asparagine in detached mature leaves of barley but no significant correlation between these two amides was found in intact seedlings or in their detached leaves; he concluded that ammonia whether produced by deamination within tissues or by introduction from without led to accumulation of asparagine in much greater quantity than that of glutamine. Kretovich & Yestigneyeva (120) infiltrated lupin and pumpkin seedlings with solution of ammonium aspartate, ammonium glutamate, potassium aspartate, and ammonium sulphate; with lupin they observed increase in asparagine content in all cases, the amount being greatest with ammonium aspartate, but none of the treatments led to glutamine synthesis; with pumpkin ammonium aspartate gave increase in asparagine content, but there was no increase with ammonium glutamate.

A feature of the work described above is marked accumulation of asparagine but not of glutamine under a variety of conditions. A number of workers have reported increase in amounts of both glutamine and asparagine following supply of nitrogenous salts to intact plants or to detached leaves; it has also been shown that during starvation of leaves of several species glutamine content reaches a maximum and then decreases earlier than does asparagine; in some plants glutamine but not asparagine accumulates (cf. 121).

Several factors may be operating to cause these apparent contradictions. Amount of enzymes may vary in different species. Accumulation of asparagine described above may be due to rapid production of aspartic acid from glutamic acid by transamination with oxalacetic acid and its subsequent amidation; under these circumstances glutamic acid could be maintained in approximately constant amount by its slower production from α-ketoglutaric acid and other animo acids e.g. alanine.

A second factor operating in wheat seedlings may be different capacities for synthesis of different organs. Reference has already been made to the lack of correlation between glutamine and asparagine contents in intact barley seedlings though such a correlation is found in mature leaves. Willis (122) reports that in excised barley roots from 10-day old seedlings glutamine accumulated as the primary synthetic product whilst asparagine was produced only slowly. It is possible that transphorases, respiratory enzymes, or enzymes concerned with amidation differ in roots and in aerial organs.

Further evidence for difference in enzyme equipment between species comes from the work of Moyse (123) who carried out detailed and extensive investigations of the changes in nitrogenous compounds and in respiration of detached leaves of wheat, haricot (*Phaseolus multiflorus*), buckwheat, and sorrel. During starvation or during culture in the light a temporary accumulation of asparagine and of glutamine occurred in wheat and of asparagine in haricot. On the other hand, in buckwheat and in sorrel the glutamine and asparagine contents remained at a low level and in each

case could be accounted for by the amount formed by hydrolysis of proteins; instead of amides, ammonia accumulated in quantity and was neutralized by acids, chiefly oxalic acid. It is also of interest that in these two plants the "climacteric phase" of respiration (124) is lacking. These two plants appear to be "ammonium" plants in the sense of Ruhland & Wetzel (125) and differ from rhubarb which Vickery *et al.* (126) have shown to produce glutamine only. These plants appear to be suitable subjects for investigation of their amino acid contents and their ability to bring about transamination and amide synthesis.

Elliott (127) has shown that extracts from lupin seedlings in the presence of ATP[2] and of magnesium and manganese ions catalyze the production of glutamine from L-glutamate and ammonia; the reaction was inhibited by calcium ions, adenosinediphosphate, fluoride, and chlorammonbenzoate. The system in these seedlings is similar to that first described by Speck (128) in the mammalian kidney. Elliott found no analogous asparagine-forming system in lupin seedlings.

Stumpf, Loomis & Michelson (129) report the presence in pumpkin seeds of a glutamyl transphorase which in the presence of phosphate, ATP[2], and manganese ions labilizes γ-amide linkages making possible exchange relations with either hydroxylamine or ammonium salts e.g., in production of glutamohydroxamic acid from glutamine. Some of the "bound" hydroxylamine reported in plant tissues may be present in this form.

Peptides.—The reductive amination of keto acids to form amino acids, the formation of amino acid amides, and the formation of the peptide bond from amino acids are all reactions by which the free energy of the system is increased. In these enzymatic syntheses the role of ATP as an energy donor is now well-established and Lipmann (130) has suggested that a single energy-rich phosphate bond is used to effect a single peptide link. Marston (131) has pointed out that this is a wasteful process; the cost of synthesizing a single peptide bond between two amino acids which effects a free energy increase of about 3 kg. cal./mole, involves the expenditure of the whole of the resonance energy of the pyrophosphate group of ATP with the dissipation of 75 per cent of it. Doby & Sturtevant (132) have published heats of hydrolysis of various synthetic peptides.

The occurrence and also the metabolism of peptides in green plants has received some long-awaited attention which promises to throw light on the problem as to whether protein synthesis takes place by condensation of amino acids or by some alternative path.

Dekker, Stone & Fruton (133) report the isolation of L-pyrrolidonyl-L-glutamyl-L-glutamine derived from L-glutamyl-L-glutamyl-L-glutamine in the alga *Pelvetia fastigiata.* Synge (102) investigating the "bound" amino acids (peptide-N) of rye-grass found that these included simple open-chain peptides but that the greater proportion consisted of a fraction from which relatively few amino acids were set free on hydrolysis and which might consist of cyclic peptides or of amino acids bound to carbohydrate.

In animal tissues Braunstein & Yefimochkina (134) found that the

biological synthesis of hippuric acid and of glutathione involves utilization of the energy of the labile phosphate of ATP. Johnston & Bloch (135) report synthesis of glutathione from glutamic acid, cysteine and glycine by liver homogenate in the presence of ATP, magnesium ions and inorganic phosphate, and Hanes, Hird & Isherwood (136), using the technique of paper chromatography, have demonstrated the formation of γ-glutamyl peptides during incubation of glutathione and free amino acids in the presence of kidney extracts. Spiegelman & Kamen (137, 138) showed that organic phosphorus fractions, including nucleoproteins, show high rate of phosphorus exchange during protein synthesis.

Fruton (139) showed that inactive glycine was replaced by radioactive glycine when the latter was incubated with glycyl-glycine in the presence of dipeptidase; and Johnston *et al.* (140) and Fruton *et al.* (141) showed (*a*) that papain activated with cysteine catalysed the replacement of the amide-nitrogen in benzoylglycine amide with isotopic N of $N^{15}H_3$ on incubation together and (*b*) that the same enzyme system also catalyzed the replacement of the amide group in carbobenzoxylglycinamide to form carbobenzoxyglycyl-L-phenylalanine.

However, the most outstanding contribution in this field comes from the work of Hanes, Hird & Isherwood (142) who describe two types of transpeptidation catalyzed by different enzymes. The first is a γ-glutamyl transpeptidase from the kidney and the second a gylcyl transpeptidase from cabbage leaves. These enzymes catalyze the reversible transfer of glutamyl and glycyl groups respectively from linkage with an amino acid residue of a donor peptide to linkage with the amino acid group of a suitable acceptor peptide.

These enzymes hydrolyze peptides as well as catalyze the transfer of a glycyl or glutamyl group. The widespread occurrence of transpeptidation catalyzed by the proteolytic group of enzymes is of widespread interest and supports the view that protein synthesis represents a reversal of protein hydrolysis i.e., the condensation of amino acids with input of energy derived from high energy phosphate.

Theories of protein synthesis.—Protein metabolism is a dynamic process involving continuous synthesis and degradation and is associated with other metabolic processes, notably respiration and photosynthesis, which provide the non-nitrogenous carbon chains and energy for synthesis. At the present time there are two theories concerning the path of protein synthesis.

The first, and most generally accepted, view postulates that protein synthesis proceeds by stepwise condensation of preformed amino acids according to the polypeptide theory first proposed by Emil Fischer. Earlier evidence supporting this view has been reviewed by Wood (121). According to the second hypothesis, for which Steward (3, 11, 112) and Street (1) are the chief protagonists, amino acids are regarded as products of protein hydrolysis and as the form in which soluble nitrogen is stored and eventually mobilized when synthesis proceeds. This hypothesis postulates building up of tissue proteins around a nucleus in one operation from (*a*) sugars or other metabolic intermediates derived from either respiration or photosynthesis

and (*b*) nitrate or ammonia or other preformed organic molecules containing nitrogen. In this hypothesis a key role is assigned to glutamic acid: nitrogen either as ammonia supplied from without or from nitrate reduction or released from oxidative deamination of amino acids or transferred as amino groups by transamination, is canalized through glutamic acid or glutamine which are considered to be the ultimate donors to the carbon chains which constitute the framework of the protein molecule.

A third hypothesis, which represents a compromise between the two views described above, was suggested earlier by Linderstrom-Lang (143) who claimed that the pre-existence of all amino acids in peptide bond synthesis of protein was not necessary but that condensation of keto-aldehydes or keto-acids with amino acids could produce ketopeptides which by transamination and further condensation would yield proteins.

It will be clear from work described earlier in this review that glutamic and aspartic acids comprise the bulk of the free amino acids in plant tissues and that they are active in transamination; furthermore, all amino acids known to be constituents of the protein molecule occur free in tissues and decrease in amount during protein synthesis. This evidence does not permit discrimination between either the polypeptide or the *en bloc* hypothesis. However, Roine (107) and Virtanen *et al.* (108) using yeast and Rautenan (105) using green plants have shown that during early stages of assimilation of nitrate or ammonia the dicarboxylic amino acids accumulate in quantity but that prior to protein formation accumulation of other amino acids has not been observed. They concluded that the formation of these other amino acids is relatively slow and that their synthesis is the limiting factor in protein formation. This work, therefore, is more consistent with the polypeptide theory or with Linderstrom-Lang's views than with the *en bloc* hypothesis.

Synge (102), as reported earlier, has determined free and "bound" amino acids in rye-grass extracts employing quantitative fractionation by electrical transport methods combined with chromatography; among the "bound" amino acids some occurred as simple open-chain peptides, some bound with basic substances, but the majority were combined in compounds in such a fashion that they were devoid of ionizable groupings and from which only a few amino acids were liberated on hydrolysis. The latter compounds might be cyclic peptides but since carbohydrates were also present in quantity in the same fraction Synge points out that the possibility of amino acid residues being bound to carbohydartes cannot be excluded. If the latter alternative is correct the evidence can be construed in support of the *en bloc* or of the ketopeptide hypothesis.

The work of Beadle and his school, and of other workers too numerous to mention here, has shown that different strains of *Neurospora* lack the necessary enzyme equipment to synthesize a particular amino acid and that consequently protein formation and growth do not take place. This is strong presumptive evidence for the polypeptide hypothesis, but it is not conclusive since Steward & Street (11) point out that a single amino acid might be

needed to fit a final molecular pattern. The argument seems weak since different strains need a different amino acid; in any case it approaches the ketopeptide theory.

Benson & Calvin (144) and Calvin (111) state that the major portion of the insoluble products formed during the first few minutes of photosynthesis of $C^{14}O_2$ was protein and that acid hydrolysis produced radioactive amino acids in approximately the same relative amounts as in the cell extract. Glutamic acid formed the largest moiety of the free amino acids but was not converted into protein in amounts commensurate with its concentration. This appears to be evidence for the polypeptide hypothesis, but Steward & Thompson (112) have pointed out that although the carbon of the glutamic acid does not go into the protein the nitrogen, which was unlabelled, may do so. This work, though suggesting the polypeptide hypothesis, does not provide conclusive evidence for it.

Hokin (145) found that of the 16 amino acid acids found in crystalline α-amylase 10 have been found essential for maximal synthesis of the protein in pigeon pancreas slices. Of these, four: tryptophan, tyrosine, valine, and leucine can be replaced by their respective ketonic acids. Glutamine which is also present in quantity but is not present in the amylase gives rise to alanine and aspartic acid by transamination and presumably to proline since the latter can be oxidized to glutamic acid by an enzyme system (146). The source of glycine and serine was not known. In this case the essential nature of certain amino acids for protein synthesis is evident and their incorporation into protein via glutamic acid not possible. It is unlikely that so many amino acids are required to fit into a final molecular pattern as suggested by Steward & Street (11) in explanation of work on *Neurospora*. The work suggests the polypeptide hypothesis including the possibility of some keto groups in the chain.

Strongest support for the polypeptide hypothesis comes from the work described in the last section viz., evidence that formation of peptides and of transpeptidation is brought about by the same enzymes which catalyze protein hydrolysis.

In concluding this section it may be said that the weight of evidence suggests that protein synthesis takes place by condensation of amino acids but that the possibility exists that some keto-peptides are initially formed and subsequently aminated.

Protein synthesis in different plant organs.—In concluding this review there is one aspect of protein metabolism which warrants attention viz., the ability of different isolated plant organs to synthesize proteins. Many workers have described protein formation in cultured isolated roots (cf. 147). Turner (148) has shown that appreciable synthesis of protein occurred during storage of "Granny Smith" apples over a period of 400 days and that the amount of protein formed was dependent on the level of respiratory activity. Christiansen & Thimann (100, 101) found increase in protein content, accompanied by decrease in amounts of free amino acids, during growth of isolated pea internodes cultures in water.

In contrast with the synthesis of protein in the isolated organs described above is work reported by Moyse (123) who made detailed investigations of changes in nitrogenous compounds in leaves of four species of plants under the following conditions: (*a*) detached leaves starved in darkness; (*b*) detached leaves cultured in light; and (*c*) attached leaves in light. The striking feature was the failure of detached leaves to achieve protein synthesis even under optimal conditions of carbon and nitrogen nutrition. Energy for synthesis was not lacking since amino acid-N and amide-N increased markedly. On the other hand in leaves attached to the plant protein synthesis occurred.

These observations are not new but confirm earlier work by Vickery, Yemm, and Wood (cf. 121). The failure to achieve protein synthesis in detached leaves led Chibnall (5) to suggest that protein level in leaves was subject to hormonal control. With the exception of Phillis & Mason (149) who described protein formation in discs punched from young cotton leaves floated on nutrient solutions, other observers have failed to show protein synthesis in detached leaves. The reason is not known; it is possible that some amino acids are formed in other organs and translocated to leaves, but the problem needs investigation.

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PHOTOSYNTHESIS[1,2]

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Introduction

Within the last two years there have appeared upwards of a dozen review articles or monographs devoted entirely or in large part to considerations of research progress in photosynthesis (1 to 12, 78). The most ambitious of these is the long awaited Volume II of *Photosynthesis and Related Processes* by Rabinowitch. This monograph now is effectively divided into three volumes, having grown so much by postwar accretion that Volume II had to be published in two parts. Whereas Rabinowitch's first volume (44) dealt mostly with the chemistry of photosynthesis, the second volume is devoted primarily to the physico-chemical aspects of the subject: to spectroscopy and the fluorescence of chlorophyll and other pigments, and to the kinetics of the photosynthetic process. This most competent presentation and analysis of numerous heterogeneous contributions is a reference work of forbidding length. Nevertheless it achieves a remarkable readability. For those who require a general and balanced but much more condensed treatment of the whole subject, Whittingham's review (4) is recommended.

In view of the number and detailed nature of relatively recent reviews on several aspects of photosynthesis, it seems justifiable to direct the present discussion chiefly toward the nonspecialist who may appreciate a general treatment of some of the major problems from a new or at least different viewpoint. Since it is necessary to comply, however painfully, with strict space limitations, a review restricted to a few topics should be more useful to the general reader and certainly more interesting to prepare, than a comprehensive tabulation of all recent contributions presenting only a vestigial discussion of any of them. This review will discuss five topics which coincide with certain interests of the authors. These topics are: (*a*) The efficiency of photosynthesis (minimal quantum requirement); (*b*) The biochemical pathway of carbon during photosynthetic assimilation; (*c*) Enzymes in some direct way implicated in the photosynthetic process; (*d*) Photosynthesis and phosphorus metabolism; (*e*) The source of photosynthetic oxygen.

The Quantum Requirement of Photosynthesis

Even assuming perfect efficiency, from simple energetic considerations, the quantum requirement of photosynthesis cannot be less than about three

[1] The survey of the literature pertaining to this review was concluded in December, 1952.

[2] The following abbreviations will be used: DNP, 2,4-dinitrophenol; ATP, adenosinetriphosphate; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; TPNH, reduced TPN; HCN, hydrogen cyanide.

although Franck (13) has presented cogent arguments against the possibility of the photosynthetic efficiency approaching 100 per cent.

Partly by reason of reaction stoichiometry, various theories of mechanism have suggested a minimal photosynthetic quantum requirement of either exactly four or exactly eight, as seemed in keeping with experimental determinations accepted at the time. Whether one thinks of carbon dioxide reduction in terms of a unique photochemical reduction occurring in close association with the chlorophyll or, as some biochemists now do, in terms of a purely thermochemical reaction sequence driven by quite nonspecific reductants chemically and physically remote from the pigment system, there is broad agreement that the photochemical parts of ordinary photosynthesis, photoreduction, or the Hill reaction, have in common the photolysis of water and the generation of an oxygen precursor whose nature does not depend upon the ultimate oxidant. Accurate measurements on the quantum requirements of several of the "aberrant" photosyntheses are now available (14, 15, 16). Understandably, the requirements were found to be essentially the same regardless of the large differences in energetic efficiency which this involves.

The experimental determination of the minimal quantum requirement of *Chlorella* photosynthesis has become one of the most strenuously contested problems in all of biology. The history of this controversy goes back to the earliest determinations when a quantum requirement of about four (energetic efficiency above 70 per cent) was first computed by Warburg & Negelein (17, 18) on the basis of manometric studies. Working in acid medium they employed the "two vessel" method to measure simultaneous oxygen and carbon dioxide pressure changes. The basic assumption was made that light had no effect on the respiratory rate and light readings were corrected by the rate of respiration in the dark. Very dense *Chlorella* suspensions were used, thereby simplifying the photometry since all incident light was assumed to be absorbed by the suspension. Subsequent work by Warburg and collaborators has shown that the high efficiencies they reported were not dependent on any of the above special conditions but were perhaps the result of certain special combinations of experimental conditions.

A group of workers at the University of Wisconsin was unable to confirm Warburg's initial result by several independent methods (19, 20, 21). Rieke (22), and Emerson & Lewis (23) found that Warburg's results were indeed reproducible if the latter's methods of measurement and computation were exactly followed, particularly with regard to the "sequence and duration" of illumination. If light and dark periods were of greater duration or if computations were based on different parts of the respective intervals, lower efficiencies invariably were obtained, a circumstance suggestive of some systematic error in the particular method leading to the calculation of exceptionally high efficiency. Emerson & Lewis (23, 24) elucidated certain transitory phenomena which occurred at the beginning and end of an illumination, and which caused the quotient of gas exchange to deviate

temporarily and considerably from unity. This effect, combined with a somewhat different equilibration lag in the two vessels of different gas-liquid volume ratios, apparently was sufficient to account for systematic errors of several hundred per cent, as well as for a marked dependence of computed efficiency upon the particular schedule of manometer readings and times of light exposure. There then seemed little doubt that the technically elegant work of Emerson & Lewis had explained the discrepancy between the findings of Warburg & Negelein and the results of other laboratories. However, Warburg rejected the explanation based upon errors in his experimental design or interpretation and, returning to the problem but largely discounting criticisms of his earlier work, he repeated those measurements with minor modifications in technique and obtained the same end result (25). There soon appeared a flood of contributions, none of which confirmed the low requirement of about four quanta but, in general, ranged upward from a value of about eight or nine (15, 26, 27, 28). The argument has swung back and forth between Warburg and co-workers on the one side (29, 30, 31, 32, 33), and Emerson and co-workers on the other side (27, 34). An attempt by Emerson and Warburg to arrange collaborative experiments proved fruitless. By the latest experiments, the breach has only been widened as Warburg's laboratory reports a quantum requirement as low as about 2.8 for *Chlorella* photosynthesis under special experimental conditions (35).

The underlying disagreement relates to methodology. Like all quantitative procedures, the manometric method has definite limitations and the quantum efficiency problem requires that the method be used under conditions where the magnitude of systematic error becomes uncertain, debatable, and possibly very large. It is not surprising that opposing schools should find grounds for disagreement in each other's experimental designs. While it may be unduly pessimistic to suggest that the argument is at an impasse, it now seems most unlikely that anything approaching agreement will be achieved by the protagonists of these two schools, in as much as the profound difference in their positions rests upon subtle points of experimental design and interpretation.

The present reviewers suggest that the versatile, two vessel, manometric method is neither adequately sensitive nor capable of sufficiently unequivocal results to obviate various arguments over the appropriate interpretations of these results. Specifically, disagreement exists over the quotient, $\Delta CO_2/\Delta O_2$, in light and in darkness, over reported anomalies in the time course of gas exchange during the first moments after transition from dark to light or the reverse (and corresponding transitory fluctuations in the quotient), and over the significance of different time lags in the gaseous equilibration between phases in the two vessels. Since the basic controversy thus concerns the method and the instrumentation itself, a persistent loyalty to manometric methods now seems contraindicated. To provide decisive information, methods based on other physical or chemical principles could be resorted to advantageously. The usefulness of the more elaborate

physical methods generally stands in inverse ratio to their complexity and it is understandable why none has achieved the popularity of the simple and versatile manometric or volumetric procedures. Nevertheless, it seems particularly desirable in this case to bring different methods to bear upon the problem. There are special physical methods with excellent specificity and very small inertia. Uncertainty over the composition of the gases responsible for pressure changes observed with a manometer (i.e., fluctuations of the quotient) can be eliminated by unambiguous assay methods such as infra-red spectrometry (36), electrical impedance measurements (37), and thermal conductivity measurements [(38, 39) for carbon dioxide], or measurements of magnetic susceptibility (40, 41) and polarography [(20) for oxygen], or mass spectrometry [(42) for both gases].

What is desired most is an analytical method specific for oxygen and showing no significant time lags in response to changes in oxygen tension. Time lags for instrumental response alone can be made negligible in each of the above methods but the major lag, that of gaseous equilibration across the interface between the suspension and the gas phase, is abolished only by the elimination of the gas phase. This can be done with the oxygen electrode. Therefore, among the most convincing determinations of the photosynthetic efficiency of *Chlorella* suspensions are those made with the dropping mercury electrode (28) and, more recently, those in which the platinum electrode was employed (43). Values of the minimal quantum requirement for *Chlorella* photosynthesis determined by these methods were in the range of about six to ten. While it is, of course, axiomatic that only the minimal values (within the range of experimental error) are of interest for the solution of this problem, it is equally true that a valid result should be confirmable by independent methods of measurement and the burden of proof clearly rests with those who claim the lowest quantum requirement.

For those not intimately concerned with the problem of minimal quantum requirement, the literature of this controversy has reached a forbidding level of technical complication and of concern with experimental detail.

Preoccupation with the polemic between Emerson and Warburg does not justify losing sight of the underlying assumptions which are made by those who attempt absolute photosynthetic rate determinations. One assumption made, or at least implied, is that respiration is a function which proceeds concomitantly with assimilation at the same rate as in the dark. Measurements of the photosynthetic rate customarily are corrected for respiration as measured in the dark and, since light measurements are carried out at intensities near or even below compensation, this correction is large. The use of the dark control, involving the often tacit assumption that light is without effect on the respiratory rate, has been a generally accepted device and its validity usually has not been specificially questioned although, as Rabinowitch points out, the possibility of a photoeffect on respiration "is a nightmare oppressing all who are concerned with the exact measurement of photosynthesis" (44, p. 569).

Warburg *et al.* (45), ignoring previous literature dealing with the question

of a light effect on respiration, reported that oxygen uptake by a dense *Chlorella* suspension was unaffected by red light of low intensity when the suspension was severely depleted of CO_2 by the use of NaOH in the side arms of the reaction vessel. According to the data given, gas uptake was, in fact, reduced by 2.5 per cent in the light. This small effect, apparently within experimental error, was ignored. However, since only an estimated 5 per cent of the cells were exposed to the light beam due to prevailing conditions of illumination and suspension density, it is doubtful if the result was as significant as the authors claimed. From this single type of experiment on but one algal strain, sweeping conclusions appear unjustified. Nevertheless these authors believed their result demonstrated a general lack of dependence of the respiration of green cells upon illumination. Actually, the more reasonable interpretation of their data taken at face value is that it demonstrated just the opposite. While the low CO_2 tension prevailing in their experiments severely inhibited photosynthesis, the suggestion that inhibition was complete is implausible as was pointed out by Whittingham (4), Rabinowitch (1), and by Kok (46) who also studied the matter experimentally. Kok (46), using a technically superior manometric method, could not reproduce Warburg's result. Not only was he unable to suppress photosynthesis completely by absorbing CO_2 efficiently, but he also observed induction effects consisting of enhanced oxygen utilization in the light. If there remained any residual photosynthesis under Warburg's experimental conditions then the observed lack of an effect of light must have been the result of respiratory stimulation. The manometric technique is, in principle, incapable of determining the true intensity of respiration if the photosynthetic rate is in doubt. The present reviewers find the circular reasoning of Warburg *et al.* unconvincing.

There is an extensive literature on the effects of light on respiration most recently reviewed by Weintraub (47), Rabinowitch (44), and Whittingham (4), but, until a few years ago, there was no convincing evidence that red or yellow light, as employed in critical quantum requirement studies, influenced *Chlorella* respiration in the steady state. However Rieke (22), Kok (46, 48, 49) and Van der Veen (50) have published data showing a nonlinear relation between light intensity and rate of oxygen exchange in the region of compensation. The data are fitted by two straight lines intersecting near or somewhat above compensation. Also, light curves measured by Burk and by Warburg are interpreted by these authors as curvelinear but they could as easily be interpreted as Kok does his curves, viz., as intersecting straight lines. Quantum requirements for photosynthesis calculated for data obtained below compensation often were in the neighborhood of four while above compensation the slope of the light curve indicated that the requirement was about twice this value. The "Kok effect" as it has come to be called, is readily interpreted as a photoinhibition of respiration which is proportional to light intensity and reaches its maximum approximately at compensation.

It seems that the effect is not as highly reproducible as might be desired

and its reality is not yet universally accepted. Also, the most convincing evidence in its favor applies to experiments in a medium of pH 7 or higher so that its application to most of the critical quantum requirement studies in other laboratories is uncertain. Emerson (1, p. 1118) has not observed the Kok effect, for his measurements of *Chlorella* gas exchange were almost linear with light intensity. However, Warburg (25, 51) and others report more curvilinear relations with points sufficiently scattered to prevent differentiating with certainty between a smooth curve and one of intersecting linear segments as suggested by Kok.

In an attempt to reconcile conflicting quantum requirement claims, Franck (52) revived the concept of photoreversal of respiratory reactions. He proposed that, under certain conditions affecting chloroplast permeability, the photoreduction of respiratory intermediates takes precedence over CO_2 assimilation. This process, energetically feasible in Franck's scheme with about half the quantum requirement of photosynthesis, was visualized as accounting for the Kok effect and for the exceptionally high calculated efficiencies of photosynthesis under Warburg's special conditions. At the time of its publication, Franck's proposal admittedly was not entirely consistent with all the known experimental results which apparently were germane. Later his proposal was modified and amplified (13), taking all data into consideration. There is not space here to develop Franck's arguments in favor of a photoreversal of catabolism under special conditions as an explanation of the quantum requirement anomaly.

Recently a direct approach was made to the problem of a photoeffect on *Chlorella* respiration. Brown (53) used tracer oxygen in the gas phase to tag the respiratory gas uptake of the algal suspension. Photosynthetic oxygen was untagged. Accordingly, the rate of tracer oxygen uptake, followed with a mass spectrometer (42), was a measure of the respiratory rate regardless of illumination of the cells. In slightly acid medium and at various intensities of white and of red light from intensities below up to several times compensation, there was evidence of neither photoinhibition nor photoenhancement of the respiration of several strains of *Chlorella*. Therefore, at least under the conditions used in these studies (which purposely were made comparable to those of published quantum requirement determinations), the use of a dark control to correct for respiration during the light exposure was found to be valid. Of course, there remains the possibility that circumstances requisite to demonstrating photoinhibition of respiration were not obtained.

A recent development in the field of quantum requirement measurements was contributed by Burk, Warburg, and various co-workers and concerns the reported kinetic separation of photochemical oxygen production from a nonphotochemical but light-augmented "combustion" process (35, 54 to 59). Using familiar manometric principles they measured *Chlorella* gas exchange in equal and alternating light and dark intervals of short duration. With periods as short as one or two minutes, their results were interpreted

as showing a rate of oxygen production in the light far greater than any previously encountered. In the dark, a combustion process (presumably not identical with ordinary respiration) proceeded at an accelerated rate, in extreme cases at tenfold the normal respiratory rate. It was proposed that for an integrated series of dark and light intervals (or for low intensity continuous light) the minimal quantum requirement is about three. However, for the photochemical partial reaction, the quantum requirement of one was suggested. Warburg and others thus considered the photosynthetic cycle as turning over at least three times as rapidly as they had proposed previously with the high rate of back reaction keeping the average energetic efficiency of the whole process just within the overall limit of 100 per cent, and concealing the one-quantum process until revealed by the device of light intermittency which was visualized as effecting sufficient time separation of the two parts of the cycle to allow for their detection.

Calvin *et al.* (9) criticized Burk & Warburg's concept of light intermittency separating partial reactions in a photosynthetic cycle by suggesting that, even taking the computed time course of gas exchange at face value, theirs is not the only possible interpretation of the manometric results. Warburg's equations (55), describing his postulated cyclic reaction mechanism in terms of gas exchange by forward and back reactions of a cycle, were found to apply as well to a very different model involving no back reactions in the Warburg sense. Presumably, the alternate model of Calvin *et al.* was not to be taken seriously for, if it is to fit the observations of Burk & Warburg, it apparently also involves a quantum requirement so low as to demand its rejection. In addition, it postulates that the oxygen production and carbon dioxide absorbing reactions are rapidly reversible. The consequences of these postulates were not explored in Calvin's brief review but are difficult to reconcile with other work cited therein.

From the standpoint of the Burk-Warburg experiments alone, it does not seem certain that the gas exchange measurements can be unambiguously interpreted in the manometric studies of Warburg & Burk and their collaborators. These reports of a one-quantum oxygen liberation or an accelerated combustion in experiments with intermittent illumination of short duration have not been confirmed outside of the laboratories of Warburg & Burk. Brackett (43) looked for such an effect with the oxygen electrode which has the advantage of much better time resolution than the manometric method and is also quite specific for oxygen. Brown (42) attempted to confirm the accelerated combustion by the use of tracer oxygen in the gas phase but not in the water of the cell suspension. Using a period of light intermittency of one minute there was no indication of enhanced rate of tracer oxygen uptake in such experiments.

The Chemical Pathway of Assimilated Carbon

The "first product" concept.—Before the advent of tracer carbon the only apparently feasible approach to the problem of identifying the so-called

"first product" of photosynthesis was to determine what substances accumulate during a period of assimilation. Such attempts were conditioned by the reasonable speculation, based on the stoichiometry of measured gas exchange, that the "first product" should be some kind of carbohydrate. Several workers had found light-dark dry weight differences in experiments on various plants to be attributable chiefly, but by no means entirely, to known carbohydrates. Using modern analytical methods, Smith (60, 61) was able to recover as carbohydrate practically all carbon assimilated by sunflower leaf tissue during light exposures of about 0.5 to over 2.5 hr. The fact that photosynthesis by mature sunflower leaf cells almost quantitatively yields carbohydrate as an ultimate translocation or storage product leads inevitably to the result obtained by Smith. Comparable studies by others on different species implicated other products as well. But there is no way to be sure that any of these substances represents a product formed immediately by the photosynthetic process, or whether the product is the end result of perhaps a long series of metabolic transformations only remotely connected with reactions of photosynthesis proper. Manifestly, the basic problem is one which cannot even be hopefully studied without employing isotopes and, since the required tracer experiments involve only brief exposures of assimilating cells to tagged CO_2, tracer dilution factors are enormous precluding the profitable use of any but radioisotopes.

The advent of radiocarbon.—Research on photosynthesis entered upon a new era with the advent of readily available, long-lived, radioactive carbon. The frequently reviewed, exciting, pioneer studies of Ruben and coworkers with C^{11} had given promise of greater things to come and, in fact, the search for C^{14} was inititated by Ruben largely for use as a tracer in photosynthetic studies. The results of Ruben's preliminary work with C^{11} were so unexpected as to excite the interest of other chemists and physiologists who renewed the attack on this problem with the much more convenient tracer, C^{14}, when it became generally available in 1946. Many students of photosynthetic problems believed, for the first time, that their goal of understanding the fundamental mechanism of photosynthesis was then in sight.

Almost overnight, interest shifted from the kinetic approach to the application of tracer methods, and a small army of biochemists, at long last provided with what seemed to be the necessary weapons, charged ahead in a frontal attack on chemical aspects of the problem. Previous kinetic studies were at first largely ignored, apparently in the belief that a delineation of the chemical pathway of assimilated carbon from CO_2 to the major organic constituents of the photosynthesizing cell would provide the clue, if not the complete solution, to the central problem of photosynthesis: how light energy is transformed into chemical energy. A healthy optimism prevailed concerning the potential fruitfulness of tracer methods combined with newly elaborated techniques such as paper chromatography.

The first results obtained with C^{14} published in the postwar era apparently were only indirectly related to photosynthesis. Studies were carried

out in Calvin's laboratory at Berkeley on the dark uptake of tracer carbon by algae which were exposed to $C^{14}O_2$ immediately after a period of illumination during which photosynthetic assimilation had been prevented by the absence of CO_2. The technique was described as "preillumination" and lately the term has also been used in publications from other laboratories with this restricted meaning.

Another research group at Chicago, comprised of Gaffron, Fager, and others, began studies of $C^{14}O_2$ incorporation during photosynthesis in experiments patterned after the earlier studies of Ruben. Subsequently, Calvin's laboratory also carried out experiments immediately concerned with photosynthesis and began a separation of products of photosynthetic assimilation of tracer carbon particularly by the green algae, *Chlorella* and *Scenedesmus*. It soon became clear that photosynthesis incorporates tracer carbon into many compounds in a very short time. Apparently, to identify a single tagged product (or intermediate) requires exposure times not of hours (Smith), or minutes (Ruben), but of seconds.

Phosphoglyceric acid as the first stable product of carbon assimilation.— From experiments in which the tagging time was 30 sec., Calvin & Benson (62) proposed that a major early product of tracer carbon assimilation was triose phosphate, since glyceric acid (presumably phosphoglyceric acid *in vivo*) was reported to be the major product in which tracer carbon was recovered. Data substantiating the identification were reported later (63). The identity of phosphoglyceric acid as the major product of very brief photosynthetic tagging experiments with algae was amply documented by Benson *et al.* (63) and confirmed by Fager *et al.* (64, 65). Additional confirmation for a higher plant was provided by Aronoff (66, 66a). The key compound probably is 2-phospho-D-glyceric acid. Although much of the tracer occurs in the 3-isomer, Calvin *et al.* (67) arrived at the provisional conclusion that the labelling of 3-phosphoglyceric acid was secondary. In view of this, it is unfortunate that the identification of the 2-isomer among the early photosynthetic products is still tentative.

Varying the time of exposure of illuminated algae to $C^{14}O_2$, the spread of tracer into a large number of compounds was studied in a series of contributions from Calvin's laboratory and by the Chicago group. These papers generally contained appropriate speculations concerning possible chemical pathways by which the tracer carbon spread into all the cellular constituents. Calvin's studies were aided by the exploitation of paper chromatography using radioautography to locate radioactive spots on the chromatograms. In addition, degradation studies on tagged compounds (e.g., phosphoglyceric acid) have provided useful information restricting speculations about probable reaction mechanisms. As new experimental results were acquired hypotheses concerning the pathway(s) of carbon assimilation have multiplied. However, conclusions which can safely be reached from incontrovertible evidence are relatively limited and it seems desirable to separate the intriguing speculations from well established facts.

At present, there is general agreement that the chief port of photosynthetic entry of tracer carbon into the metabolic machinery of the cell is the carboxyl group of phosphoglyceric acid. This suggests the existence of a 2-carbon acceptor for the $C^{14}O_2$ undergoing assimilation. The alpha and beta carbons of phosphoglyceric acid rapidly become radioactive but significantly less rapidly than the carboxyl carbon. This argues in favor of some kind of cyclic regeneration of the acceptor from assimilated carbon. Speculations concerning the postulated cycle are further restricted by the fact that the 2-carbon precursor of phosphoglyceric acid apparently must be symmetrical with respect to tracer since the alpha and beta carbons of phosphoglyceric acid acquire radioactivity at approximately equal rates. Glycine and glycolic acid are the only rapidly tagged 2-carbon compounds which have been identified on the Berkeley chromatograms. Both compounds are said to be symmetrically labelled. These compounds are thought to be related to but not identical with the 2-carbon acceptor (68, 69). Of course, the acceptor may not occur as such but only as a 2-carbon fragment of a larger molecule. There is no direct evidence concerning the identity of the acceptor and no consensus has been reached concerning its probable origin.

Appearance of tracer in compounds other than phosphoglyceric acid.— Tracer appears in pyruvate about as fast as it does in phosphoglycerate although, upon isolation, the ratio of total radioactivity in glycerate to that in pyruvate was found to exceed significantly the equilibrium value for the interconversion of these compounds, suggesting that the phosphoglycerate was not derived from pyruvate but rather the reverse (64, 70). This conclusion was further substantiated by studies with cell-free, leaf macerates described below (71, 71a). The radioactive pyruvate probably was present originally as phosphopyruvic acid. The studies reported by Fager *et al.* (64, 70) applied to tagged pyruvate obtained after hydrolysis. The Berkeley group reported radioactivity in phosphopyruvic acid and so labelled certain spots on their chromatograms, although information which supports this identification has not yet been reported in detail.

The Calvin group has also reported the very early appearance of tracer in alanine, malic acid, and aspartic acid. Other compounds become tagged much more slowly. They are thought to represent tracer incorporation via reactions not directly concerned in photosynthesis. As tagging times increase the spread of tracer into many compounds becomes accountable by a multiplicity of pathways. No particular over-all scheme seems to be especially superior to others.

It cannot be claimed that consensus in this field goes far beyond the above points. At present, there is no general agreement about the existence of ports of rapid CO_2 entry besides the carboxyl group of phosphoglyceric acid. Other primary carboxylations have been postulated without good experimental support. One such hypothesis concerns the origin of tagged malate, another early product of C^{14} incorporation. While malate (or some related C_4 product) has frequently been suggested by Calvin's group as the

product of a primary $C^{14}O_2$ fixation reaction with some C_3 acceptor, the critical experiments (72) in favor of this hypothesis were unconvincing as Gaffron *et al.* have pointed out (70). Additional evidence from Calvin's laboratory makes the hypothesis improbable with regard to malate itself (73). At the present time there seems to be no compelling reason to postulate more than one significant entry port for $C^{14}O_2$ incorporation in photosynthesis.

Dark uptake after illumination.—In early "preillumination" experiments Calvin & Benson (62, 74) reported that illuminating algae in the absence of any carbon dioxide resulted in greatly increased uptake of tracer when $C^{14}O_2$ was added in the dark period after the illumination. By varying the interval between the end of the illumination and the introduction of $C^{14}O_2$, it was possible to measure the dark uptake as a function of time and to determine the course of its decay. Curves representing the decline of the dark pickup of tracer indicate a half-life of about 4 min. although we cannot appraise the reliability of this value since the curves were given without any experimental points. These results were reminiscent of the dark pickup studied by Aufdemgarten (39) and McAlister (75, 76) but their relation to photosynthesis was initially questioned by the Chicago group (77). The "preillumination" experiments reported by Calvin *et al.* were designed with the concept that photochemical reactions of photosynthesis should be separate from the thermochemical reduction of carbon dioxide, that the light reactions produce a relatively long-lived "reducing power" which then can function in a rather nonspecific fashion. Therefore, it was not surprising that Calvin's group interpreted its results in the light of this basic assumption. For a number of reasons the Chicago group, on the other hand, considered the photochemical reduction of a carbon dioxide-acceptor complex a more likely possibility, and rejected the concept of light-generated, nonspecific reducing power. This fairly clearcut distinction between the fundamental viewpoints of the Chicago and Berkeley groups has been maintained up through the latest publications from the respective laboratories. The Chicago workers have studied dark pickup reactions systematically and have provided an interpretation of the phenomenon which differs from that of the Berkeley group.

Fager *et al.* (64) confirmed the relatively long half-life of the enhanced tracer uptake in the dark after preillumination. Two minutes was the value suggested but here, too, experimental points were not shown (78).

By measuring total $C^{14}O_2$ dark uptake as a function of time after the end of illumination, Gaffron *et al.* (70) determined the time course of tracer incorporation both after a period of steady state photosynthesis and after "preillumination" without CO_2. This dark uptake was, in both cases, cyanide sensitive and, also in both cases, the tracer was recovered almost entirely as phosphoglyceric and pyruvic acids.

The Chicago group called attention to the difference between the rapid (half-life, 3 sec.) disappearance of dark pickup activity after photosynthesis

as compared with the much slower (half-life, 2 to 4 min.) decline in such activity after "preillumination" when tested by the Berkeley method of exposing the cells to $C^{14}O_2$ for a standard interval as a function of time after the light was turned off. Their interpretation (64) was based on the difference in prevailing CO_2 concentrations in the two types of experiments. However, this explanation should be amended by noting that the different experimental procedures measured different processes. The rapid dark uptake of tracer after photosynthesis (half-life, 3 sec.) was measured as the time course of a reaction running to completion in 20 to 30 sec., viz., as interpreted by the Chicago group, the fixation of $C^{14}O_2$ by an acceptor substance produced in the light but surviving in the dark. The longer survival of dark pickup activity after "preillumination" represented, on the other hand, the time course of disappearance of surviving acceptor when no exogenous carbon dioxide was supplied.

If we assume that the Chicago and Berkeley "preillumination" experiments are in other respects comparable, the light induced ability of algae to fix CO_2 decreases more rapidly in the dark when exogenous CO_2 is present during the entire course of its decline (half-life, 15 sec.) (70, 78) than when it is supplied at various times after the end of the light period (74). It is this difference which may be accounted for by different CO_2 tensions. However, comparing by the same method the time course of dark pickup for each type of experiment, the data of Gaffron *et al.* (70) clearly distinguished the 3 sec. half-life of enhanced dark uptake after photosynthesis from a 15 sec. half-life for similarly enhanced dark uptake after "preillumination." Considering all the evidence, it appears unlikely that different CO_2 tensions (prior to adding tracer) can explain this discrepancy which is suggestive of a more fundamental difference between dark pickup of tracer after photosynthesis and tagging after "preillumination."

The Chicago group stressed the point that during the dark pickup which they observed, tracer was incorporated only into carboxyl groups of phosphoglyceric (to a lesser extent pyruvic) acid, whereas, an equivalent amount of tracer incorporation during normal photosynthesis carries much of the C^{14} beyond this initial stage. According to their view, the carbon dioxide acceptor is produced in the light and survives in the dark until it reacts with either exogenous $C^{14}O_2$ or endogenous carbon dixoide; in the former case leading primarily to carboxyl-labelled phosphoglyceric acid. The further metabolism of this compound is normally dependent upon photochemical reduction. Phosphoglyceric acid is thus considered a true intermediate of photosynthesis.

In order to arrive at the simplest reasonable explanation of these kinetic results, Gaffron *et al.* (64, 70, 78) postulated an unstable 3-carbon precursor to phosphoglyceric acid. Accordingly, the dark pickup curves represent the time course of stabilization of the precursor as phosphoglyceric acid (a dark reaction). The hypothesis is illustrated as follows:

$$[C_2] + CO_2 \rightleftarrows [C_3] \rightarrow \text{phosphoglycerate.}$$

The hypothetical precursor, [C_3], was conceived as a compound which rapidly exchanges its terminal (carboxyl?) carbon with that of CO_2. Thus it could become tagged very rapidly by exchange. It is also postulated to be unstable so that, unless transformed into phosphoglyceric acid, any C^{14} it may have incorporated would be lost as $C^{14}O_2$, thus, never appearing among the isolated products of photosynthesis or after-pickup. While this hypothetical unstable 3-carbon precursor is not required as the only possible explanation of the kinetic data on dark pickup after an illumination, it does provide a simple and self consistent mechanism by which to account for the tracer incorporation under these experimental conditions.

Controversial experiments and interpretations.—The rationale of photosynthetic tagging experiments both at Berkeley and at Chicago involved the basic asssumption that tracer assimilation was carried out under steady-state conditions. That such conditions did not obtain for "preillumination" tagging experiments was, and is, a most serious impediment in the way of their proper interpretation. At both Berkeley and Chicago laboratories, the contention that tagging was carried out under steady state conditions was not at first supported by convincing evidence nor subjected to critical scrutiny. However, the basic assumption was not called into question until Calvin *et al.* published a time curve for total radioactivity assimilated by photosynthesizing algae which departed severely from linearity. The rate of tagging in the first moments was at least five times the subsequent rate (67, cf. Fig. 2). The nonlinearity was explained by suggesting that it was an initial response to increased tension of carbon dioxide; in other words, that a steady state did not prevail. The implications of such a circumstance were ignored. Utter & Wood called specific attention to these data in a critical review of carbon dioxide fixation mechanisms (79, cf. p. 137). These authors criticized the view that phosphoglycerate must be an important early product or intermediate of photosynthesis simply because it incorporates tracer initially more rapidly than other constituents. Utter & Wood elaborated on the familiar concept of accelerated tagging through exchange reactions whereby tracer could be incorporated at a rate greatly in excess of the net assimilation rate into a compound which may or may not be on the main pathway of assimilation. The time course of total assimilation under such conditions would be concave toward the abscissa and it is little wonder that Utter & Wood saw in Calvin's data vindication of their skepticism (79).

However, the experimental curves of tracer uptake with time as Calvin reported were said not to be characteristic of tagging experiments in Chicago (70), and recently Fager & Rosenberg (80) presented very convincing confirmatory data. The time course of total tracer uptake observed by them was very slightly convex toward the abscissa. Most significant was the observation that the curve for incorporation of C^{14} into the carboxyl group of phosphoglycerate was strictly linear during the first minute of tagging and extrapolated to $+1 \pm 0.5$ sec. It is thus reassuring to have the necessary

confirmation concerning the basic assumptions implied in our evaluation of the importance of phosphoglyceric acid on the main pathway of photosynthetic assimilation.

It is not certain whether the difference between the linear time course of tracer assimilation demonstrated by Fager & Rosenberg and the remarkably nonlinear curves published by the Berkeley group (67, 81) can be explained on the basis of what has been published. However, it appears that the difference may reside in whether or not truly steady-state conditions prevailed in the critical experiments. Obviously it is essential that a steady-state be maintained during tagging but this condition has not always prevailed in either the Berkeley or Chicago laboratories. Fager (64, 65) suggested that a steady-state may not have obtained in some of the early tagging experiments at Chicago. Calvin *et al.* (67) indicated the absence of steady-state conditions (changing pCO_2) during tagging in at least some Berkeley experiments. Among the earliest descriptions (May, 1948) of the Berkeley methods of photosynthetic tagging, it was reported that the algae were maintained under conditions of steady-state photosynthesis with excess carbon dioxide for a time; *the suspension was then flushed with air for 5 minutes*, and the tracer was added (82). A similar statement was made with reference to more recent tagging experiments (83, 84, 85). In other cases the details of photosynthetic tagging were not given but the experimental points show the time curve of tagging to be grossly nonlinear over its entire course in spite of the lines drawn on the graphs (72, cf. Fig. 1). In still other cases the data are fitted reasonably well by straight lines (84, cf. Fig. 1), but, in some instances where such lines can be extrapolated back to the early moments of tagging, it is clear that linear extrapolation does not run through the origin at zero time (72, cf. Figs. 1 to 4).

It is implied in various reports that tagging was carried out at Berkeley under steady-state conditions (69, 73, 86), but even in a contribution entitled *Kinetic relationships of the intermediates in steady state photosynthesis* (84) the data themselves constitute incontestable proof that serious departure from steady-state conditions occurred. In these experiments, departure from steady-state conditions due to flushing with air just before adding tracer was responsible, perhaps, for the initial surge of radioactivity into the cells. Subsequent tracer assimilation at about 40 per cent of the initial rate may or may not have been limited by carbon dioxide tension as suggested by Benson and collaborators. The *decline* in radioactivity in phosphorylated sugars, glycine, alanine after only 4 min., and in all compounds tested (except sucrose and glutamate) after 10 to 15 min. of tagging is convincing evidence of nonsteady-state conditions.

However, the shortcomings of technique apparently were later corrected by the Berkeley group in the few months prior to a subsequent report entitled *The steady state* (87). Data therein show total tracer uptake to proceed linearly over a 30 min. period of photosynthesis with no suggestion of a concavity toward the abscissa found in curves previously reported from that laboratory. This latest result is quite in line with the data of

Fager & Rosenberg (80) and satisfactorily confirms their findings. Thus, the disagreement seems to have been resolved.

The most important area of factual disagreement relating to the dark pickup experiments now lies in whether or not the tracer spreads significantly beyond the carboxyl groups of phosphoglyceric and pyruvic acids. That it should do so, seems required of a general, reducing-power hypothesis in the light of which the Berkeley results are interpreted. That it does not do so is concluded from findings published by the Chicago group. Only further studies can decide whether there is really a significant and consistent qualitative difference between the results of the two laboratories.

Results giving promise of future progress.—At this point we shall exercise a reviewer's prerogative of predicting future trends. We wish to call special attention to three quite separate developments, each of which appears to be important and promising.

(*a*) Recently, Benson *et al.* (88, 89, 90, 91) found it possible to identify as phosphates of ribulose and sedoheptulose certain previously unidentified spots on chromatograms displaying products of short term photosynthesis. Adequate localization of C^{14} within the molecules of these sugar phosphates is eagerly awaited. The existing evidence is such that the presumed photosynthetic roles of these compounds remain a matter of speculation. This subject was discussed authoritatively in a recent review (9). It is mentioned here chiefly because there is some reason to believe that these C_5 and C_7 compounds may be closely related to the much sought after 2-carbon acceptor discussed above. As yet, however, the data reported are only strongly suggestive of this possibility.

(*b*) By exposing an illuminated algal suspension to a nearly constant excess concentration of $C^{14}O_2$ for a relatively long time, Calvin & Massini (87) found that the level of incorporated tracer either reached a saturation level in a very few minutes (e.g., phosphoglyceric acid) or continued to rise with time as for a final storage product accumulating in amount during illumination (e.g., sucrose). This is a particularly noteworthy contribution for it represents the exploratory application of a technique which will allow an estimate of the prevailing sizes of metabolic pools of key compounds in photosynthetic assimilation. The abrupt changes in levels of radioactivity in phosphoglycerate and in a fraction containing mainly ribulose diphosphate upon the transition from light to dark or the reverse, are interesting since they are possibly the consequences of abrupt changes in the active pool sizes of the respective constituents. As yet this technique is only in the preliminary stage of exploitation for photosynthetic studies. However it holds forth promise of providing data on specific activities of tagged intermediates, the need for which has been stressed by Wood (92) and Utter & Wood (79). By an obvious extension of the method it is possible, at least in principle, to distinguish, for a given intermediate, between its total quantity present and the fraction which is intimately involved in photosynthesis.

(*c*) Fager (71) prepared cell-free spinach leaf macerates which exhibited

increased $C^{14}O_2$ fixation upon illumination. The rate of this light-enhanced fixation was no greater than 1 per cent of the photosynthetic rate of the original tissue under comparable conditions. The rate also decreased with time. A particularly noteworthy feature of the fixation was that the major part of the radioactivity was recovered as phosphoglyceric and phosphopyruvic acids just as in short term photosynthesis with intact cells. Other reports (93, 94, 95, 96, 97, 98) of photochemical CO_2 fixation *in vitro* (accomplished by coupling known reductive carboxylations to a Hill reaction) do not provide for the incorporation of C^{14} in a pattern like that of photosynthesis.

Fager (71a) achieved some fractionation of his preparation by acetone precipitation. The proteinaceous precipitate from 47.5 to 60 vol. per cent acetone contained over one-half of the original activity and about two-thirds of the activity responsible for $C^{14}O_2$ fixation as phosphoglyceric and phosphopyruvic acids. The concentration of activity achieved over that of the original spinach juice preparation was not specified but apparently it represented a considerable purification in terms of either dry weight or total protein. Tracer fixation in the chlorophyll-free "purified" fraction was not photosensitive.

In both crude and "purified" preparations Fager concluded that tracer fixation in phosphoglycerate apparently preceded that in phosphopyruvate. Ratios of total activity in phosphoglycerate to that in phosphopyruvate were as high as 10:1. In the case of the "purified" fraction, ratios of 12:1 and 19:1 were reported. Fluoride ($10^{-2}M$) which should inhibit effectively the enolase promoting equilibration of these compounds, was found to increase the ratio to 72:1. These results confirm similar findings with regard to activity ratios of these compounds tagged by short term photosynthesis.

Fager tested various additives on the purified fraction. Adenosinetriphosphate ($10^{-3}M$) only reduced total fixation. Arsenate ($2\times10^{-3}M$) caused a small shift in fixation from ether-extractable acids to neutral substances. Ribose-5-phosphate ($1.5\times10^{-3}M$) and phosphoglyceric acid ($10^{-2}M$) were strongly inhibitory. Sedoheptulose ($1.5\times10^{-3}M$) was without significant effect. Glyoxal ($10^{-2}M$) also was inhibitory contrary to its stimulative effect on unfractionated material. Glyoxal and other 2-carbon compounds were reported (71) to enhance fixation by crude preparations but, in view of the anomalous results with glyoxal, the influence of other 2-carbon compounds on the activity of purified preparations cannot easily be predicted. Cysteine ($2\times10^{-3}M$), when present during the fractionation, enhanced the activity several fold.

The activity of Fager's preparations can be explained if it is assumed that they contain traces of the normal 2-carbon acceptor functional in photosynthesis and that more acceptor is produced at a slow rate in the light. The very low activity of the preparations relative to comparable photosynthetic fixation may be due either to rate limitation imposed upon reactions involving production of the acceptor or to reactions relating to the

oxidation of the primary photochemical oxidant to molecular oxygen. Since these spinach juice preparations are not active in a conventional Hill reaction, the latter alternative is deemed likely.

While the CO_2 fixing ability of Fager's preparations was very low it seems possible that modifications in the preparatory procedure may yield cell-free material more active in both $C^{14}O_2$ fixing ability and photochemical oxygen liberation, a hope which encourages continued study of this approach to photosynthesis *in vitro*.

In appraising the current status of our knowledge of the chemical pathway of carbon assimilation, it is clear that work with tracer carbon has produced, during its short and turbulent history, a wealth of information which is just beginning to assume some aspects of coordination. These results are both quantitatively and qualitatively encouraging. On the other hand, real progress toward an understanding of the fundamental mechanism of photosynthesis has been somewhat less than most of us would have predicted. New techniques bring new problems and in photosynthesis, as in other research fields, it has been demonstrated that one can arrive at wrong conclusions even more easily using tracers than without their aid.

Enzymes in Photosynthesis

The following section will deal primarily with the existing evidence for the participation of certain enzymes and enzyme systems which have been postulated to play a role in photosynthesis. Most of the information has been derived from studies on the intact photosynthetic apparatus or from studies on partial reactions of photosynthesis, as in the Hill reaction and its various modifications. Partial systems, such as the Hill reaction, most likely should be considered as multienzyme systems in the sense of Dixon (99). Actually, little information is available on specific enzyme systems in photosynthesis, but an effort is made to survey our present knowledge of such systems. Enzymes which are concerned with the glycolytic transformation of phosphoglyceric acid into respiratory intermediates and sugars will not be dealt with in this review, even though it is well realized that some of the intermediates of glycolysis may be of importance as precursors in the reaction system involved in photosynthetic carbon dioxide fixation.

Catalysts of the photochemical system: chlorophyll as hydrogen donor.— Chlorophyll may be considered as a photocatalyst concerned with the transformation of light energy into chemical energy in the process of photosynthesis (44). The role of chlorophyll and of accessory pigments in the process of energy transfer has been reviewed by Franck (7, 100), and by Rabinowitch (1, 3) who also has reviewed the theories of photochemical oxidation and reduction of chlorophyll during photosynthesis (44, pp. 554–57). At this point, only the evidence for and against hydrogen transfer by chlorophyll will be reviewed. Franck & Herzfeld (101) had postulated that chlorophyll in photosynthesis oscillated between an oxidized and reduced form, denoting the reduced form as H-chlorophyll. Norris *et al.* (102) tested the

hypothesis that a proton was involved in such a postulated reversible oxidation reduction cycle by incubating *Chlorella* cells in tritium water in the dark and in the light. No incorporation of tritium into chlorophyll could be detected. These investigators, however, pointed out that isotope discrimination between hydrogen and tritium might be so large that the experiment should be carried out at high concentrations of deuterium water. Norris *et al.* also demonstrated the absence of thermal exchange between purified chlorophyll and tritium water. The experiments *in vivo* were repeated by Calvin & Aronoff with heavy water, and the incorporation of C^{14} into the chlorophyll of algae was studied simultaneously (103, cf. 104 p. 559). They reported that only one fourth of the theoretically expected amount of deuterium was incorporated into chlorophyll, which they believed could be attributed to synthesis of chlorophyll. Frenkel (105) has tested the procedure of Calvin & Aronoff to determine possible exchange of hydrogen isotopes during the extraction of chlorophyll. *Scenedesmus* cells incubated in the dark were extracted with acetone containing heavy water. Also under these conditions no incorporation of deuterium into chlorophyll was observed. It has been suggested that there is a large reservoir of nonexchangeable hydrogen which is acted upon photochemically (44, 104). The data on hand do not permit a conclusion as to the feasibility of this hypothesis.

Another suggestion is that the extracted chlorophyll is identical with the oxidized rather than with the reduced form of chlorophyll (44). This may well be possible, judging from experiments on reversible bleaching of chlorophyll (106). It is not known, however, whether this reaction involves transfer of protons.

Calvin & Aronoff (103) have suggested that chlorophyll functions via an enolizable hydrogen. The experiments of Weigl & Livingston (107), however, appear to speak against such an assumption. It has also been proposed that only a small part of all the chlorophyll molecules are active in photosynthesis. This is unlikely because of the high efficiency of photosynthesis at low light intensities. Weigl & Livingston have redetermined the possibility of an exchange reaction between chlorophyll and heavy water in a number of solvents and could not observe any exchange.

Weigl & Livingston (108) also studied the possibility of hydrogen transfer in the chlorophyll sensitized reduction of butter yellow by deuterated ascorbic acid in dry dioxane. When four to seven butter yellow molecules had reacted for each molecule of sensitizer present, with about 75 per cent reduction of the butter yellow, the chlorophyll was separated and analyzed for deuterium. On the assumption that chlorophyll contained one active hydrogen which could be transferred, and that one hydrogen would be transferred for each molecule of butter yellow reduced, not more than 4 per cent of the expected amount of deuterium could be detected in chlorophyll. These authors conclude that in this particular reaction hydrogen transfer is not involved, but point out that this may not be necessarily true for other chlorophyll sensitized reactions.

In conclusion it may be said that we have no evidence that chlorophyll transfers protons in photosynthesis. The experiments performed thus far have not tested all the postulated reaction mechanisms, and a possible proton transfer by chlorophyll may have eluded investigators to the present time.

Catalysts of the photochemical system: catalyst B of Franck & Herzfeld.—In an effort to establish a consistent theory of photosynthesis which would take into account all of the available kinetic data, Franck & Herzfeld (101, 109) postulated a catalyst active in the stabilization of intermediate labile photo products (catalyst B). This catalyst accounts for the magnitude of flash saturation when only about one chlorophyll molecule out of a thousand is absorbing light, and under some conditions for the value of the saturation rate of photosynthesis in continuous light. From a consideration of the high quantum yield of photosynthesis at low light intensities and because of the absence of long induction periods, Franck & Herzfeld concluded that photosensitive substrate was probably present in a concentration equal to that of chlorophyll. Franck & Herzfeld then postulated that unstable photoproducts are formed by the photochemical reaction and the capacity of catalyst B to stabilize these products determines the amount of photosynthesis per flash at saturation. At the same time back reactions are postulated to occur which transform the unstable photoproducts to their original energy level in case catalytic stabilization does not keep pace with their formation. At low light intensities, catalyst B is effective in stabilizing practically all unstable photoproducts; back reactions become important only at higher light intensities (near and beyond light saturation). Another consequence of this theory is the interpretation of the effect of cyanide on the yield per flash in Emerson & Arnold's experiments (110). The yield per flash is independent of cyanide concentration if the period between flashes is made long enough. Weller & Franck (111) have repeated these experiments in more detail. They interpret their results to mean that cyanide has no effect on the working period of catalyst B; however, cyanide affects the rate of formation of photosensitive substrate (inhibition of catalyst A of Franck & Herzfeld) Rieke & Gaffron (112) also have demonstrated in more intricate flashing light experiments that the catalyst which limits photosynthesis per flash at light saturation is not the cyanide sensitive catalyst of photosynthesis. There are no known inhibitors of catalyst B.

Clendenning & Ehrmantraut (113) studying the photochemical reduction of Hill reagents by isolated chloroplasts measured the working period of the dark catalyst as about 1/100 sec., thus supporting Franck & Herzfeld's view that the catalyst contributing to flash saturation is not concerned with reactions immediately involved in carbon dioxide fixation.

Enzymes and co enzymes involved in transfer of reducing energy to Hill oxidants.—The discovery that isolated chloroplasts in the presence of pyridine-nucleotides and the proper enzymes and substrates will carry out reductive carboxylation (93, 94, 95, 96, 97, 98) has lent strong support to the view of either coenzyme I or II, or both, being the natural Hill oxidants

in photosynthesis (114, 115, p. 586). This concept, however, has been criticized by Franck (7, 100) and by Tolmach (96). Franck has pointed out that the natural oxidant in photosynthesis has to be present in high enough concentration to suppress the photochemical reduction of molecular oxygen, which was demonstrated by Mehler (116) to be an effective Hill oxidant. The concentration of pyridine-nucleotides in leaves, as determined thus far, seems to be several magnitudes smaller (117). Furthermore, the reduction of pyridine-nucleotides in chloroplast preparations occurs only with small yields, and no direct spectroscopic reduction has been observed (116). Also, there is no evidence of the normal Hill reaction requiring either coenzyme I or II.

Ehrmantraut & Rabinowitch (16) have observed an inhibition by malonate of the photochemical reduction of quinone by intact *Chlorella* cells. This inhibition is reversed by an equimolar amount of fumarate, thus pointing to an inhibition of succinic dehydrogenase. The authors point out that this conclusion is contrary to the hypothesis of Franck (7, 100) which assumes a direct reduction by chlorophyll of such oxidants as quinone. Ehrmantraut & Rabinowitch cite the possibility of an unspecific narcotization by malonate, but no further experimental evidence is available on this point. Other enzyme systems are inhibited by malonate besides succinic dehydrogenase (118), although the latter enzyme seems to be the one most sensitive to this inhibitor. The effect of malonate on intermediates in photosynthesis was studied by Bassham *et al.* (73) who could not find any effect on the formation of phosphoglyceric acid, but observed an inhibition of the formation of C^{14}-labelled malic acid.

The effect of a number of other metabolic inhibitors on the Hill reaction and its modifications has been studied (16, 119, 120). Spikes *et al.* (121) have studied the effect of univalent ions on the photochemical activity of isolated chloroplasts. A number of univalent anions act as noncompetitive inhibitors; fluoride, however, gave a different type of inhibition than any other of the univalent ions tested (122). No critical analysis is available to indicate at which points these inhibitors interfere in this complex reaction system.

Enzymes involved in carbon dioxide fixation.—The initial fixation of carbon dioxide has usually been assumed to be a carboxylation reaction. Kinetic data obtained prior to the use of tracer carbon as well as results of isotope experiments are consistent with the hypothesis that this carboxylation reaction is inhibited by cyanide. In the theory of Franck & Herzfeld (101, 109), this cyanide-sensitive enzyme system has been referred to as "catalyst A."

Specific enzyme systems suggested to take part in carbon dioxide fixation are coenzyme-linked systems active in reductive carboxylation (114), and carbonic anhydrase which has been implicated at various times to take part in the photosynthetic sequence of reactions (44, 123).

Enzymes involved in carbon dioxide fixation: carboxylases.—The topic of "carboxylating enzymes in plants" has been reviewed recently (124). Only certain problems relating to photosynthesis will be discussed here. Of the

known carboxylases, the bacterial amino acid decarboxylases, particularly glutamic acid decarboyxlase, shows high sensitivity toward cyanide (125). For other known carboxylases no high sensitivity to cyanide has been established except for the "malic" enzyme system discussed below.

Clendenning *et al.* (126) have looked for an enzyme in plant extracts decarboxylating phosphoglyceric acid and have found no evidence for direct decarboxylation or indirect decarboxylation through pyruvate. They also have studied the relative distribution of different carboxylases in plants and demonstrated a much higher abundance of the malic system in green tissues in contrast to nonphotosynthetic tissues. In their test system involving reduced TPN^2, pyruvate, carbon dioxide, and "malic" enzyme they could not demonstrate any cyanide sensitivity as long as the enzyme was present in excess. At low enzyme concentrations $5 \times 10^{-4} M$ HCN^2 produced a definite inhibition. This observation is of interest even though we have no direct evidence for the participation of the above system in photosynthesis.

In the cell free system prepared by Fager (71, 71a), described above, the addition of DPN^2 or TPN had no effect on the light fixation of carbon dioxide into phosphoglyceric acid.

Enzymes involved in carbon dioxide fixation: carbonic anhydrase.—Most of the work on this enzyme relating to plant material has been reviewed by Weier & Stocking (127, p. 67) and by Rabinowitch (44). Since then several papers on the isolation and properties of carbonic anhydrase from plants have been published (128, 129, 130, 131, 132). A recent comprehensive review of the properties of the enzyme was prepared by Roughton & Clark (133).

Experimental evidence strongly favors the view of carbon dioxide penetrating most readily into living plant cells in the anhydrous form (44), although Steemann Nielsen (134, 135) has presented evidence for uptake of bicarbonate ion by several water plants. Österlind (136, 137) has reported utilization of bicarbonate ion by *Scenedesmus quadricauda* to be more effective under certain conditions than the utilization of anhydrous carbon dioxide. Some of these results have been discussed by Steemann Nielsen & Kristiansen (138).

Waygood & Clendenning (123, 139) critically examined conflicting evidence in the literature on the distribution of carbonic anhydrase within plant cells. They concluded from their experiments that the enzyme is localized in the cytoplasm. Furthermore, the enzyme is generally present in photosynthetic tissues and is usually absent in nonphotosynthetic tissues of the same plants. The measured activity of carbonic anhydrase (139) would be sufficient to hydrate carbon dioxide in the cell at rates comparable to those of maximum photosynthesis, based on the calculations of Burr (140). It is difficult to understand, however, why such hydration should be required in photosynthesis, in view of the evidence of anhydrous carbon dioxide being produced by a number of carboxylases (128, 141, 142).

A good deal of the evidence for participation of carbonic anhydrase in photosynthesis is based on the cyanide sensitivity of carbonic anhydrase

and of photosynthesis in certain organisms. Wood & Sibly (130) from a survey of the literature have noted a good deal of variation in the cyanide sensitivity of plant carbonic anhydrase preparations which have been examined. Differences in the cyanide sensitivity of photosynthesis in a variety of organisms could then be possibly explained by differences in the sensitivity of their carbonic anhydrases.

Wood & Sibly (130) have pointed out a similar sensitivity to azide for most plant preparations of carbonic anhydrase tested; this observation, however, may be fortuitous as the pH was not given by all investigators. Gaffron (143, p. 491) has reported an azide inhibition of photosynthesis, but it is not indicated which partial reaction is affected. Arnon & Whatley (144) and Macdowall (120) have reported azide inhibitions of photochemical reactions of chloroplasts. Thus, the available studies on azide inhibition of photosynthesis appear to contribute little to the question of participation of carbonic anhydrase in this process.

Sulfanilamide which inhibits carbonic anhydrase (133), but apparently is not specific for this enzyme as it inhibits "Zwischenferment" (145) and possibly other enzymes, does inhibit carbonic anhydrase from *Tradescantia* (139). Sulfanilamide also inhibits photosynthesis of *Chlamydomonas*, affecting a dark reaction as judged from the shape of the light saturation curve (105).

The evidence for participation of carbonic anhydrase in the photosynthetic process is thus based on its general abundance in green tissues and its sensitivity to certain metabolic inhibitors which unfortunately are not specific for this enzyme.

Enzymes relating to precursors of molecular oxygen: catalyst C.—Franck & Herzfeld (101) proposed this name for the catalytic system which is active in the liberation of molecular oxygen from an intermediate peroxide. Gaffron (146) suggested that more than one enzyme must be involved in the reaction path from primary photochemical oxidant to molecular oxygen. This assumption was based on the differential action of inhibitors on photosynthesis and photoreduction of anaerobically adapted algae. One proposed enzyme is involved in the transformation of the primary photochemical oxidant into an intermediate oxidant, which is not a photo-peroxide, and the other enzyme or enzymes catalyze the transformation of this intermediate into a hypothetical peroxide and finally into molecular oxygen. Gaffron (147) hypothesized that the oxygen liberating system is activated by light. This hypothesis has been used by Weller & Franck (111) to explain the nature of hydroxylamine inhibition of photosynthesis. The free enzyme, not inhibited by hydroxylamine, will be activated by light in the same proportion at each light intensity as the enzyme in a system not subjected to this inhibitor. Weller & Franck believe more experimental evidence should be adduced to confirm this hypothesis.

Enzymes relating to precursors of molecular oxygen: catalase.—Catalase has often been implicated as the "oxygen liberating enzyme" of photosyn-

thesis. Rabinowitch (44) has reviewed the earlier literature and feels the evidence is not in favor of such an hypothesis, but adds that the problem should be reinvestigated.

The strongest argument against the catalase theory is found in experiments by Gaffron (148), in which catalase activity in a certain strain of *Scenedesmus* was completely inhibited, while photosynthesis was not appreciably affected. Cyanide concentrations which strongly inhibit catalase activity also have little effect on the photochemical evolution of oxygen (16, 149). Tamiya (150) has criticized the objections raised against the catalase theory. He has pointed out that the measurement of hydrogen peroxide decomposition by photosynthetic tissues may not be a true measure of the catalase present in the photosynthetic apparatus. He cites experiments in which an increased catalase activity can be observed after heating of living cells; consequently, catalase activity and photosynthetic activity of living cells should not necessarily go hand in hand. Mehler (116) has raised the same point and set out to determine if hydrogen peroxide is actually produced during photosynthesis. He employed the reaction system of Keilin & Hartree (151) in which ethyl alcohol is peroxydatively oxidized to acetaldehyde by hydrogen peroxide in the presence of excess catalase. Chloroplasts, actively producing oxygen in the light in the presence of Hill reagents, will not produce acetaldehyde when excess ethyl alcohol and catalase are added. In the absence of Hill reagents, however, and in the presence of oxygen acetaldehyde is produced. This observation led Mehler to the discovery of molecular oxygen as an effective Hill oxidant under the appropriate conditions. Hydrogen peroxide does not appear to be an intermediate of photosynthesis or of the Hill reaction under conditions where other oxidant is present. Catalase from plant sources has been isolated in highly purified form by Galston *et al.* (152).

Enzymes relating to precursors of molecular oxygen: hydrogenase.—Hydrogenase by definition is an enzyme which reversibly catalyzes reactions involving molecular hydrogen. Whether hydrogenase can catalyze direct reduction of metabolic substrate by molecular hydrogen or always requires intermediate enzymes remains to be elucidated. Conversely, hydrogenase is concerned with the evolution of molecular hydrogen from certain substrates, probably not directly but as the terminal enzyme of a system active in hydrogen transport. It remains to be seen whether there is more than one enzyme which fits the above definition.

The enzyme has received a good deal of attention in the last several years and our knowledge of its properties has been reviewed by Umbreit (153) and by Gest (154). Recent work on requirements of growth factors which appear to be necessary for the production of hydrogenase in microorganisms has been reported by Billen & Lichstein (155).

Hydrogenase is present among the photosynthetic organisms in the purple bacteria (44), very probably in the green bacteria (156), and in *Rhodomicrobium vaniellii* [an unassigned photosynthetic organism (156a)],

and in certain algae (146, 157). Its presence has been reported in higher plants (158), but this observation has not yet been corroborated by other workers. There is no evidence of an essential role of hydrogenase in the "normal" process of photosynthesis in which molecular oxygen is liberated, or in bacterial photosynthesis when hydrogen donors other than molecular hydrogen are consumed in the photosynthetic process.

In algae, hydrogenase, strictly speaking, is an "adaptive enzyme." Gaffron demonstrated, in the green alga *Scenedesmus*, that incubation in hydrogen in the dark from one to several hours is required and even then complete activation may not be achieved unless the algae are carefully manipulated. No investigations are available so far to indicate the nature of the precursor of the active enzyme.

Hydrogenase from *Rhodospirillum rubrum* has been partially purified by Gest (159).

Other enzymes: cytochrome-f.—Hill & Scarisbrick (160) discovered a new cytochrome component present only in photosynthetic tissues which they named cytochrome-*f*. Davenport & Hill (161) have described the properties of this component in more detail. Its electrode potential between pH 6 and 7.7 is 0.365 v. which is slightly more oxidizing than the potential of cytochrome-*c*. Davenport & Hill have pointed out that cytochrome-*f*, in many of its properties examined thus far, is very similar to cytochrome-*c*. They have demonstrated a similarity of the bonding of the heme to protein, a considerable likeness in their spectra; also both cytochrome components in the ferro form are unable to combine at pH 7 with oxygen or carbon monoxide. Cytochrome-*f*, however, is more readily heat denatured than cytochrome-*c*.

Hill (8, 162) has made the suggestion that cytochrome-*f* may play a role in photosynthesis, because of its general presence in photosynthetic tissues, and its localization within the chloroplasts. Davenport & Hill (161) have indicated that its chemical energy could possibly be utilized in photosynthesis in a stepwise manner. On the basis of Strehler's evidence (163) that ester phosphate is formed and utilized at an accelerated rate during photosynthesis, one might postulate that cytochrome-*f* could play a role in oxidative phosphorylation. The potential between cytochrome-*f* and the oxygen electrode corresponds to a free energy change per electron which is approximately equal to the free energy of hydrolysis of an energy rich phosphate. Thus, one mole of ester phosphate could possibly be formed per mole of cytochrome oxidized. One might speculate, on the other hand, that cytochrome-*f* could play a role in the oxygen liberating system.

Thus far, at any rate, no direct experimental evidence appears to be available which points to the participation of cytochrome-*f* in photosynthesis.

Photosynthesis and Phosphorus Metabolism

Most of the work summarized here covers the last two years. The reader is referred to a number of reviews dealing with the literature previous to

1950 (12, 44, 154), also to a review on the metabolism of phosphorylated compounds in plants (164), and a recent monograph on phosphorus metabolism (165).

The most important contributions to this field have been those of Benson & Calvin and of Fager & Gaffron and their collaborators on phosphorylated intermediates in photosynthesis, and the work of Kandler (166), Strehler (163, 167) and of Goodman *et al.* (168) on transformation of phosphorus fractions during photosynthesis. The work of Benson, Calvin *et al.* and of Fager *et al.*, discussed in the preceding section, which led to the discovery of phosphorylated intermediates in photosynthesis, has laid the groundwork for further studies relating phosphorus metabolism and photosynthesis. Thus far, however, nothing is known about the reactions by which phosphorus enters into phosphoglycerate, ribulose-phosphate, or seduheptulose-phosphate which have been suggested as playing an active role in photosynthesis.

The transformation of orthophosphate during photosynthesis has been studied by Kandler (166), who worked with *Chlorella pyrenoidosa*, suspended in a phosphate free medium. Kandler observed definite changes in cellular orthophosphate levels upon illumination and when the cells were returned to darkness. The most striking changes were observed during the first minute of illumination when induction phenomena, ordinarily observed in measurement of gas exchange, also were reflected in changes of orthophosphate concentrations. The level of orthophosphate dropped sharply immediately upon illumination and rapidly recovered to a new steady-state; this steady-state value in the light was never higher than the one in the dark. When illumination was stopped the phosphate level rose to a maximum after $1\frac{1}{2}$ min. and rapidly fell again to a new steady-state. Kandler saw proof in these experiments that part of the light energy in photosynthesis is transformed into energy-rich phosphate. Kandler proposed two hypothetical schemes to account for the role of phosphorylation in photosynthesis. The first one postulates the degradation of the primary photochemical reductant to a reductant of intermediate energy level with the production of one energy-rich phosphate per molecule of reductant. The intermediate reductant and the energy-rich phosphate are then utilized in the process of carbon dioxide reduction. The second scheme of Kandler proposes a degradation of a fraction of the primary reductant to energy-rich phosphate, and this energy-rich phosphate in combination with the primary reductant is active in carbon dioxide reduction.

Strehler & Totter (167) have developed an elegant method for the determination of adenosinetriphosphate (ATP)[2] by means of measurement of the luminescence of firefly extracts, and this method was applied to a study of ATP levels in *Chlorella* cells under a variety of conditions (163). Strehler observed that upon illumination of anaerobically incubated cells the ATP level rose to a maximum within one minute and then fell off to a steady-state. When oxygen was admitted in the dark to anaerobically incubated cells, the ATP level rose to a steady-state value in about 30 seconds; this value at

25°C. was always higher than the ATP level in the light. However, at 4°C. the steady state value of ATP in the light was higher than in the dark in the presence of oxygen. Strehler has interpreted this observation to the effect that at low temperatures the aerobic production of ATP by *Chlorella* is inhibited to a greater extent than the light-induced formation of ATP; the rate of ATP formation (of cells previously incubated under anaerobic conditions), measured after 25 sec. illumination, saturated at high light intensities. The steady-state level of ATP, however, measured after 10 min. of illumination, reached a maximum and fell off at higher light intensities. The last two results were obtained whether carbon dioxide was present or not. When, after several minutes of illumination, the cells were returned to darkness a rapid rise in the ATP level could be observed. In the presence of carbon dioxide it levelled off within 3 min.; in the absence of carbon dioxide, however, it continued to rise for some time. Strehler has calculated a minimum ratio of 1:6 for energy-rich phosphate (as terminal phosphate of ATP) formed per molecule of oxygen produced during photosynthesis, but suggested that the actual value for the rate of formation of energy-rich phosphate may be considerably higher. Strehler proposed that ATP is generated and utilized by "light driven" reactions. Because the presence or absence of carbon dioixde had little effect on the steady state level of ATP in the light, Strehler believed that ATP is not directly concerned with CO_2 fixation but rather with the production of a reductant of the oxidation reduction level of carbohydrate. His theory otherwise appears to be similar to Kandler's first scheme of phosphate metabolism in photosynthesis.

It is of interest that in Kandler's and in Strehler's experiments the known induction phenomena in photosynthesis were reflected respectively in the inorganic phosphate levels and in the levels of ATP. In fact, Kandler's curves for phosphate levels are more or less the mirror image of Strehler's curves for ATP levels.

Goodman *et al.* (168) have studied incorporation of radiophosphate in the light and in the dark into a number of phosphorylated compounds of *Scenedesmus obliquus*. They found a rapid incorporation of P^{32} labelled phosphate into ATP in the dark and a rapid incorporation of P^{32} into phosphoglyceric acid in the light. From the rate of incorporation of P^{32} into ATP they concluded that under their conditions ATP was the first detectable product formed from inorganic phosphate.

Experiments by Kamen & Spiegelman (169) and by Gest & Kamen (170) with labelled phosphate had demonstrated that in *Chlorella* and in *Rhodospirillum rubrum* the phosphorus turnover in the fraction insoluble in trichloracetic acid (TCA) is greater in the light than in the dark, and they also had observed that light stimulated the flow of low specific activity phosphate from the insoluble phosphate into soluble phosphate, with a simultaneous flow in the opposite direction. Kamen & Spiegelman considered their observations as strong evidence that "phosphorylation is mediated by light directly or indirectly in photosynthetic organisms." Simonis & Grube (171)

could not confirm the increased uptake of labelled phosphate into the TCA-insoluble fraction by leaves of *Helodea densa*. They observed an increased uptake of P^{32} into the TCA-soluble fraction in the light which was greater in the presence of carbon dioxide than in its absence. They also observed that the level of inorganic phosphate drops in the light. Wassink *et al.* (172) on the other hand have obtained results similar to those of Kamen & Spiegelman. They found in *Chlorella* an increased conversion of TCA-soluble phosphate into TCA-insoluble phosphate in the light, particularly in the absence of carbon dioxide. In the absence of carbon dioxide they observed a conversion amounting to 30 per cent of the phosphate content of the cells into the TCA-insoluble fraction. Wassink *et al.* (173) noticed an increased uptake of orthophosphate by cells in the light in the absence of carbon dioxide. They fractionated (5 min. hydrolysis with *N* HCl at 100°C.) the TCA-soluble phosphate into a labile fraction and a stable fraction and found that the conversion of the labile fraction into the stable fraction was reduced in the presence of carbon dioxide. Addition of glucose had quantitatively the same effect as carbon dioxide in diminishing the conversion of labile into stable phosphate. Similar observations were made by Wintermans & Tjia (174), who concluded that the extra phosphate which was taken up in the absence of carbon dioxide was largely stored in labile phosphate compounds.

Holzer (175) has found that a great part of the TCA-soluble, 7 min. hydrolyzable, phosphate in *Chlorella* is metaphosphate and has suggested that the ester phosphate fraction may be small. He observed differences in the amount of metaphosphate in the light and in the dark. However, it is not apparent from the data if these differences were significant.

Phosphorylation in cell-free preparations in the light has been demonstrated by Vishniac & Ochoa (176) who incubated spinach chloroplast preparations with DPN, ATP^2, labelled phosphate, and mitochondria. Radioactive phosphate was incorporated into ATP, the incorporation depending upon the complete system and light and was active with mitochondria from either plants (mung bean) or animals (rat).

In the cell-free system of Fager (71a) described previously, addition of ATP actually caused an inhibition of carbon dioxide fixation into phosphoglyceric acid in the light and in the dark. This inhibition, Fager suggested, possibly may have been caused by ATP promoting other carbon dioxide fixations reactions, or it may have been due to ATP having reduced the amount of precursor which could be carboxylated to form phosphoglyceric acid. Fager has indicated that acylphosphate bonds were not involved in the fixation of carbon dioxide into phosphoglyceric acid in this sytem.

In conclusion, it may be said that the investigations of Strehler clearly show that oxidative production and utilization of energy-rich phosphate (as ATP^2) are accelerated during photosynthesis. From the studies reviewed it does not seem possible to say as to whether production of energy-rich phosphate is tied to reactions involving the primary reductant of photosynthesis. Neither do we know as yet of the possible fate of energy-rich phosphate in

photosynthetic carbon dioxide fixation. Nonphotochemical metabolic transformations appear to be accelerated during photosynthesis. Thus, the increased rate of production and consumption of energy-rich phosphate may be a reflection of this increased metabolism, which, strictly speaking, may not be part of photosynthesis. The work of Fager on cell-free preparations which are capable of fixing carbon dioxide into phosphoglyceric acid would indicate that ATP is not necessary for such fixation. However, no final conclusion can be drawn from these experiments, as the activity of this system is small compared to normal photosynthesis.

The effect of certain metabolic inhibitors on photosynthesis: inhibitors which in other systems, are known to interfere with the metabolism of energy-rich phosphate.—Loomis & Lipmann (177) observed that low concentrations of 2,4-dinitrophenol (DNP)[2] would inhibit the formation of energy-rich phosphate bonds in rabbit kidney homogenates without affecting oxidation and would stimulate oxidation, in phosphate-deficient systems. In the meanwhile, this observation has been extended to numerous organisms and tissue preparations. Substances other than DNP will produce similar effects, although in each case the mechanism of this action is far from elucidated (115, p. 578).

No systematic study has been carried out on the effect of substances which might interfere with the metabolism of energy-rich phosphate in photosynthesis. Gaffron (178) demonstrated that photosynthesis and photoreduction in *Scenedesmus* (strain D-3) were strongly inhibited by DNP. Dark production of molecular hydrogen was completely inhibited by $2\times10^{-4}M$ DNP (pH 6.2) although light production of hydrogen was observed under these conditions (178a).

Holzer (175) has studied the effect of DNP on photosynthesis and respiration of *Chlorella pyrenoidosa* and obtained results similar to those of Gaffron. Holzer concluded that ATP is necessary for photosynthesis but did not report any analyses indicating an effect of DNP on the distribution of phosphate fractions. According to Macdowall (120), the photochemical reduction of indophenol by isolated chloroplasts is inhibited by low concentrations of DNP.

Azide, which has been shown to interfere with transfer of energy-rich phosphate in yeast fermentation (179), also has been shown to be inhibitory to photosynthesis (143, 180), and various degrees of inhibition of the Hill reaction have been observed (16, 120, 144). No evidence has been published, however, which would indicate that either DNP[2] or azide are inhibitory because of their interference with phosphate metabolism in these systems.

Gest & Kamen (170) made the interesting observation that in *Chlorella* the turnover of P^{32} was inhibited in the presence of cyanide in the light and in the dark. Photosynthesis was strongly inhibited; respiration, however, was stimulated by the cyanide concentrations employed. The dark effect is thus suggestive of the action of DNP described by Loomis & Lipmann (177).

Warburg (56) has reported a reversible arsenate inhibition of photosynthesis. He interpreted this effect as an inhibition of normal ATP formation. Fager (71a) has studied the effect of arsenate in the cell-free preparation mentioned in the preceding section, and actually observed a slight stimulation of carbon dioxide fixation into phosphoglyceric acid.

It is apparent from the evidence cited, that work with inhibitors which are known to interfere with the metabolism of energy-rich phosphate in certain systems, thus far has thrown little light on the problem of formation and utilization of energy-rich phosphate in photosynthesis.

The behavior of plants grown on phosphorus-deficient media.—Pirson (181) has described an inhibition of photosynthesis in *Ankistrodesmus* cultures grown on phosphorus-deficient media. This inhibition was only apparent at saturating light intensities, and it was readily reversed by the addition of phosphate within one hour. Lindeman (182) has obtained similar results with *Lemna minor* and he observed that addition of phosphate to phosphorus-deficient cultures brought about an increase in photosynthetic rates which were not accompanied by an increased chlorophyll concentration. These authors have stated that the phosphate effect described must be on a dark reaction of photosynthesis.

The Source of Photosynthetic Oxygen

Fundamental to our understanding of the mechanism of photosynthesis is the source of the evolved oxygen. Does it come from water, carbon dioxide, or both? This matter has been brought up in several reviews (183, 184) and treated in a fashion which seems to us not altogether satisfactory. Reviewers and authors of original research contributions (86, 185) habitually cite the initial application of the tracer technique to this problem by Ruben *et al.* (186) and imply that the matter was then settled. This work has even been presented for consumption by beginning students in elementary text books (187, 188, 189). Without deprecating the ingenuity of Ruben and his co-workers in their pioneer use of isotopically enriched oxygen or disputing the validity of their very clear cut experimental results, it should nevertheless be pointed out that their experiments were in no sense conclusive and their results were admittedly quite ambiguous. In fact, one of the authors of the original work has retracted any claim to the contrary and it may be worthwhile to quote from the discussion of Kamen & Barker (190) their comments on the earlier conclusion that water rather than carbon dioxide was the ultimate precursor of photosynthetic oxygen:

> Such a conclusion is reasonable, but by no means certain since it depends on the unproven assumption that isotope exchange is no more rapid inside the cells . . . than in the outside medium . . . ." "It is quite possible and, indeed, even probable that this assumption is incorrect. . . . " "It can be calculated that at pH 6, the randomization of O^{18} is rapid enough to invalidate the conclusion that carbon dioxide is not a source of oxygen." " . . . These experiments do not provide proof of the role of water as the [sole] precursor of oxygen.

The difficulty was believed to be circumvented by employing the oxygen isotopes at their equilibrium concentrations for the reaction, $H_2O^* + CO_2 \leftrightarrows H_2O + CO_2^*$, which leads to a slight but significant enrichment of the heavy oxygen isotope in the CO_2. This was done by Vinogradov & Teis in 1941 (191), by Yoshida *et al.* in 1942 (193), by Dole & Jenks in 1944 (192), and again by Vinogradov and Teis in 1947 (194). Generally only the work of Dole & Jenks has been cited in this connection. However, there has not appeared an adequately critical comparison of the techniques employed in the several laboratories which might serve as a basis for preference of some results over those of other investigators. It is illuminating to tabulate the various results in terms of the fractions of the photosynthetic oxygen apparently originating from water and carbon dioxide respectively (Table 1). Rejecting the hypothesis that the source of photosynthetic oxygen depends upon whether it is investigated in Moscow, Evanston, or Tokyo, we feel that the above array of experimental results does little to bolster our confidence in the theoretically well-nigh inescapable conclusion that *all* the photosynthetic oxygen should originate from the water.

TABLE I

ORIGIN OF PHOTOSYNTHETIC OXYGEN

Per cent of photosynthetic oxygen from		Reference
H_2O	CO_2	
61	39	191
67	33	193
85	15	192
81	19	194

The situation has a further discouraging complication. It so happens that the calculated equilibrium constant at 20°C. for the reaction, $O^{16}O^{18}{}_{(g)} + H_2O^{16}{}_{(l)} \leftrightarrows O^{16}O^{16}{}_{(g)} + H_2O^{18}{}_{(l)}$, is such as to account for a small concentration of heavy isotope in gaseous oxygen. If the photosynthetic oxygen were to equilibrate with the cellular water before being evolved, then its isotopic composition would indicate (perhaps incorrectly) that some had originated from carbon dioxide. Dole & Jenks (192) suggested very tentatively that this might be the explanation for their finding that about one oxygen atom in seven apparently came from carbon dioxide. Since the isotope exchange reaction in question is extremely slow, Kamen & Barker (190) and Kamen (195) seriously considered the possibility that it might be accelerated by living cells. More recently Bentley (183) virtually accepted it as probable. It is remarkable to find in one and the same paper (192) a set of results which are cited as definitive confirmation of water as the sole source of photosynthetic oxygen and also a suggestion of biologically catalyzed isotope exchange be-

tween water and molecular oxygen which, if true, completely invalidates the method. We are forced to conclude that the only kind of result which, in principle could not be seriously questioned on the basis of the effect of exchange reactions, would have been the finding that all or nearly all the photosynthetic oxygen arose from carbon dioxide! Probably investigators of this problem would have defended such a skeptical attitude all along except for the fact that there are compelling indirect theoretical considerations which *a priori* predict the type of result first obtained by Ruben *et al.* The present reviewers do not wish to suggest an alternative concept but want only to emphasize that the experimental evidence from tracer oxygen experiments in several different laboratories not only shows wide disagreement but, on several counts, it is simply inadequate to support or deny the widely accepted theory of water as the sole source of photosynthetic oxygen.

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THE USE OF RESPIRATORY INHIBITORS[1,2]

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Introduction

The aim of this article is to survey the inhibitors available for respiration studies, including both well tried ones as well as those of more recent introduction. The study of war "gases" and their antidotes in the early nineteen-forties made many new additions to the list, especially among arsenicals and thiols; the organic chelating compounds for metals have also been the subject of much study.

The use of selective inhibitors has become one of the most important tools for elucidating the mechanism of respiration in living cells and tissues. The method is not infallible, and its most dangerous enemies at present are not the sceptics, but those who have accepted a limited series of results at face value. In association with other methods, and where varied applications of the inhibitor method itself lead to consistent results, it is probably the most fertile technique at present in use in this particular field.

The ideal of a specific inhibitor acting upon a single enzyme is at present unrealised; what we have is a number of substances which, in suitable dosages, will react preferentially with particular groupings in enzyme molecules. Dosages and conditions may be controlled with a view to limiting their reactions and increasing their specificity. Protein precipitants, such as alcohols, heavy metals, picric, and phosphotungstic acids, have no sufficient power of differentiation and fall outside our scope. Fixatives which prevent an enzyme's action by removing its substrate are also excluded, though the distinction may not always be easy in practice, and a single substance may be both fixative and inhibitor. Hydrogen cyanide may form cyanhydrins with carbonyl groups either in substrates such as triosephosphate or enzymes such as glutamic acid decarboxylase. A selective inhibitor may be regarded as a substance having a high affinity for one or more reactive points on the enzyme surface. At least one of the points attacked may be the prosthetic group at which the enzyme's catalytic powers centre. This is not invariable, and some inactivations seem to be due to steric hindrance by an inhibitor molecule attached not to the active group itself but to a neighbour (see e.g. p. 67).

Characters of a Useful Respiratory Inhibitor

The most important characteristic of a useful selective inhibitor is a

[1] The survey of the literature pertaining to this review was concluded in January, 1953

[2] The following abbreviations will be used in this chapter: BAL, 2,3-dimercaptopropanol; dieca, sodium diethyldithiocarbamate; ATP, adenosinetriphosphate; dh., dehydrogenase; dc., decarboxylase; ox., oxidase.

limited range of reaction; but this is not the only requirement. Many inhibitors that can be successfully applied to extracted enzymes or enzyme systems are useless for the investigation of living tissues. Thus, pyrophosphate, which has been helpful in elucidating the mechanism of cysteine oxidation *in vitro* is difficult to apply to tissues because of its high dissociation and slow rate of entry (1, 2, 3). Reasonable rates of arrival at the reacting centres and of reaction under biological conditions are needed. Hydrogen cyanide shows these two properties and is one of the most convenient of the inhibitors forming metallic complexes. Inhibitors like iodoacetic acid, that inhibit more slowly, are likely to be less convenient in use, and really slow rates may be intolerable. The rate of development of an inhibition may be controlled by the rate of the inhibitor's entry into the cell, but it may depend on other factors such as the internal distribution of the enzyme concerned. A low dissociation constant of the inhibitor usually favours rapidity of inhibition *in vivo* and is further discussed in connection with acidity on page 65.

A requirement to some extent conflicting with the foregoing is solubility in biological media. Nonpolar compounds may enter plant cells very readily but yet be difficult to apply. Thioureas are useful copper reagents and enter plant cells (4) but are generally too insoluble in water to be applied conveniently to the tissues, although occasional use has been made of them (5). Some promising candidates have failed because they are rapidly destroyed by the tissue concerned. Most inhibitors act by combining with the enzyme to form an inactive compound, either with the prosthetic group, which may be removed, or with the whole enzyme. High affinity between the inhibitor and enzyme is desirable, so that the inhibitor may be used in low concentration and yield a stable compound. This is particularly desirable where it is hoped to obtain quantitative results. Noncompetitive inhibitors, operating at low concentrations and independent of the substrate concentration for their effects, therefore, have great advantages. Nevertheless, several competitive inhibitors, whose action depends on the relative concentrations of inhibitor and substrate present, have played outstanding parts in respiration studies, notably carbon monoxide and malonic acid. What they lack in precision they make up in specificity. Even inhibitors of low specificity, if they can be used to differentiate classes of enzymes, may be valuable. The urethanes have played an important part in this way.

Reversal of inhibition.—Reversibility of the inhibition reaction may be advantageous in allowing a convenient demonstration of its selective nature. Irreversible inhibitions may be due to a general protoplasmic degradation, and it is less easy to show that this has not happened if the inhibition cannot be removed. Too ready reversal may, on the other hand, create practical difficulties, as when hydrogen cyanide has to be used in the presence of alkalis.

Occasionally an inhibitor may react reversibly with one system and irreversibly with another. Cyanide inhibitions are nearly always reversible,

but succinic dehydrogenase is only inhibited under relatively drastic conditions and then irreversibly (6). The competitive inhibition of succinic dehydrogenase by malonic acid is reversible by adjustment of the succinic acid concentration, but the respiration of some cells (p. 81) is inhibited by moderate doses of malonic acid irreversibly. The site of the irreversible inhibition has not yet been identified.

Reversal of an inhibition may be brought about in several ways. If the inhibitor-enzyme complex is readily dissociable, it may be sufficient, even with intact tissues, merely to take away the external supply of inhibitor. A familiar example is the disappearance of cyanide inhibition when cyanide is removed from the tissue surface by washing or by diffusion into an alkali. The cyanide inhibition of extracted belladonna polyphenol oxidase disappears merely on standing at mildly acid pH, as the cyanide diffuses off into the air. Similar recovery may be exhibited by a tissue, after which a second dosage of cyanide will renew the inhibition. It is claimed that the respiration of wheat roots shows a recovery from cyanide or azide inhibition after an hour or so and that inhibition can then only be brought about by much higher concentrations of inhibitor (7). If this is so, the secondary respiration cannot be identical with the original, and it must be supposed that a change of enzyme mechanism has occurred, not a simple reversal of inhibition.

Inhibition may be caused by an actual removal of the prosthetic group after its reaction with the inhibitor. Prolonged dialysis against 0.01*M* HCN will remove the prosthetic copper from potato polyphenoloxidase (3, 8, 9) or cucumber ascorbic oxidase (10). The resulting inhibition may be reversed by treatment with dilute copper sulphate. An excess of copper must be supplied, because some is taken up at catalytically inactive positions on the apoenzyme. Inhibition may also be reversed by the use of reagents which combine more vigorously than the enzyme with the inhibitor. For example, the inhibition of yeast invertase by silver ions is readily reversed by H_2S which does not itself poison the enzyme at low concentrations (11).

The means of reversal may afford valuable confirmation of the nature and specificity of the inhibition. Lewisite is an efficient poison for enzymes containing -SH groups with which it combines in pairs. Its action is more efficiently reversed by dithiols such as BAL[2](2,3-dimercaptopropanol), capable of forming stable rings (p. 78), than by monothiols such as cysteine or glutathione (12 to 15). BAL itself inhibits mushroom polyphenol oxidase by combining with its copper, and the inhibition is reversed by addition of excess copper sulphate (16).

Another type of reversal, and still perhaps the most elegantly diagnostic that we have, is due to the decomposition of iron carbonyl compounds by light of absorbed wave lengths. The reaction has been extensively investigated by Warburg (3) and has been one of the most important tools for the inhibition of respiratory oxidations.

When the oxygen consumption of a tissue is stopped by an enzyme inhibitor, it may be possible to cause renewed oxygen uptake by giving the

tissue a substrate that occurs at a later stage than the enzyme block in the respiratory series of reactions. For example, if enolase is inhibited with sodium fluoride, it may be possible to restore oxygen consumption by giving pyruvic acid. This appears to happen with spinach leaves (17). Such manipulations are sometimes referred to as reversals of inhibition but are not so in reality. The enolase remains inhibited and the new oxygen consumption comes from the oxidation of the pyruvic acid. When fumarate is added to malonate-inhibited tissues, again increased oxygen consumption is often observed. It is possible here that cyclic oxidation of the fumarate may lead to succinate formation as in spinach leaves (18) and that the succinate may accumulate sufficiently to reverse the malonate inhibition. The term reversal is however, much more frequently applied to the fumarate effect than proved. Such loose applications are likely to be misleading, and in the absence of proof it would probably be better to forego them.

Identity of the Enzyme Inhibited

It is a necessary precaution that the action of an inhibitor should be tested upon the enzyme as extracted from the tissue in question as well as being applied to the tissue itself. This is not always done, the tacit assumption being made that the enzyme as identified by function will always react with inhibitors in the same way from whatever tissue it may have come. Yet there is abundant evidence that this does not always happen. Alcohol dehydrogenases from animal tissues are not inhibited by low concentrations of iodoacetate (19) but the alcohol dehydrogenases of yeast (20), *Bacterium coli* (21), and oat coleoptiles (22) are. Lactic dehydrogenases are more resistant; but, at the relatively high concentration of 10 mM iodoacetate, the muscle enzyme is heavily inhibited, whereas the yeast enzyme is not (20), and the heart enzyme is still uninhibited at 20 mM (23). The ascorbic oxidase of cucurbits is reported to be inhibited by carbon monoxide (24), but not that of barley seedlings (25). The polyphenol oxidases of potatoes and apples differ greatly in their susceptibility to hydrogen cyanide (26, 27): whereas the potato enzyme is inhibited by *p*-nitrophenol (28), that of belladonna was not inhibited over a wide range of concentrations (29). Pyrophosphate inhibits the zymohexase of yeast, but not that of muscle (3, 15, 22, 30). The warning is clear that direct investigation of their enzymes is generally necessary before the results of experiments with living tissues can be satisfactorily interpreted.

Fortunately, serious variations seem comparatively rare, as will be seen in the following pages, and some investigators have taken considerable pains to show similarity of results with given enzymes of different origin. Thus, the cytochrome oxidases of wheat embryos and carrots were shown by Goddard (31) and Weeks & Robertson (32) respectively, to approximate closely in their behaviour towards carbon monoxide to Warburg's yeast enzyme (33).

Effect of Cell Structure on Selective Inhibitions

There is an evident risk that an enzyme incorporated in the living cell structure may not react with an inhibitor in precisely the same way as after extraction. It is well known that enzyme molecules contain masked -SH groups, incapable of reacting with any of the known thiol reagents, but which become reactive by partial denaturation of the proteins during extraction (34). Similarly, the existence of masked iron in plant cells was realised by Molisch as early as 1893 (35). It may, however, be doubted whether groups protected from powerful reagents by their molecular configuration are capable of playing a role with natural substrates. There are very striking examples available in which the action of inhibitors on extracted enzymes has paralleled their effects on cell respiration, and none are more telling than the classic investigations of Warburg (3) and Keilin (36) on yeast respiration and cytochrome oxidase. Their inhibitions by hydrogen cyanide, hydrogen sulphide, and carbon monoxide ran closely together, neither was affected by pyrophosphate or ethyl isocyanide. These results played a considerable part in revealing the respiratory role of cytochrome oxidase.

Rather surprisingly, an enzyme may be more protected in homogenates then *in situ*. It has been noted that cytochrome oxidase of yeast is less vulnerable to sodium azide after extraction than in the cell, and it has been suggested that the azide is partly lost on the excessive amounts of protein in the extracts (37). Similarly, the effect of glyceraldehyde upon tissue glycolyses is much stronger than its effect in extracts where the vulnerable hexokinase is commonly protected by excess glucose (38). It would appear highly improbable that ascorbic oxidase should be unaffected by azide, while barley roots which depend upon it, show the expected inhibition. Yet, in numerous experiments I have been unable to demonstrate any poisoning of the extracted enzyme. Since azide reacts only with cupric and not with cuprous copper, it is possible that the excess reducing agent in the form of the ascorbic acid used as substrate, may be partly responsible. Another possibility of variation is illustrated by fluoroacetate which does not affect aconitase in extracts but which causes its inhibition *in vivo*. This results from the conversion of fluoroacetate in the living cells to the actual inhibitor (39) as described on page 82.

Differentiating concentrations.—The least concentration of an inhibitor that will suppress an enzyme activity completely is not a quantity that can be accurately expressed, even when the inhibition is noncompetitive. On the other hand, the concentration reducing activity to one-half can be fixed and is the index generally used in comparing the effectiveness of different inhibitors (see e.g. 3, 27, 40). For the special purpose of selective inhibition *in vivo* it is not suitable, and a compromise must be made. The aim is usually to suppress the enzyme's activity within the limits of error of its measurement with the least possible disturbance of other enzymes. For

a large number of noncompetitive inhibitors, as detailed in a later section, the most suitable concentration is of millimolar order. Occasionally, as with cyanide acting upon uricase (41) or hydroxylamine upon lysine decarboxylase (42), the concentration may be brought as low as tenth or hundredth millimolar. When the dimensions of the problem are to some extent known, and it is desired to distinguish between simple alternatives, it may be worth while to aim at an inhibition a little less than total. It was found that sodium diethyldithiocarbamate (dieca)[2] totally inhibited barley ascorbic oxidase at millimolar concentration but also inhibited its cytochrome oxidase by about 25 per cent. At 0.2 m*M* the two inhibitions fell to about 90 and 9 per cent, respectively. The rise of the ratio, ascorbic inhibition/cytochrome inhibition, from about 4 to 10 allowed a better chance of distinguishing the contributions of the two systems to barley respiration (43).

If a qualitative identification only is sought, it may be possible to err on the safe side by using a dosage considerably below the minimum for full inhibition. More often the practice seems to have been to increase the dose to allow for difficulties and failures of entry. Since enzymes may be less protected in the cell than in impure extracts, this may produce very misleading results, and the question of access needs to be approached along different lines (p. 60).

The relation of the effective internal concentration to the external concentration applied raises many problems at present unsolved, but it may be noted that very few respiratory inhibitors are strong electrolytes. Their entry appears to be independent of respiratory metabolism and occurs under anaerobic as well as aerobic conditions. Arisz observed that the uptake of weak or non-electrolytes was much less effected than that of strong electrolytes by the presence of oxygen (44). Many inhibitors are nonionizing, and those that dissociate are most effective when presented to the cells as undissociated molecules. It is unlikely, therefore, that they should have high accumulation ratios with internal concentrations at equilibrium greatly in excess of those outside.

With competitive inhibitors, the question of dosages is in theory more complex, because it needs to take account of the internal concentration of substrate. In practice, the difference is not very noticeable, because the substrate concentration in the partial reactions of respiration is nearly always low. Nevertheless, the usual inhibitor concentrations in this class tend to run rather higher than with the noncompetitive types. To inhibit the succinoxidase activity of carrot discs completely, Hanley *et al.* (45) found it necessary to use 10 m*M* malonate. The concentration of carbon monoxide commonly used corresponds to about 4 m*M*, and to use this it is necessary to reduce the oxygen concentration to one quarter of the normal concentration in air. Only substances with a high intrinsic specificity are, therefore, likely to be useful as competitive inhibitors. Many of them are closely related structurally to the normal substrates they displace.

Acidity of the medium.—After concentration, the most important factor

to control in applying inhibitors to tissues is the reaction of the medium. The proper treatment has only recently been understood, and it is clear that some earlier work needs reconsideration and that some troublesome discrepancies, e.g. with malonic acid, can now be attributed to pH differences.

Lowering the pH from 7.0 to 4.5 usually has a relatively small effect on the rate of respiration. Tips of barley roots in 0.07*M* phosphate buffers show about 25 per cent reduction, with a lower trough at pH 5.0. Turner & Hanley (46) found that carrot discs in phosphate buffers had about the same rates of respiration at pH 7.5 and 6.5 but showed a drop of about 45 per cent at pH 4.5. The oxygen uptake of yeast, also in the same buffers, rose 10 per cent in passing from pH 6.9 to pH 3.9 (40). It is not certain whether these changes are caused by the change in pH or by the substitution of $H_2PO_4^-$ for HPO_4^{--} ions, but the important point here is that they are relatively small. Within the same range and using the same buffers, the change of activity of an inhibitor may be very much greater.

The effect seems to depend primarily upon the dissociation of the active substance and is not limited to respiratory inhibitors. The extensive study by Simon & Beevers (40) has shown closely similar pH effects with respiratory accelerators (often the same substances as inhibitors at lower concentration ranges), growth accelerators and inhibitors, germination inhibitors, some types of bacteriostasis, and yeast fermentation. Three main types of effect are distinguishable, corresponding with three types of dissociation. They are shown by (*a*) weak acids, the effect increasing with acidity; (*b*) weak bases, the effect increasing with alkalinity; and (*c*) nonelectrolytes, the effect being independent of pH. Strong acids and alkalis can only be present in the biological pH range, as fully dissociated salts, and are also unaffected by pH changes.

Weak acids and nonelectrolytes are about equally represented among commonly used respiratory inhibitors; weak bases, represented by phenylhydrazine; zwitterions, represented by thiourea; and strong electrolytes, are rare. The correct acidity to use will vary with the class to which the inhibitor belongs. For a nonelectrolyte, the pH within the biological range is unimportant and may be determined by other considerations. For weak acids Simon & Beevers state the useful generalisation (40) that activity is virtually independent of pH below the pK value but diminishes above it. In other words, to obtain maximal inhibition with a weak acid, the pH should be at least as low as the pK. Stenlid (47) found that the inhibition of mature carrot leaf respiration by millimolar hydrazoic acid (azide) and 2,4-dinitrophenol was much greater at pH 4.5 than at pH 7; whereas the inhibition by *o*-phenanthroline, which is not an electrolyte, was unchanged. The results of Simon & Beevers show that the inhibition of yeast respiration by ethylphenylurethane (nondissociating) and hydrogen cyanide (pK = 8.9) are unchanged in the pH range 8 to 3; whereas the activities of hydrazoic acid (pK = 4.7) and iodoacetic acid (pK = 3.1) increase with acidity, the effect

TABLE I

pK VALUES OF SOME WEAK ACIDS AND BASES WHICH INHIBIT RESPIRATION

Acids	pK	pK_1	pK_2
Pyrophosphoric acid	—	0.9	2.0
Maleic acid	—	1.8	6.6
Arsenic acid	—	2.3	—
Malonic acid	—	2.5	2.7
Iodoacetic acid	3.1	—	—
Hydrogen fluoride	3.2	—	—
2,4-dinitrophenol	4.0	—	—
Hydrazoic acid	4.7	—	—
8-hydroxyquinoline	4.7	—	—
p-nitrophenol	7.2	—	—
Hydrogen cyanide	8.9	—	—
Arsenious acid	9.2	—	—
Bases	pK_a		
Semicarbazide	3.6		
Phenylhydrazine	5.2		
Hydroxylamine	6.2		

being greater with the iodoacetate. Comparing two phenolic substances, *p*-nitrophenol (pK = 7.2) with 3,5-dinitro-*o*-cresol (pK = 4.4) they found pH changes ineffective below about pH 6 and 4 respectively. Conversely, the activity of the base phenylhydrazine rose sharply with pH to about pH 5 and thereafter very slowly. Its pK_a equalled 5.2. With the stronger base, *n*-butylamine ($pK_a = 10.6$), the inhibitory activity rose continuously to pH 8.5, the pK_a value being too alkaline to reach. The behaviour of substances forming zwitterions does not seem to have been tested in this way; but their activity would seem likely to decrease in either direction from the isoelectric point. Esterification, by converting a weak acid to a nonelectrolyte, may render its effect independent of pH. Thus, it has been shown that inhibition of the respiration of maize roots by the diethyl ester of malonic acid is almost the same at pH 8 and pH 4 (47a).

It does not seem possible to provide any complete explanation for the pH effect. Simon & Beevers point out that, although inhibition runs roughly parallel with the concentration of undissociated molecules, it does not follow it exactly. It has to be remembered that the pH in question is applied externally and that there is little knowledge of how the internal pH reacts towards it. A degree of acidity such as pH 4, that reduces the respiration of intact cells only moderately, destroys the activity of some respiratory enzymes almost entirely. Cell-free extracts of *Arum* spadix which consume oxygen rapidly at pH 7 are totally inactive at pH 3.5 (48). Extracted

enzymes, whose activity is known to be reduced to a very low ebb at pH 4, include such diverse compounds as succinic dehydrogenase of carrots (45), malic dehydrogenase, and ascorbic oxidase of wheat seedlings (49), xanthine oxidase (50), invertase from some sources (51), tyramine oxidase (52), glutamic decarboxylase of barley (53), and polyphenoloxidase of apples (54). Since respiration continues when pH 4 is applied externally to plant tissues, it is scarcely possible to suppose that the respiratory enzymes are subjected to it *in situ* or that the greater effect of inhibitors at the lower external pH is due to its direct access to the cell enzymes. In confirmation of this Simon & Beevers found that the concentration of iodoacetate required to halve the activity of zymase remained unaltered between pH 3 and pH 6. The same change of pH necessitated a 13-fold increase of iodoacetate concentration to halve the respiration of intact yeast.

There remains the possibility that the pH effect depends upon a control of inhibitor entry. Since inhibition by acids is greatest at pH values below pK and inhibition by bases above pK_a, maximal inhibition is associated with a high proportion of undissociated molecules. It is frequently argued that the molecules of weak electrolytes enter cells by diffusion more readily than the ions (55 to 58). Inhibition is usually established rapidly and, even with inhibitors like iodoacetate that are relatively slow in action, the experimental aim is usually to determine the final degree of inhibition attained. That is to say, we are not concerned with the rate of entry of the inhibitor but with the equilibrium concentration established. If we assume that only molecules can pass into the interior and that ions are virtually barred, then equilibrium will be reached when the concentration of molecules is equal inside and out. Starting with à large volume of inhibitor of given concentration outside, the internal concentration of molecules will come to equal the concentration of molecules provided externally, though the ionic concentrations may be very different, as determined by the internal and external pH values. A change of external pH increasing the proportion of undissociated molecules will tend to increase the total internal concentration, even if the internal pH remains unaltered. The effectiveness of the inhibitor will thus be increased whether it reacts with the enzyme as molecule or ion. In the foregoing, the word internal may be applied to the sites of enzyme action and not necessarily to the cytoplasm as a whole.

The above rules appear to govern the biological activities of most weak electrolytes, but there are known exceptions (59). One of the most important in the present context might be afforded by competitive inhibitors when the substrates with which they compete are also weak electrolytes. If the pK of a weak acid substrate lies below the pK of its competitive inhibitor, minimal amounts of inhibitor needed to obtain a given degree of inhibition will only be reached at the substrate pK. In the most familiar example, the competition between added succinate ($pK_1 = 4.2$) and malonate, the pK of the inhibitor is the lower and sets the limit in the usual way.

The stability of some respiratory inhibitors is affected by the acidity of

the solution. Phenols, such as hydroquinone and resorcinol, oxidise rapidly in mildly alkaline solutions and even, though more slowly, at neutrality. The toxicity of hydroquinone increases with oxidation, probably due to the formation of *p*-quinone. Smith *et al.* showed that the toxicities of hydroquinine and *p*-quinone to *Colletotrichum circinans* at pH 7 were about equal (60). Dieca[2] is stable at pH 7, but on being converted to the acid form at pH 5 it rapidly breaks down with the liberation of carbonylsulphide (43). In both these examples the gas changes due to the alteration of the inhibitors are great enough to affect manometric measurements of respiration rates, so causing a further complication.

Analysis of Respiration by Partial Inhibition

The inhibition of respiration is frequently incomplete even when a fully differentiating dosage of inhibitor is applied. The question arises whether this can be taken to indicate that respiration is proceeding by more than one channel. It was first posed by experiments with m*M* cyanide which completely inhibits cytochrome and other terminal oxidases. Warburg (3) found that if n=uninhibited activity, the quantity n[HCN]/1-n remained fairly constant for a given tissue. On this basis he proposed that the amount of inhibition depended on the dissociation of the enzyme-inhibitor complex, and was "as complete as is required by the dissociation law."

The range of tissues and inhibitor concentrations investigated was small and, even so, different "dissociation constants" were obtained for each tissue, varying from 2.3×10^{-5} for red blood cells to 4.4×10^{-4} for spleen. Although not calculated, the value for the extracted enzyme would be different again. A serious objection to the theory was raised by Commoner (61) who considered the absolute rates, Q_{0_2}, of a number of animal tissues. He was able to show that, whereas the cyanide-inhibited fraction varied widely, the cyanide-resistant fraction remained remarkably constant. This situation, he pointed out, is most easily interpreted as due to two independently varying fractions of respiration. He was further able to show that the cyanide-resistant fraction had characteristics, such as dependence upon a flavoprotein and possibly upon different substrates, which distinguished it from the more conspicuous cyanide-inhibited respiration. So far as the higher plants are concerned, Warburg's theory could not account for the observed inhibitions which, with m*M* cyanide, vary all the way from 90 per cent or higher in cereal embryos (62, 63) down to nil in *Arum* spadix (48).

When partial inhibitions are observed, it is important to show that increasing the inhibitor concentration above the differentiating dose does not materially increase the inhibition. When surviving respiration is plotted against inhibitor dosage, there should be a shelf in the curve for some reasonable range above the differentiating concentration. In the absence of such there is no very convincing demonstration of selection by the inhibitor. Curves of this type have been published for cyanide inhibition of yeast (61), and wheat respirations with horizontal curve in the range 0.1 to 1.0 m*M*

(64) and have also been obtained for the dieca[2] inhibition of barley embryos (0.2 to 1.0 mM) and the $\alpha\alpha'$-dipyridyl inhibition of barley embryos—horizontal from 1.0 to 4.0 mM (65).

It may be argued that the surviving respiration in the presence of the inhibitor is due to a shunt mechanism that was not in active operation before the inhibitor was applied. There is some evidence that the respiration of added substrates by such microorganisms as *Prototheca* (66) and *Scenedesmus* (67) suppresses the endogenous respiration. The saprophytic respiration is inhibited by low concentrations of cyanide and azide but the endogenous respiration is not (67, 68). It is therefore possible that inhibition of the external substrate respiration might release the endogenous.

The quantitative significance of a partial inhibition can only be determined if both components can be separately inhibited. Unfortunately, no specific inhibitors are known for the flavoprotein oxidases which are the most probable terminal oxidases of cyanide-resistant respiration. Experiments to be published elsewhere have shown that the cyanide-inhibited respiration of very young barley roots is itself composite. In seven-day-old root tips, the cyanide-stable respiration is very slight, but, although the cyanide inhibition accounts for 90 per cent or more, inhibition by carbon monoxide reversed by light accounts for much less. Inhibition by 0.2 mM dieca[2] in separate experiments is more considerable and accounts approximately for the difference. Since the iron inhibitor and the copper inhibitor were applied to different batches of roots, it cannot be argued that the copper system only became active after the suppression of the iron system or vice versa. Unless the iron and copper systems are operating in series, which there is no reason to suspect, the inhibitors in this experiment do perform an analysis of the terminal oxidation.

List of Selective Inhibitors of Respiration

The most commonly used inhibitors may be arranged for convenience as follows. Cyanide and azide each appear under two headings and others may eventually do the same. The arrangement is based on the known or major reaction of the inhibitor, and on secondary effects, usually of unknown mechanism, which occur. The last two categories are physiological for want of the necessary chemical information.

Thiol reagents.—Alkylating agents (iodoacetic acid, iodoacetamide, methyl and ethyl iodoacetate, methyl bromide); trivalent arsenicals (sodium arsenite, lewisite, mapharside); mercaptide-formers (*p*-chlormercuribenzoate, phenylmercuric nitrate); sodium maleate; mild oxidising agents.

Carbonyl reagents.—Hydroxylamine, semicarbazide, hydrogen cyanide.

Reagent forming a fluorophosphate.—Sodium fluoride.

Metal reagents.—Aromatic sulphonic acids (1-naphthol-2-sulphonic acid, suramin); reagents forming complexes with transition metals (hydrogen cyanide, hydrogen or sodium sulphide, sodium azide, sodium pyrophosphate, 8-hydroxyquinoline); other reagents for transition metals (BAL[2], carbon

monoxide); reagents chelating iron ($\alpha\alpha'$-dipyridyl, *o*-phenanthroline); reagents chelating copper (dieca,[2] potassium ethylxanthate, salicylaldoxime).

Phenols forming inactive complexes with copper proteins.—*p*-Nitrophenol, resorcinol.

Structural inhibitors.—Pyruvic acid, oxaloacetic acid, acetaldehyde, glyceraldehyde, malonic acid.

Reagent for oxaloacetic acid.—Sodium fluoroacetate.

Reagents affecting phosphate transfers.—Sodium arsenate, sodium azide, 2,4-dinitrophenol, phloridzin.

Narcotics.—Urethane, octyl alcohol, malachite green.

In the following paragraphs citations without attribution to a species refer to enzymes from animal tissues or bacteria, or of unstated origin; dh. stands for a dehydrogenase, dc. a decarboxylase, and ox, for an oxidase.

Thiol-alkylating Reagents

Reactive -SH groups occur on many enzymes (34, 69, 70), but there is considerable variation in the reactions between different types of enzymes and different types of thiol reagents, and a useful degree of specificity results. The alkylating agents are relatively slow in action and react with some, not all, -SH groups on native proteins.

$$\text{Alkyl I} + \text{HS}\cdot\text{R} \rightarrow \text{Alkyl S}\cdot\text{R} + \text{HI}$$

If the thiol group is essential to the activity of a catalytic protein, or possibly merely adjacent to an essential group of a catalytic protein, the formation of the alkyl compound abolishes the activity. As thiol reagents, the alkylators are unsatisfactory being slow, incomplete, and unspecific (34), but the weakness of their reaction reduces the number of enzymes with which they are likely to combine. In the past they have been much used, but there is now a well-marked tendency to abandon them for quicker-acting and more certain thiol reagents such as those forming mercaptides.

Iodoacetic acid.—Monoiodoacetic acid, introduced by Lundsgaard in 1930 as an inhibitor of muscle glycolysis (71), has been the most used of the alkylating reagents. Its first satisfactory application to plant tissue was reported by Turner (72, 73) who emphasised the necessity for prolonged application, using periods up to 12 hours. The usual concentrations used have been around mM, at which inhibition may be more or less complete. There is evidence that phosphorylation in yeast (74) or zymin preparations (75) is nearly inhibited at 0.1mM, but the enzyme affected has not yet been identified. There is fairly general agreement that the most susceptible glycolytic enzyme is triosephosphate dehydrogenase.

Inhibited at mM concentration: Triosephosphate dh.[2] (21, 76, 77), of yeast (20), and of barley (78); Alcohol dh. (21), of yeast (20), and of oat coleoptile (22); not from animal tissues.

Slightly inhibited at 1 to 10 mM: Succinic dh. of *Neurospora* (79); Sorbitol dh. (80); Lactic dh. (20); Carboxylase of yeast (70); β-Amylase (34);

Protease of tobacco leaf (81); Papain (34); Pyrophosphatase (82); of yeast (83); Urease (77).

Not inhibited at 5 to 10 m*M*: Malic dh. (84), of oats (22); Lactic dh. (23), of yeast (20); Isocitric dh. (85), of oats (86); Succinic dh. of potato (87); α-Glycerophosphate dh. (88); Glutamic dh. (34); Formic dh. of peas and beans (89); Glucose ox.[2] of *Aspergillus* (90, 91); Amine ox. (92); Xanthine ox. (20, 77); D-Amino acid ox. (34); Tyrosine dc.[2] (93); Lysine dc. (94); Zymohexase (95); Transaminase (34); Lipase (34).

Iodoacetamide.—The amide inhibits similarly to iodoacetic acid, though it may affect other than -SH groups (34). It has recently been shown that two types of triosephosphate dehydrogenase occur in peas (96) and leaves (97). One requires diphosphopyridine nucleotide and the other triphosphopyridine nucleotide; both are inhibited by iodoacetamide. It does not inhibit the glucose-6-phosphate dehydrogenase of pea (96) nor animal tissues (98), but inhibits urease (34) and the pyruvic oxidase system (76) even more strongly than iodoacetic acid. Other known effects are similar to those of the acid (70, 85).

Methyl and ethyl iodoacetates.—Mackworth (77) has compared the action of a number of lachrymators, including methyl and ethyl iodoacetates, with that of iodoacetic acid. There was a general similarity of action, but the esters were frequently the more powerful. It was necessary to dissolve them in ethylene glycol monoethyl ether (0.25 *M*) and to add a corresponding amount of the solvent to controls. Fermentation and respiration of yeast were inhibited almost completely in about 30 minutes at 0.2 m*M*. The inhibition developed slowly even with extracted enzymes and protection was afforded by excess cysteine or glutathione.

Inhibited at 0.66 m*M*: Succinic dh.[2]; Choline dh.; Alcohol dh., yeast; Triosephosphate dh.

Slightly inhibited at 0.66 to 2 m*M*: Carboxylase of yeast; Urease; Xanthine ox.[2]

Not inhibited at 0.66 m*M*: Malic dh.; Lactic dh.; Diaphorase.; Glucose ox.; D-Amino acid ox.; Zymohexase; Carbonic anhydrase; Choline esterase.

Methyl bromide.—Methyl bromide has been shown to alkylate organic sulphur and nitrogen compounds at room temperature. N-methylation occurs on primary, secondary and tertiary bases to give quaternary compounds, but the basic group must not be ionised. Compounds containing only carbon, hydrogen, and oxygen are not methylated (99). Thiol groups are methylated at 25°C. and neutral reaction, probably according to the equation

$$R \cdot SH + CH_3Br \rightarrow R \cdot S \cdot CH_3 + HBr.$$

Yeast respiration is inhibited 66 per cent by m*M* methyl bromide with

accumulation of acetaldehyde. Development of the inhibition is slow and relatively high concentrations are needed (100).

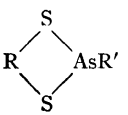
Inhibited at 10 m*M*: Succinic dh.[2]; Papain (100); Hexokinase (101); Urease, weakly (100).

Trivalent Arsenicals

Trivalent arsenicals react with -SH groups on proteins under physiological conditions. The reaction with monothiols is reversible:

$$2R \cdot SH + R'AsO \leftrightarrows (RS)_2AsR' + H_2O$$

but the reaction with dithiols or with two -SH groups close enough together upon a protein gives a stable ring of the type



Pentavalent arsenic is not bound in this way (12, 14). Protection from arsenical inhibition is afforded weakly by monothiols such as cysteine and glutathione and more strongly by dithiols, of which BAL[2] has been intensively studied. Reversal may also occur (12 to 15, 102).

Sodium arsenite.—Onaka in 1911 showed that 0.029 m*M* sodium arsenite inhibited the respiration of red blood cells (103), and Dresel later extended the observation to yeast respiration and fermentation (104, 105). Arsenite (0.1 m*M*) totally inhibits the oxidative decarboxylation of pyruvic acid, sometimes spoken of as the pyruvic oxidase system (106). It has been shown that it does not affect any of the coenzymes involved, cocarboxylase, diphosphopyridine nucleotide, nor adenylic acid, and the anaerobic oxidation of pyruvic acid is also less affected than the aerobic. The enzyme most vigorously attacked has not yet been identified. The succinic oxidase system is much less affected (106).

Not inhibited at 0.1 m*M*: Succinic dh.[2]; α-Glycerophosphate dh.; Lactic dh. (106) at 330 m*M* (23); Malic dh. (84), oat coleoptile (22); Triosephosphate dh. (106); Glutamic dh. of peas and beans at 20 m*M* (107); Isocitric dh. (85); Amino acid ox. at 1.2 m*M* (106); Amine ox. at m*M* (108); Zymohexase; Carboxylase of yeast (106).

Lewisite.—Lewisite (2-chlorovinyl dichlorarsine, $CHCl:CHAsCl_2$) is fat-soluble and hydrolyses to the toxic oxide on entering aqueous solution. After neutralisation it is a more powerful inhibitor than sodium arsenite (15, 106). It has been found to inhibit the anaerobic CO_2-production of slices of tomato stem by 37 to 40 per cent at m*M* concentration (109). Like arsenite, it appears to affect the oxidative decarboxylation of pyruvic acid more powerfully than any other system, though it is still uncertain what enzyme is the most vulnerable. Partial reversal is effected with excess BAL[2]; very little by cysteine and glutathione (13, 15).

Inhibited at 0.1 to 1.0 mM: Alcohol dh.[2] of yeast (15); Succinic dh.; α-Glycerophosphate dh.; Triosephosphate dh. (106); Choline ox.[2]; Arginase (15).

Not inhibited at 0.1 to 1.0 mM: Isocitric dh. (85); Alcohol dh. of liver; Lactic dh. (15); Malic dh. stimulated (84, 106); Diamine ox.[2]; D-Amino acid ox.; Cytochrome ox.; Catalase; Starch phosphorylase; Carboxylase of yeast (15, 106); Transaminase; Urease; Uricase; Pepsin; Trypsin (15).

Mapharside.—Mapharside (*m*-amino-*p*-hydroxyphenylarsenoxide) is a trypanocidal drug and has been investigated as an enzyme inhibitor by Gordon & Quastel (102), who find it generally to be more effective than arsenite. Inhibition is rapid. The pyruvic oxidase system is heavily inhibited at 0.1 to 1.0 mM; excess glutathione (102), or slight excess of BAL[2] (110) gives good protection.

Inhibited at mM: Urease (102); Pyrophosphatase (111).

Slightly inhibited at mM: Esterase; Choline esterase; Choline dh. (102).

Not inhibited at mM: Lactic dh.; Cytochrome ox.; Catalase; Invertase of yeast; Lipase (102).

Other trivalent arsenicals have been employed and show similar properties (102, 106, 110, 112, 113). Disubstituted compounds, particularly diphenylchloroarsine, $\phi As(Cl)\phi$, have been shown by Lotspeich & Peters (85) to inhibit isocitric dehydrogenase which is not inhibited by arsenite and lewisite. They attributed the effect to the formation of a monothioarsenite of an unusual stability, which it may owe to the presence of the two benzene rings. The inhibition is largely reversed by monothiols.

Mercaptide Formers

Traces of copper ($<$mM) react with -SH groups to form stable copper mercaptides and have frequently been used as a thiol reagent, or to inhibit thiol enzymes. The inhibition may be reversed by soluble thiols (85).

Organic mercurials react strongly with single -SH groups and apparently with all -SH groups on native proteins. They, therefore, inhibit enzymes which are not attacked by alkylating reagents and arsenicals. The inhibition may be reversed by reducing agents such as glutathione, BAL,[2] hydrogen cyanide, and hydrogen sulphide.

p-Chloromercuribenzoate.—

Inhibited at $<$mM: Isocitric dh.[2] (85); Succinic dh. (70, 113), oat and pea seedlings (114); Malic dh. (70); Formic dh. (89).

Similar effects have been obtained with phenylmercuric nitrate (85, 114) and phenylmercuric acetate [Succinic dh. of potatoes (87)].

Maleic Acid

Morgan & Friedmann (115, 116) showed that maleic acid reacts with thiols under physiological conditions to form addition compounds.

$$R \cdot SH + CH(COOH):CH \cdot COOH \rightarrow R \cdot SCH(COOH) \cdot CH_2COOH$$

At concentrations of maleate around 100 mM the succinoxidase system is half inhibited (115). The pyruvic dehydrogenase and pyruvic oxidase systems are more sensitive, and the latter is completely inhibited at 4 mM (76). Preliminary incubation is necessary.

Not inhibited at mM: Isocitric dh.[2] (85); Malic dh. (84); Triosephosphate dh. (76).

Mild Oxidising Agents

Mild oxidising agents react only with pairs of —SH groups on proteins near enough to allow —S—S— formation. A number have been used as thiol reagents and inhibitors, including ferricyanide, porphyrindin, iodosobenzoate and alloxan (see 34).

Carbonyl Reagents

A few enzymes appear to possess carbonyl groups whose integrity is essential to their functioning. The following reagents are commonly used as inhibitors but may also be used to remove carbonyl end-products that would otherwise inactivate.

Hydroxylamine.—Hydroxylamine inhibits a series of enzymes which apparently contain necessary carbonyl groups but no metal. It also inhibits catalase by combining with its haematin (117). It does not inhibit iron enzymes generally. Inhibition by hydroxylamine may be reversed by pyruvic acid (118).

Inhibited at <mM: Amino acid decarboxylases (93, 94, 118, 119, 120); Glutamic dc.[2]; of barley (53); Diamine ox.[2] (118, 121).

Not inhibited at mM: (Mono)amine ox. (92); Invertase of yeast (122).

Semicarbazide.—

Inhibited at mM: Amino acid decarboxylases (93, 94, 118, 119, 120); Glutamic dc. of barley (53); Diamine ox. (118, 123).

Not inhibited at mM: Glutamic dc. of bacteria (120); Glucose ox. of *Penicillium* (124); Invertase of yeast (122); Formic dh. of beans (89).

Hydrogen cyanide.—Cyanhydrins are formed rapidly at low concentrations under biological conditions with methyl glyoxal, glyceraldehyde phosphate (3), pyruvic and oxaloacetic acids (125). Cyanide combines with such substances more rapidly than either hydroxylamine or semicarbazide. It inactivates enzymes containing essential carbonyl groups, and the inhibition is reversible spontaneously (94) or by addition of pyruvic acid (118). To distinguish inhibition by cyanhydrin formation from inhibition due to complex formation with metals, it is necessary to show that the enzyme is also inhibited by other carbonyl reagents and not by reagents forming metal complexes, such as azide and carbon monoxide.

Inhibited at <mM: Amino acid decarboxylases, except histidine dc.[2], of

bacteria (93, 94, 118, 120, 126); Diamine oxidase (118, 123).
Enzymes known not to be inhibited by cyanide are listed on page 76.

Reagent Forming a Fluorophosphate

Sodium fluoride.—Lohmann & Meyerhof (127, 128) showed that sodium fluoride inhibits phosphoglyceric acid⇄phosphopyruvic acid but not the subsequent dephosphorylation to pyruvic acid. The mechanism was elucidated by Warburg & Christian (129) who showed that enolase, the enzyme concerned, is a metal protein, the metal probably being magnesium. Inhibition by fluoride occurs only in the presence of phosphate. Magnesium is withdrawn from the enzyme and a complex magnesium fluorophosphate is formed.

Succinic dehydrogenase is inactivated in the presence of phosphate and of 40 to 60 m*M* fluoride. Slater & Bonner suggest the formation of an inactive fluorophosphoenzyme (130). Succinic dehydrogenase is also inhibited by suramin in a manner to suggest the participation of a metal (see below). Adenosine triphosphotase is inhibited at 10 m*M* (130a).

Not inhibited at 10 m*M*: Isocitric dh.[2], oats (86); Malic dh. (131); Glutamic dh., peas and beans (107); Glucose-6-phosphate dh. (98); α-Glycerophosphate dh. (88); Cytochrome ox.(36); Uricase (132); Tyrosine dc. (93); Lysine dc. (94); Catalase (117).

Metal Reagents—Aromatic Sulphonic Acids

A number of naphthyl and anthracyl sulphonic acids have been used as inhibitors of enzymes requiring metals, particularly magnesium and calcium. Their action appears to be twofold and has been analysed by Wormall *et al.*, using the complex derivative suramin (germanin, Bayer 205). The reaction with metals is largely independent of pH, but, below the isoelectric point of the enzyme, there may also be reaction with the protein itself. In acid media, nonmetallic enzymes may be inactivated as well as metallic ones not inactivated in neutral solution (133, 134).

1-naphthol-2-sulphonic acid.—This acid, at m*M* concentration, strongly inhibits yeast carboxylase (135), which is probably linked to its coenzyme through magnesium (136, 137). It inhibits carboxylase activity in barley extracts and tissues, so causing accumulation of pyruvic acid (138, 139).

1-amino-2-naphthol-6-sulphonic acid.—Uricase, an iron-containing enzyme, is inhibited 45 per cent at 4 m*M* (132). Ascorbic oxidase, a copper enzyme, is inhibited by 1-amino-8-naphthol-4-sulphonic acid (140).

Suramin.—Although suramin is an important trypanosidal drug and inhibits the fermentation of yeast juices, it does not inhibit fermentation by intact cells. Its capacity for entering plant cells does not seem to have been tested.

Inhibited at $<$m*M* at neutral pH: Carboxylase of yeast (135); Hexokinase of yeast (requires magnesium); Succinic dh.; Choline dh.; Ascorbic ox. of cucumber, weakly; Cytochrome ox. (133, 134).

Inhibited only at pH <5.5: Urease of jack bean; Amylase of malt; Invertase; β-Glucosidase; Polyphenol ox. of potato and mushroom; Peroxidase; Catalase; Carbonic anhydrase (133, 134).

Reagents Forming Complexes with Transition Metals

Under this heading are gathered the reagents which react with both iron and copper and which were largely introduced by the pioneer work of Warburg.

Hydrogen cyanide.—Hydrogen cyanide forms catalytically inactive complexes with iron and copper in numerous metal-enzymes. The undissociated molecule may react, as with cytochrome oxidase (141); or the CN^- ion, as with cytochrome-*c*, ferrihaemoglobin and ferrimyoglobin (142). The complexes are reversible, though rather more stable than those formed by azide (117, 142). Action is rapid with extracted enzymes and tissues.

Some nonmetallic systems are accelerated by cyanide, and increased oxygen consumption results from cyanide treatment of potatoes (143), and of beech mycorrhizas which have been excised for several days. The latter show about 50 per cent increase when bathed in 5 m*M* hydrogen cyanide (144). Barley amylase (143), α-glycerophosphate dehydrogenase (88), glucose-6-phosphate dehydrogenase (98), and papain (116) are accelerated. The activation of papain is attributed to reduction of —S—S— groups; the same may apply to the other thiol enzymes.

Crude polyphenol oxidase extracts are very variable in the concentration of cyanide needed for inhibition (26, 27, 145 to 148); the purified enzyme prepared by Kubowitz (8) from potatoes was only slowly inhibited. The inhibition of xanthine oxidase may be largely indirect and due to hydrogen peroxide after suppression of catalase (149). The technique of cyanide application in manometers has caused some trouble, and various solutions are proposed (48, 150). The best, but most troublesome, is probably the use of calcium cyanide suggested by Robbie (151, 152).

Inhibited at m*M*: Formic dh.[2] of peas and beans (89); Cytochrome ox. (153) of yeast (36), wheat embryos (62), and maize embryos (154); Ascorbic ox. of cauliflower (155), barley (156), drumstick (157), and of apples (158); Catalase of yeast (159); Peroxidase of horseradish (159), and of wheat (49); Uricase (41, 132).

Partially inhibited at 1–10 m*M*: Polyphenol ox. of potato (27, 36), apple (27, 54), belladonna (29, 145), and of tea leaf (146), *Lactarius* (27), and of mushroom (160); Laccase (161). Extent for last two not stated.

Not inhibited at m*M*: Lactic dh. (23); Malic dh. (84) of wheat (49); Succinic dh. of potato (87), *Neurospora* (79); Alcohol dh. (19) of yeast (162), and of peas (163); α-Glycerophosphate dh. (164); Glutamic dh. of yeast (165), and of peas and beans (107); Proline dh. (166); Glucose ox. of *Aspergillus* (90, 91), and of *Penicillium* (124); Xanthine ox. (149, 167); D-Amino acid ox. (168); Oxalic ox.

of moss (169); Amine ox. (92, 108, 170); Pyrophosphatase (111) of yeast (83); Zymohexase of muscle (95); Urease (171); Pectin esterase (172).

Hydrogen sulphide.—Hydrogen (or sodium) sulphide has been used usually as a means of confirming the action of cyanide. It has the disadvantage of being itself rapidly oxidised by some enzymes and tissues. It inhibits D-Amino acid oxidase irreversibly (132).

Inhibited at m*M*: Cytochrome ox.[2] (36); Ascorbic ox. of drumstick (157); Polyphenol ox. [partially? (36)]; Catalase of yeast; Peroxidase of horse radish (159); Laccase (161).

Not inhibited at <m*M*: Sorbitol dh. (80); Xanthine ox. (173); Glucose ox., *Penicillium* (124); Uricase (41, 132); Amino acid dc. of bacteria (93, 94, 119).

Sodium azide.—Sodium azide resembles cyanide in its capacity for forming complexes with metals, and, like it, combines as undissociated (hydrazoic) acid with cytochrome oxidase and as N_3^- ion with cytochrome-*c*. Its complexes are slightly less stable (141, 142), but the degree of inhibition obtained is usually very similar. It has been shown to combine with the haematin of catalase with a slight change in the absorption spectrum (117). Its action is noticeably affected by pH in the range 5 to 8, even with extracted enzymes. Mushroom catechol oxidase is inhibited 68 per cent by 2 m*M* azide at pH 5.9 and not at all at pH 7.3 (159). D-amino acid oxidase is inhibited 53 per cent at pH 6.8 and slightly accelerated at pH 7.8 (132). Presumably the HN_3 molecule is the principal agent. Acceleration is also recorded with α-phosphoglycerate dehydrogenase (88), and with impure glucose oxidase preparations, where it is said to be due to suppression of catalase activity by the azide (124). The action of azide on phosphate transfers is given on page 83.

Inhibited at m*M*: Formic dh.[2] of peas and beans (89); Cytochrome ox. (159) of wheat embryo (62), and of maize embryo (154); Catalase of yeast (159).

Partially inhibited at m*M*: Polyphenol ox. of belladonna (29), and of mushroom (159); Peroxidase of horse radish (159); Lysine dc.; Arginine dc.; Ornithine dc. of bacteria (94, 120); Carbonic anhydrase (174); Uricase (132); Laccase, extent not stated (161).

Not inhibited at m*M*: Glucose-6-phosphate dh. (98); Sorbitol dh. (80); Glucose ox. of *Aspergillus* (90), and of *Penicillium* (124); Oxalic ox. of moss (169); Xanthine ox. (173); Uricase (41); Tyrosine dc.; Histidine dc.; Glutamic dc. (93, 119, 120).

Sodium pyrophosphate.—Warburg & Sakuma (1, 2, 3) found that pyrophosphate would inhibit the oxidation of cysteine catalysed by traces of iron; but it was also found that the iron and copper complexes of pyrophos-

phate would themselves act as oxidation catalysts. The copper complex oxidised cysteine (3). The power of pyrophosphate to form iron and copper complexes appears to be weaker than that of cyanide or azide, and it inhibits fewer metallic enzymes. It is slow to enter tissues and may be hydrolysed by pyrophosphatase. Concentrations used are usually of the order of 10 m*M*. The inhibition of isocitric dehydrogenase is relieved by Mn ions, and that of zymohexase by Zn and other ions. Pyrophosphate accelerated heart malic dehydrogenase (84).

Inhibited at about 10 m*M*: Isocitric dh.[2] (85, 175) of oat coleoptile (86); Succinic dh. (6, 176) of *Neurospora* (79); Zymohexase of yeast (30).
Not inhibited: Malic dh. (84) of oats (22); Lactic dh. (23); Other dehydrogenases (176); D-Amino acid ox. (132); Xanthine ox. (132, 177); Cytochrome ox. (36); Polyphenol ox. of potato (28); Uricase (132).

8-hydroxyquinoline.—A number of metals, including copper and iron, are chelated by 8-hydroxyquinoline (oxine) under biological conditions (178), and it has been used as an inhibitor of the metal-containing enzymes. Ascorbic oxidase of squash is strongly inhibited by it at 2 m*M*; some other preparations are rather less inhibited (179). Barley ascorbic oxidase is totally inhibited at 1 m*M* (181).

The complexes with iron, copper, manganese, vanadium, and even 8-hydroxyquinoline alone within the pH range 6.8 to 7.5, catalyse the oxidation of thiol groups in cysteine and proteins (180). Addition of 8-hydroxyquinoline to cytochrome oxidase extracts of barley at neutral reaction caused large increases of O_2-uptake. Application of a m*M* solution to living root tips, which are believed to depend mainly on ascorbic oxidase, caused 66 per cent inhibition (181). The catalytic effect of the metal complexes diminishes with pH, though not so rapidly as the effects of the metal or the oxine alone (180). Further study of the pH relationships seems necessary if the compound is to be used satisfactorily as an enzyme inhibitor.

Not inhibited: Formic dh. of peas (89, 163); Glucose ox. of *Penicillium* (124); Lysine dc. (94).

Other Reagents for Transition Metals

BAL[2] *(2,3,-dimercaptopropanol).*—BAL readily forms stable complexes with copper and ferric iron but apparently not with ferrous iron, zinc, or magnesium (182). It inhibits some copper and iron enzymes by removal of their metals into mercaptides. BAL, being a dithiol, is liable to atmospheric oxidation catalysed by copper and iron protoporphyrin (183), cytochrome oxidase (182) and belladonna polyphenol oxidase (29). It is rapidly destroyed by barley root tips (181).

Inhibited at 1–5 m*M*: Polyphenol ox.[2] of mushroom; Peroxidase; Catalase; Carbonic anhydrase; Aldehyde mutase; Glyoxalase (182).
Not inhibited at 4 m*M*: Cytochrome ox.; Pyrophosphatase; Xanthine ox.;

D-Amino acid ox.; Pepsin; Trypsin; Papain; Zymohexase (182); Formic dh. of peas (89).

Carbon monoxide.—The iron carbonyls are decomposed by visible light of short wave length, but the copper carbonyls are light-stable. Formation and decomposition are both rapid under physiological conditions. Carbon monoxide reacts with iron and copper only when they are reduced, and the carbonyl formation is quenched by high concentrations of oxygen. Inhibition of the enzymes is, therefore, competitive with oxygen and usually requires a high CO/O_2 ratio to be at all considerable. The ratio most frequently used is 19/1, i.e. 5 per cent oxygen diluted with carbon monoxide or nitrogen at 1 atm. pressure. Carbon monoxide provides the most elegant method for the identification of cytochrome oxidase activity *in vivo*, since rapid alterations of inhibition and its reversal are obtained in the absence and presence of light of wavelength 430 mμ. The full "action spectrum" was demonstrated by Warburg (3). Inhibition of yeast cytochrome oxidase with $CO/O_2 = 9$ is under 50 per cent (36) and with heart cytochrome oxidase about 60 per cent (153). Inhibition of wheat cytochrome oxidase with $CO/O_2 = 19$ is about 80 per cent (62) and with barley cytochrome oxidase about 75 per cent (181). The inhibition of polyphenol oxidase under comparable conditions is stronger and not reversed by light (36). Peroxidase, with hydrogen peroxide substrate, remains in the ferric form and is not inhibited.

Inhibited, light-reversible: Cytochrome ox.[2] [as above, potatoes (183a)]; Dihydroxymaleic ox. activity of peroxidase (184).

Inhibited, light-stable: Polyphenol ox. of potato (8, 36), belladonna (145), mushroom (160), and of chloroplast preparations (185); Ascorbic ox. of cucurbits (24).

Not inhibited: Ascorbic ox. of barley (25); Laccase (161); Peroxidase (184); Catalase (184); Uricase (132); Xanthine ox. (186); Glucose ox. of *Aspergillus* (91); Glutamic dc. of barley (53); Lysine dc. (94).

Reagents Chelating Iron

αα'-Dipyridyl.—This reagent reacts vigorously with ferrous iron to form a soluble complex $[Fe(C_{10}H_8N_2)_3]^{++}$ Reaction occurs at neutral pH and biological temperatures. There is no reaction with ferric iron, copper, manganese, or any other metal tried by Albert & Gledhill (187). Ferrous dipyridyl has a stability constant, $K_s = [\text{metal complex}]/[\text{free metal}] \times [\text{chelating agent}] = 10^{15}$ in water (188). Nevertheless, it has been shown that exchange does occur slowly between free labelled Fe^{++} and ferrous dipyridyl. Such exchanges could not be demonstrated between Fe^{+++} and haematins (189), and it has been doubted whether dipyridyl could inhibit iron enzymes. Keilin has shown that it inhibits the oxidation of cysteine by haematin, and that, after about two hours, the haematin is completely destroyed and the iron may be recovered almost completely as ferrous αα'-dipyridyl. Although the presence of the dipyridyl inhibited the cysteine oxidation by

64 per cent, it was supposed that it reacted only with the "partly liberated iron" of haematin decomposition products, not with the iron porphyrin itself (159). According to Hill (190) this last effect does not occur even after reduction.

The entry of $\alpha\alpha'$-dipyridyl into barley roots becomes visible by the formation of the pink ferrous complex *in situ*. It inhibits the respiration of barley embryos by about 50 per cent but does not inhibit cytochrome oxidase extracted from them (65). Stenlid (191) has shown about 60 per cent inhibition of respiration and total inhibition of salt uptake in wheat roots.

Keilin (159) states that the ferrous $\alpha\alpha'$-dipyridyl complex is catalytically inactive in oxidation, but this does not entirely accord with the reviewer's experience. At neutral or slightly acid pH, dipyridyl, with or without addition of equivalent ferrous sulphate, vigorously catalysed the oxidation of ascorbic acid.

Yeast zymohexase, which requires the presence of iron or zinc ions, is inhibited by $\alpha\alpha'$-dipyridyl, whereas the muscle enzyme is not (3), recalling the behaviour towards pyrophosphate. D-Amino acid oxidase is not inhibited (132).

o-phenanthroline.—*o*-Phenanthroline closely resembles $\alpha\alpha'$-dipyridyl in its power of co-ordinating iron (187) and forms an even more stable complex, $K_s = 10^{17.1}$ (188). It has been applied in similar ways (47) and may also build oxidation catalysts for ascorbic acid *in vitro* (181). It does not inhibit the catalase in chloroplast preparations (3).

The locus of the respiratory block caused by *o*-phenanthroline and $\alpha\alpha'$-dipyridyl is still in doubt. Since they do not react with manganese (187, 192), isocitric dehydrogenase may be excluded. Stumpf (193) has shown that zymohexase of yeast resembles that of muscle in not requiring the presence of a metal so that this possibility is apparently ruled out also. Aconitase, which requires free ferrous ions, is a possibility at present under investigation.

Reagents Chelating Copper

Sodium diethyldithiocarbamate (dieca).—Dieca chelates copper but also a good many other metals under mild conditions (187). Although it reacts with iron, it scarcely affects cytochrome oxidase at 0.2 m*M* and appears to be the most useful chelating agent at present available for distinguishing the copper enzymes from cytochrome oxidase *in situ*. It is unstable in mildly acid solutions, but suitable conditions for its use with extracted enzymes and tissues have been described (43). Inhibition is irreversible.

Inhibited at m*M*: Ascorbic ox.[2] of squash, cauliflower, cabbage (179), barley (43), and of wheat (49); Polyphenol ox. of belladonna (145), mushroom (43), and of chloroplast preparations (185); Laccase (161).

Not inhibited at m*M*: Succinic dh. (194); Formic dh. of peas and beans (89); Uricase (41); Lysine dc. (94).

Potassium ethylxanthate.—Potassium ethylxanthate is more restricted in its reactions with metals than dieca[2] (187). It inhibits ascorbic oxidase at m*M* (179, 181) but also inhibits cytochrome oxidase to a considerable degree (43).

Salicylaldoxime.—Salicylaldoxime reacts well and exhibits good specificity for copper (187). It has been shown to inhibit potato polyphenol oxidase (8) and the respiration of *Chlorella* (5). Dialysis reversed the respiratory inhibition but not that of polyphenol oxidase.

Phenols Forming Inactive Complexes with Copper Proteins

Richter showed in 1934 that some phenols competed with catechol for polyphenol oxidase and caused a competitive inhibition (28). Occasional attempts have been made to use them as selective inhibitors of polyphenol oxidase in tissues, e.g. *p*-nitrophenol (17), resorcinol (54). The method is at present useless because no studies have been made of the specificity of the inhibitors, and substituted phenols are of a very wide toxicity (40). *p*-Nitrophenol has been shown to inhibit the respiratory O_2-uptake of yeast strongly (40), though there is good reason to believe that it is almost wholly catalysed by cytochrome oxidase and not at all by polyphenol oxidase.

Structural Inhibitors

The course of an enzyme-catalysed reaction is often inhibited by its own end product which competes for the enzyme with the substrate. This effect is distinct from the development of the back component in a reversible reaction and is exerted, for example, by pyruvic acid upon lactic dehydrogenase (23, 195) and oxaloacetic acid upon malic dehydrogenase (22, 49, 84,195). It has been turned to account where it is desired to cause an intermediate product to accumulate, e.g., acetaldehyde poisoning of carboxylase (139), but is obviously of no use if a stoppage of later stages is wanted. It cannot, of course, be assumed that a product will inhibit its own reaction specifically; pyruvic acid appears to inhibit malic dehydrogenase (84) and ascorbic oxidase (156) as well as lactic dehydrogenase.

Glyceraldehyde.—Glyceraldehyde has long been known to inhibit glycolysis, but the locus of its operation is doubtful. It now appears that two distinct effects are responsible, one due to D-glyceraldehyde, and one shown by DL-glyceraldehyde, but not by the D-isomer and, presumably, due to L-glyceraldehyde.

D-glyceraldehyde has been shown by Needham *et al.* (38) to inhibit the triosephosphate dehydrogenase of muscle; 6 m*M* D-glyceraldehyde; caused about 70 per cent inhibition.

DL-glyceraldehyde (but not D-glyceraldehyde) at similar concentrations inhibits hexokinase of muscle (38, 196, 197) and yeast (197). Glucose affords strong protection against the inhibitor.

Malonic Acid.—Malonic acid requires a low pH (p. 66) and half an hour or so to develop its inhibitory effect in plant tissues. It has been

extensively used and made to bear a great weight in supporting the occurrence of a tricarboxylic acid cycle in plant respiration. Its principal effect is the competitive inhibition of succinic dehydrogenase, first indicated by Quastel (198, 199), with an affinity ratio of about 50/1 in favour of the malonate (200). Other carboxylic acids, such as oxalic, also inhibit succinic dehydrogenase, but are generally less efficient than malonic (84, 201). They are all supposed to act by their twin carboxyl groups uniting with the dehydrogenase protein in the vicinity of the prosthetic thiol group, and so shielding it. Malonate inhibitions of plant respiration are commonly reversible with an excess of succinic acid (17, 109), but irreversible inhibitions occur also in barley (181, 202), carrots (45), and yeast (203); and cannot be referred without more investigation to a competitive inhibition of succinic dehydrogenase. The respiration of *Arum* spadix is not inhibited even at low pH (48). At concentrations above 0.01*M* inhibition of anaerobic respiration may become appreciable, and at 0.1*M* almost complete (203a). Malonic acid sometimes occurs in plant tissues, e.g., 0.005 per cent fresh weight in wheat leaves (204), and up to 0.002 in numerous leguminous leaves. Runner bean stalks yielded a sap containing 0.03*M* malonate, which inhibited the respiration of stem discs at pH 4.5 (204a), and which would complicate the extraction of an active succinic dehydrogenase.

Other inhibitions by malonic acid are usually less vigorous, but the oxidation of citrate by pigeon breast muscle is more strongly inhibited than its oxidation of succinate (205).

Inhibited: Succinic dh.[2] of potato (87), pollen (206), *Neurospora* (79), and of oat and pea seedlings (89); Lactic dh. (195, 207); Malic dh. 84); Glucose ox. of *Aspergillus* (91); Oxaloacetic dc. (208, 209).

Not inhibited: Formic dh. of peas and beans (89); Fumarase of oats (210); Isocitric dh. of oats (86); Sorbitol dh. (80).

Reagent for Oxaloacetic Acid

Sodium fluoroacetate.—Sodium fluoroacetate exerts its toxicity *in vivo* by combining with oxaloacetic acid and, in the phrase of Liébecq & Peters, jamming the citric acid cycle, (209, 211, 212, 213). Fluoroethylfluoroacetate has been shown to have twice the toxicity of the sodium salt (214). Sodium fluoroacetate has not been found to inhibit any enzyme directly. On addition to tissues or homogenates it competes with acetic acid and forms a fluorotricarboxylic acid (212), possibly fluorocitric acid (215), in place of the citric acid normally produced. Acetate may accumulate (216). The fluorocitric acid inhibits aconitase and, in the form of extracted "inhibitor fractions," has been made to do so *in vitro* (39). Citric acid accumulates in fluoroacetate-poisoned tissues (217).

Sodium fluoroacetate at 10 m*M* inhibits the O_2-uptake of potato slices which have been shown to synthesise citric acid from acetate plus oxaloacetate and to contain fumarase (218). It also inhibits O_2-uptake by oat coleoptile sections (219) and increases the accumulation of acetate in young

tomato stem slices incubated with pyruvate (109). The use of sodium fluoroacetate appears to provide a welcome possibility of inhibiting the tricarboxylic acid cycle at a new point in addition to the malonate inhibition of succinic dehydrogenase.

Reagents Affecting Phosphate Transfers

Sodium arsenate.—In the presence of the appropriate enzymes small quantities of arsenic catalyse the arsenolysis of phosphate esters. The best known example is the dephosphorylation at position 1 of 1,3-diphosphoglyceric acid in the presence of triosephosphate dehydrogenase. The rapid destruction of the linkage prevents phosphate transfer to adenosinediphosphate and, consequently, the formation of energy-rich ATP[2] (220 to 223). 3-Phosphoglyceric acid is, however, formed as usual or even at an increased speed. Glycolysis and respiration continue, though disconnected from ATP formation.

Arsenate also catalyses the removal of phosphate from acetyl phosphate in the presence of triosephosphate dehydrogenase (223) or phosphotransacetylase (224, 225), and causes the formation of glucose instead of glucose-1-phosphate when potato phosphorylase acts upon starch (226); for other effects see Stumpf (227).

Successful application may also be made to tissues. At 1.0 mM arsenate inhibits the uptake of P^{32} by beech mycorrhizas without cutting down oxygen consumption (144). Low concentrations, which do not affect O_2-uptake, also inhibit the elongation of pieces of oat coleoptile (228), suggesting a dependence of the process on the energy of high-energy phosphates.

Sodium azide.—Although introduced as an inhibitor of metal oxidases, azide also proves to be an inhibitor of phosphate transfers. Like arsenate, it may increase the rate of glycolysis, as in *Aerobacter aerogenes* (229). In tissues which probably do not depend on a metallic oxidase, such as *Arum* spadix (181), and beech mycorrhizas (144), O_2-uptake may be increased as well as CO_2 output. Phosphorylation and formation of energy-rich phosphates are, on the other hand, markedly inhibited at low concentrations (37, 230, 231, 232). The effect is reversible and, as it will occur anaerobically, is presumed to operate upon the formation of ATP[2] during triosephosphate oxidation. The reaction mechanism is still uncertain (37, 231). Anaerobic CO_2-production is also inhibited in beech mycorrhizas (144).

2,4-Dinitrophenol.—There is no inhibition of metal oxidases at low concentrations of this inhibitor. At very low concentrations, stimulation of O_2-uptake may be marked as with carrot discs (233) and leaves (47), *Arum* spadix (181), wheat roots (234), and oat coleoptiles (219). Higher concentrations inhibit; but, between 10^{-5} and $10^{-3}M$, may accelerate anaerobic respiration (234a). There is strong inhibition of phosphorylation and formation of energy-rich phosphates at concentrations which stimulate O_2-uptake (232, 235, 236, 237). The chemical mechanism of the uncoupling is still uncertain. Syntheses (238, 239) and uptakes supposed to depend on energy-

rich phosphates are also inhibited. These include phosphate uptake by yeast (240), glucose uptake by wheat roots (234), and potassium chloride accumulation by carrot discs (233).

Phloridzin.—Phloridzin has long been known to inhibit phosphorylation in concentrations between 1 to 10 m*M* (241). It inhibits the phosphorolysis of starch to glucose-1-phosphate (242) and the formation of energy-rich phosphates associated with the oxidation of triosephosphate (243, 244), pyruvate, and citrate (245). The method of action is uncertain. Phloridzin does not inhibit amylases (241, 246), zymohexase (243), phosphoglucomutase (242), or succinoxidase (245).

Narcotics

Narcotics inhibit enzymes only at relatively high concentrations. They do not react with prosthetic groups on enzyme surfaces, but more probably cause some degree of denaturation of the enzymatic protein. Many physiological functions may be deranged at narcotic concentrations too low to inhibit enzymes. In a homologous series, narcotic activity is likely to rise with molecular weight (3). At high concentrations, narcotics cause irreversible inhibitions and cell breakdown (3, 247). Specificity is low.

Ethyl urethane.—Urethane at about 10 to 500 m*M* is the narcotic most frequently used to inhibit respiration.

Inhibited: Lactic dh.[2] (23), α-Glycerophosphate dh. (88); D-Amino acid ox. [totally at 570 m*M* (132)]; Amine ox. (92).

Not inhibited: Malic dh. at 17 m*M* (84), and in oats (22); Sorbitol dh. at 200 m*M* (80); Isocitric dh. of oats at 10 m*M* (86); Glucose ox. of *Penicillium* (124); Uricase at 5 per cent (132); Cytochrome ox. at 570 m*M* (153); Histidine dc. at 100 m*M* (119); Lysine dc. (94).

Octyl alcohol.—n-Octanol has been used principally to distinguish between D and L amino acid oxidases (168).

Inhibited: α-Phosphoglycerate dh.[2] (88); L-Amino acid ox. (168).

Not inhibited: Lactic dh. (23); Sorbitol Dh. in saturated solution (80); D-Amino acid ox. (168); Polyphenol ox., potato (28).

Malachite green.—This dye was shown by Quastel & Wheatley (199) to inhibit a number of dehydrogenases. It does not inhibit polyphenol oxidase (145).

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ORGANIC ACIDS IN PLANT METABOLISM[1]

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The organic acids occupy a central position in the metabolism of plants. They are early products of photosynthesis and as such serve as precursors for the synthesis of many other compounds. They also arise as products of the degradation of the more reduced chemical entities in the plant. The close metabolic relationship of organic acids to fats, carbohydrates, and proteins emphasizes their key role in plants.

Organic acid metabolism was thoroughly reviewed by Thimann & Bonner (121) in 1950. Subsequent work has been covered under other topics in the last issue of *Annual Review of Plant Physiology* (5, 113, 127). The present review will be concerned chiefly with developments between October 1951 and October 1952.

Tricarboxylic Acid Cycle

The tricarboxylic acid cycle releases energy and interrelates fat, carbohydrate, and protein metabolism. By analogy with animals, the probable functioning of the cycle in plants had been suggested; however, for some years the evidence offered in support of the cycle in plants was incomplete. The operation of certain enzymes and the presence of certain acids of the cycle were demonstrated in particular plants, but the complex of enzymes and the complete array of acids were never shown in a single plant tissue. Studies with inhibitors strongly indicated the cycle but were hardly conclusive. These lines of evidence were summarized by Wolf (143).

The paper of Millerd *et al.* (86) constitutes a milestone in the study of the Krebs cycle in plants, because, in a particulate enzyme complex from a single tissue, the integrated cycle was demonstrated. Particles fulfilling the description for mitochondria were recovered from mung bean seedlings; these particles oxidized the intermediates of the Krebs cycle and accomplished the oxidation of pyruvate to carbon dioxide and water. Furthermore, the energy from the oxidation was not dissipated but was used for the formation of high energy phosphate bonds in adenosinetriphosphate (ATP).[1] The preparation of particles capable of effecting the reactions of the Krebs cycle was performed at a temperature of 2°C., and successful operation apparently depended upon selection of a suitable plant tissue and suspending medium (pH 7.1, 0.1 *M* phosphate, 0.4 *M* sucrose). Such preparations oxidized citrate, α-ketoglutarate, and succinate very rapidly [Q_{O_2}(N) around

[1] The following abbreviations will be used: ATP, adenosinetriphosphate; ADP, adenosinediphosphate; AMP, adenosine-5-phosphate; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; TPNH, reduced TPN; FDP, fructose-1,6-diphosphate; F-6-P, fructose-6-phosphate.

200], and fumarate and malate more slowly [Q_{O_2}(N) 23–54]. Pyruvate by itself was oxidized slowly, but the reactions were greatly enhanced by the addition of fumarate, malate, succinate, α-ketoglutarate or citrate in low concentrations. The particles required no addition of cytochrome-*c* unless phosphate was omitted from the grinding medium; although added cytochrome was not normally required, the function of the cytochrome system in the over-all oxidations was indicated. The operation of the cycle was enhanced by added ATP, adenosinediphosphate (ADP)[1] or adenosine-5-phosphate (AMP),[1] and by magnesium ions. When the oxidation of α-ketoglutarate occurred in the presence of ATP and P^{32}-labeled phosphate ions, the ATP reisolated contained P^{32} (the ATP was enzymatically degraded to ADP and then resynthesized). The P^{32} under the same conditions was incorporated into hexosephosphates.

Dow (38) confirmed the oxidative capacity of mung bean mitochondria as reported by Millerd *et al.* (86), and found comparable activity in mitochondria from the hypocotyls of etiolated white lupine seedlings. He obtained typical Q_{O_2}(N) values (μl. O_2 uptake per hour per mg. N in the preparation) with lupine mitochondria as follows: citrate, 268; α-ketoglutarate, 247; succinate, 830; fumarate, 47; *l*-malate, 133; pyruvate plus 0.001 *M* malate, 160. Dow found no plant growth substance which specifically inhibited or stimulated the individual reactions of the Krebs cycle in lupine mitochondria, but numerous nonspecific stimulations and inhibitions were evident.

Brummond (24) has supplemented the evidence for operation of the tricarboxylic acid cycle in mitochondria from the cotyledons of etiolated white lupine seedlings. When washed mitochondria were supplied pyruvate alone, it was oxidized slowly, but in the presence of small amounts of malate the oxidation was rapid. Separate experiments were performed in which equal molar amounts of pyruvate-2-C^{14} and malate, or these two acids plus another single acid of the tricarboxylic acid cycle (citrate, *cis*-aconitate, isocitrate, oxalosuccinate, succinate, fumarate, α-ketoglutarate), were jointly added to washed mitochondria. When the residual added acid was recovered after partial oxidation, the C^{14} label could be found in it. Thus, the mitochondria were able to transfer the carbonyl carbon from pyruvate to citrate, *cis*-aconitate. isocitrate, oxalosuccinate, α-ketoglutarate, succinate, fumarate, and malate. Some of the C^{14} also was recovered in the carbon dioxide evolved. Oxidation of acetate-1-C^{14} could not be demonstrated. These data, indicating the actual condensation of a derivative of pyruvate to form citrate and the conversion of this citrate to the other intermediates of the tricarboxylic acid cycle, offer substantial support to the already convincing evidence of Millerd *et al.* (86) for the operation of the tricarboxylic acid cycle in plant mitochondria.

There is now no cause to doubt the operation of the tricarboxylic acid cycle in certain seedlings. The question of the general importance of the cycle in mature plant tissues remains to be answered. Does the developing plant

retain the highly active tricarboxylic acid cycle of the seedling, or does the plant discard it for other oxidative mechanisms? Do certain tissues in the mature plant have a tricarboxylic acid cycle, whereas other tissues do not?

Mature leaves often contain demonstrable quantities of several of the acids of the tricarboxylic acid cycle (121), and certain of the enzymes required for the conversions in the cycle. Bonner & Wildman (22) found that the respiration of starved spinach leaves could be enhanced by the addition of succinate, malate, fumarate, isocitrate, citrate or pyruvate. Furthermore, succinate, fumarate, isocitrate, malate, and pyruvate could partially reverse the malonate inhibition of the respiration. In comparable fashion, sections of starved tobacco leaves responded to the addition of organic acids (65). The percentage stimulations over the endogenous uptake of oxygen were: pyruvate, 35; acetate, 26; citrate, 42; *cis*-aconitate, 51; succinate, 70; fumarate, 33; malate, 65; and oxaloacetate, 31. Only α-ketoglutarate among the acids of the cycle was inactive; perhaps it did not penetrate the intact cells, for it stimulated uptake of oxygen of a leaf brei by 14 per cent. The tissue oxidized malonate, so no valid tests could be made with it as an inhibitor. These results and others suggest the operation of the tricarboxylic acid cycle in certain mature leaves. However, experiments with C^{14} indicate that if operative the cycle must be relatively sluggish in some leaves. For example, in *Bryophyllum calycinum* supplied $C^{14}O_2$ for a short period, the C^{14} does not come to equilibrium among the organic acids of the leaves even after several days (117). The presence in leaves of the acids of the cycle is regularly cited as evidence for the operation of the cycle, but the high concentration of these acids and their nonequilibrium distribution might be offered to show that operation of the cycle is slow. The equilibrium established by aconitase between citric and isocitric acids, is strongly in favor of citric acid, but in *Bryophyllum* leaves isocitrate is far more abundant than citrate. Whatley (141) has demonstrated aconitase in extracts from leaves of chickweed, broccoli, white clover, etc., but in certain leaves, as those from elder, he could find no aconitase.

The statement of Link *et al.* (76) that acetate is oxidized via the Krebs cycle in tomato stem slices is supported by the demonstration that about 0.1 per cent of the C^{14} from 50 μm of carboxyl-labeled acetate appeared in carbon dioxide recovered after 2 hr. incubation with 61.2 mg. dry weight of tissue in a volume of about 5 ml. In the absence of any evidence that the C^{14} was incorporated into any member of the Krebs cycle, their data seem inconclusive. The acetate inhibited the rate of oxygen uptake of the tissue. The investigators also incubated tissue juice with a mixture of acetate, oxaloacetate, malonate, and ATP, and found that citrate was formed, as they had reported earlier for preparations from potato tubers (17).

Laties (73) investigated the oxidation of pyruvate by barley root segments and found it sensitive to iodoacetate and malonate. The malonate inhibition persisted for a time but eventually was reversed by pyruvate, citrate, *cis*-aconitate, α-ketoglutarate, succinate, fumarate, or *l*-malate.

Acetate was inhibitory. When fumarate plus pyruvate were oxidized by excised barley roots or spinach leaf sections in the presence of malonate, the accumulation of succinate could be demonstrated (74).

Laties (75) has found active oxidation of acids of the Krebs cycle by mitochondria from cauliflower buds. The oxidation of α-ketoglutarate was stimulated by ATP, but dinitrophenol caused no increase in respiration in the absence of ATP. Malonate inhibition was apparent at low concentrations of the organic acids. Evidence also indicates operation of the tricarboxylic acid cycle in *Avena* coleoptiles [Bonner (21)]. Hanley *et al.* (50) have demonstrated malonate inhibition of the respiration of carrot root slices and reversal of the inhibition with succinate. Now that certain plant tissues have been clearly shown to possess the Krebs cycle, it will be interesting to follow the inevitable exploration of plants in general for the reactions.

In addition to the work done directly with plants, the results of recent investigations with animal tissues are pertinent for interpretation of possible plant reactions. Ochoa and co-workers (94) have crystallized the condensing enzyme from pig heart. They have shown the presence of the enzyme in liver, kidney, brain, skeletal muscle, and heart muscle of several animals, and in a number of species of bacterial cells. The anaerobic *Clostridium butylicum* was devoid of the enzyme. Stern *et al.* (107) have discussed the action of the condensing enzyme in detail; the condensing enzyme catalyzes the reaction between oxaloacetate and acetyl coenzyme A to yield citrate and coenzyme A, the reaction is reversible, and the equilibrium lies far toward citrate synthesis.

Oxidation of acetate via the Krebs cycle in plants has not been as clearly shown as the oxidation of pyruvate, so the report by Korkes *et al.* (68) on the mechanism of the oxidation with pyruvate as the acetyl donor is particularly pertinent. The fact that in the presence of suitable preparations pyruvate plus oxaloacetate could yield citrate in the absence of phosphate indicated that the conversion of pyruvate to acetyl phosphate was not obligatory. Instead, pyruvate, DPN[1] (diphosphopyridine nucleotide), and coenzyme A yielded acetyl coenzyme A directly (plus carbon dioxide and reduced DPN), and this condensed with oxaloacetate in the presence of the condensing enzyme to yield citrate. When present, orthophosphate competed with oxaloacetate as an acetyl acceptor and decreased the yield of citrate.

Other Oxidations of Organic Acids

Mapson & Goddard (78) demonstrated a glutathione reductase in pea seeds, and simultaneously Conn & Vennesland (32) reported the enzyme in wheat germ. Subsequently they have described the enzyme in greater detail (6, 33, 79). The enzyme catalyzes the reaction of reduced TPN[1] (triphosphopyridine nucleotide) with oxidized glutathione and hydrogen ions to yield oxidized TPN and reduced glutathione. It will not react with DPN. Anderson *et al.* (6) found that glutathione reductase is widely distributed in plants,

as were malic enzyme, isocitric dehydrogenase, and glucose-6-phosphate dehydrogenase. These enzymes reduce TPN which, in turn, will reduce oxidized glutathione in the presence of glutathione reductase; the system furnishes a convenient test for the presence of any enzyme requiring TPN. Yamaguchi & Joslyn (144) observed that an enzyme in immature pea seeds, similar to or identical with glutathione reductase, catalyzed the reduction of dehydroascorbic acid, glutathione serving as the hydrogen donor.

In recently described reactions of plant respiration and photosynthesis, TPN has played a more prominent role than DPN. Conn *et al.* (31) reported that extracts of wheat germ contain an enzyme system which oxidizes reduced TPN. This TPN oxidase is a soluble enzyme and is rather stable. It is not completely specific, for it will oxidize reduced DPN at about a fourth the rate of reduced TPN. Substances which inhibit heme and copper enzymes inhibit the oxidase, as do ascorbic acid and catalase. The oxidase can be separated into two components by electrophoresis. One component is a peroxidase, and horseradish peroxidase can be substituted for it. The nature of the other component and the general importance of the enzyme system in plant respiration have not been established.

In their study of the respiration of tomato stem slices, Link *et al.* (76) found that pyruvate was oxidized directly to acetate, and also was converted by dismutation to lactate and acetate plus carbon dioxide. Although they cite these two mechanisms for pyruvate conversion, they are of the opinion that carbohydrate and acetate are oxidized via the Krebs cycle. Other reactions reported for tomato stem slices included the splitting of malic acid to glycolic acid, the oxidation of the glycolic, glyoxylic, and formic acids, and the oxidation of amino acids. It was suggested that both tyrosinase and cytochrome oxidase might function in the respiration of tomato stems.

Formic dehydrogenase, demonstrated some years ago in plants, was systematically investigated by Mathews & Vennesland (81). They were able to resolve the system into two factors, formic dehydrogenase, which catalyzes the reduction of DPN[1] by formate, and an enzyme for the oxidation of reduced DPN by methylene blue. Production of formate by reduction of CO_2 by reduced DPN was very slight. Davison (35), with preparations from bean seeds, has tested the effect of a variety of inhibitors. Inhibition by cyanide suggests an active heavy metal, whereas inhibition by *p*-chloromercuribenzoate suggests the activity of a sulfhydryl group.

The study of microorganisms has added recently to our information on the oxidation of organic acids; Ajl (1) has reviewed their terminal respiratory patterns. He cites reports suggesting the function of the Krebs cycle in yeasts and bacteria. Ajl *et al.* (3, 4), found *Escherichia coli* unable to incorporate C^{14} from acetate-2-C^{14} into α-ketoglutarate, although *Micrococcus lysodeikticus* did produce labeled α-ketoglutarate under similar conditions. Ajl (2) suggested the possible functioning of a four carbon acid cycle involving a condensation of acetate to succinate in the cells. Apparently

glycolate and glyoxylate are not produced in the oxidation of acetate. The difficulties encountered by Karlsson & Barker (61) in demonstrating a tricarboxylic acid cycle in intact *Azotobacter agilis* cells may have arisen largely from lack of permeability of the cells to the substrates. This seems likely, for Stone & Wilson (108, 109) have been able to demonstrate the operation of the cycle in cell-free preparations of *Azotobacter vinelandii*.

Interconversion of Organic Acids

The organic acids are readily interconverted in many plant tissues. The production of citrate from malate in darkened tobacco leaves is a classical example of such a change (135). Vickery and co-workers have recently investigated the changes in the organic acid balance in tobacco leaves when the salt of an organic acid is supplied to the detached leaves through the petiole. The first two papers in this series were reviewed earlier (121). Detached leaves of tobacco cultured on water showed little change in total acidity but a marked conversion of malate to citrate (98). Culture on malate enhanced the formation of citrate, and the isocitrate metabolized was converted chiefly to citrate (130). Much of the acetate taken in was completely oxidized. Oxalate caused some injury to the leaves, but considerable amounts of the oxalate did enter the detached leaves (131); citrate increased at the expense of malate, but the change was slower in the presence of oxalate than in control leaves. The relative inertness of oxalate indicated it to be an end product of plant oxidation rather than an active metabolite.

Tobacco leaves cultured on citrate solution (132) increased in malic as well as citric acid. The citrate ion was taken up more readily from culture solution at pH 4 but accumulated more in the tissue from solutions at pH 7. The citrate stimulated respiration of the leaves, and over half of the citrate taken up was respired or converted to other compounds. Succinate and *l*-malate were likewise furnished to tobacco leaves (133). Succinate was rapidly metabolized, and 90 per cent of it disappeared within two days; about 50 to 60 per cent of the malate taken up disappeared. Malate increased either when succinate or malate was supplied in the culture medium, and the increase was such as to suggest that the succinate was converted readily to malate. In the opposite direction, the succinate content of leaves cultured on malate increased. Evidently succinate is a highly active metabolite in the tobacco leaf. Malate and succinate were about equally effective in the production of citrate. *dl*-Malate was furnished to tobacco leaves and the malic acid isolated was tested for its specific rotation (134). The results indicated that the *d* enantiomorph was unused and accumulated. As oxalate, *d*-malate appeared to be inert in the tobacco leaf.

Zbinovsky & Burris (146) have vacuum-infiltrated C^{14}-labeled organic acids into tobacco leaves to follow the transfer of carbon among the organic acids. The organic acids from the leaves were recovered by partition chromatography on silica gel and analyzed for their C^{14} content. Formate proved to be a very active metabolite, and it was quickly assimilated into a number

of the organic acids. After 40 min. in the light, far more total C^{14} was in malate than in the residual formate, and somewhat more was in citrate than in formate. The fact that malate had over half the specific activity per mg. carbon of the residual formate indicated that at least two of its carbon atoms must have been labeled. Degradation (unpublished) has, in fact, shown both carboxyls almost equally labeled, and carbons two and three with specific activities only about a fourth less than the carboxyls. Much shorter exposures to formate-C^{14} may reveal more concerning the mode of the malate synthesis. Mosbach *et al.* (87) suggest that oxidation of formate to CO_2 precedes its incorporation into citrate by *Aspergillus niger*. Although this may also be true with tobacco leaves, it does not seem likely. Strecker (110) has followed the incorporation of formate into pyruvate by extracts of *E. coli;* an analogous condensation may occur in the green leaf.

Results with the C^{14} tracer (146) verified, as indicated by Vickery & Abrahams (131), that oxalate is almost inert in the tobacco leaf. In the buckwheat leaf, labeled oxalate transferred its C^{14} somewhat more actively than in tobacco, but the transfer was still decidedly sluggish. When labeled glycolic acid was infiltrated, oxalic acid had a higher specific activity than acetate or citrate; evidently in the intact tobacco leaf oxalic acid can arise from the oxidation of glycolic acid either in light or dark. Whereas, in the light, malic acid derived twice as high a specific activity as citric acid from labeled glycolate, in the dark the specific activity of citric acid was four times that of malate. This result (and comparable results with dark infiltrated acetate) is compatible with the suggestion of Calvin (27) that a tricarboxylic acid cycle active in the dark may be suppressed in the light. Labeled acetate gave rise to very little labeling in oxalate, so acetate evidently is not metabolized via glycolate. The transfer of C^{14} from labeled malate to citrate in the darkened tobacco leaf confirms the extensive data of Vickery *et al.* (135), indicating a substantial conversion of malate to citrate. Citric acid was surprisingly sluggish in its conversion to other organic acids, although it did transfer its label to the acids recovered.

Citric acid is 5 to 10 times as abundant in legume seeds as in cereal seeds, according to Täufel & Krusen (118). It is uniformly distributed within legume seeds, but in cereals the citric acid is concentrated in the embryo and is almost lacking in the endosperm. Citric acid in rye and barley doubles within the first five days after the start of germination.

The mode of formation of citrate in *A. niger* has been the subject of much investigation and disagreement. Foster & Carson (42) cite results with C^{14}-labeled acetate and $C^{13}O_2$ which they interpret as indicating the condensation of 3 two carbon fragments to form citrate. Foster *et al.* (43) had earlier reported a C_2, C_2 condensation to form fumarate via succinate in *Rhizopus nigricans*. Martin & Wilson (80) found that in their surface cultures of *A. niger* CO_2 fixation could account for 4.4 to 7.3 per cent of the citric acid carbon; the middle carboxyl carbon was more radioactive than the terminal carboxyl carbons. Bomstein & Johnson (20) have conducted a

careful study of the formation of citrate by *A. niger*, and their yields of citrate are much higher than those achieved by others in their study of the mechanism of the acid synthesis. By the use of labeled acetate, with appropriate isolations and degradations, they were led to conclude that citrate probably was formed by condensation of active acetate and a four carbon dicarboxylic acid. The C-4 acid apparently was formed chiefly by condensation of carbon dioxide with a C-3 compound, but that this was a simple reversal of oxaloacetic decarboxylase seemed unlikely. The condensation of two acetate molecules could account for the formation of a minor portion of the C-4 acid. Oxalate did not arise by direct oxidation of acetate, but perhaps by splitting of oxaloacetate or oxalosuccinate. These results clarify the mechanism for citrate synthesis, but emphasize that the synthetic pathway is not simple, for intermediates may arise from more than one source.

A wide variety of crude plant meals (pea, soybean, lupine, clover) produce acetylmethyl carbinol from pyruvate and acetaldehyde. Singer (102) has purified the enzyme from wheat germ about 11,000 fold and has obtained a preparation which is free of racemase and appears homogeneous by electrophoresis. When this enzyme synthesizes acetylmethyl carbinol from pyruvate plus acetaldehyde or from acetaldehyde alone, the acetylmethyl carbinol is a mixture of optical isomers with a predominance of the dextro form. This partial asymmetric enzymatic synthesis is unique, and Singer discusses possible explanations for its mode of operation.

A considerable amount of new information concerning the lactic and glycolic acid dehydrogenases has been discovered since the last coverage of the subject by Vennesland & Conn (127). Zelitch & Ochoa (147) have succeeded in obtaining a highly purified preparation from spinach leaves which will oxidize both lactic and glycolic acids. It was possible to demonstrate that the purified enzyme was a flavoprotein with flavinmononucleotide as the prosthetic group. The similarity of the enzyme to L-amino acid oxidase and its insensitivity to cyanide had early suggested to Clagett *et al.* (29) that it might be a flavoprotein enzyme, but in their crude preparations they had been unable to demonstrate the formation of hydrogen peroxide, to show any influence of added catalase or catalase inhibitors, or to show any effect of added alcohol on the total uptake of oxygen. Zelitch & Ochoa's discovery of the flavoprotein nature of the enzyme now resolves the earlier puzzling results, and has revealed the production of hydrogen peroxide by the enzyme. They are of the opinion that the oxidation of glyoxylate formed from the glycolate is at least partially a nonenzymatic reaction utilizing the hydrogen peroxide formed in the oxidation of glyoxylate; the reaction can be inhibited by the addition of catalase to the purified enzyme preparations. In the purified preparations, in the absence of catalase, the glycolate is oxidized cleanly to formate and carbon dioxide, but in the intact cell glycolate gives rise also to glycine, and serine [Tolbert & Cohan (123)]. Glycolate also is a logical precursor of oxalate, which is such an abundant acid in plants, for labeled glycolate infiltrated into tobacco leaves gives oxalate of rather high specific activity (146).

Kenten & Mann (62, 63) have independently obtained essentially the same results as Zelitch & Ochoa (147). It was apparent to them from tests of manganese oxidation that the alpha-hydroxy acid dehydrogenase formed hydrogen peroxide during the oxidation of lactate or glycolate. Following this lead, they showed that catalase decreased the oxygen uptake on glycolate or lactate to about half that observed in the absence of catalase. Kenten & Mann indicated that the normal course of glycolate oxidation yields glyoxylate which in the absence of catalase is oxidized by peroxide to formate and carbon dioxide. In the presence of catalase the oxidation of glyoxylate produced oxalate which was recovered from the reaction mixture. In a similar manner, lactate was oxidized to pyruvate and the pyruvate, in the presence of the hydrogen peroxide formed, was oxidized to acetate and carbon dioxide.

Another particularly puzzling aspect of the metabolism of glycolate has been its apparent catalytic role in supporting greater uptake of oxygen than can be accounted for by its complete oxidation to carbon dioxide and water (67, 122). Zelitch & Ochoa also have discovered an explanation for this in their observation that glycolate oxidation and glyoxylate reduction can be coupled with the oxidation of other substrates and can thus serve in a terminal oxidation system (147); (the glyoxylic acid reductase was described in the oral presentation but not in the abstract cited). Specifically, they found that glucose dehydrogenase could transfer hydrogen from glucose to glyoxylate via DPN,[1] in the presence of glyoxylic acid reductase from spinach leaves, to form glycolate, which in turn could transfer the hydrogen to molecular oxygen to form hydrogen peroxide in the presence of glycolic acid dehydrogenase. With such a reconstructed system, uptake of oxygen well in excess of that needed for complete oxidation of added glycolate was demonstrated.

Noll (89) surveyed a number of plants and found representatives of 16 families of plants which could oxidize lactic and glycolic acids. He could demonstrate the reduction of 2,6-dichlorophenolindophenol anaerobically by the enzyme, so it seems approrpriate to designate the enzyme as glycolic (or lactic) acid dehydrogenase. It is a flavoprotein enzyme producing hydrogen peroxide and has none of the characteristics of the classical metalloprotein oxidases which produce water rather than hydrogen peroxide. Clagett *et al.* (29) had reported the remarkably low energy of activation of 2.3 kcal./mole for glycolic acid dehydrogenase. Although tyrosinase is in this range, no dehydrogenases or flavoprotein enzymes have been reported with such low energies of activation. Suspecting that the complications attending the oxidation of glyoxylate in the crude preparations studied earlier by Clagett *et al.* (29) might have confused the results, Noll (89) redetermined the energy of activation with purified preparations. He used the reduction of dye to measure activity under anaerobic conditions which would preclude the formation of peroxide. The molar energy of activation of glycolic acid was 12.3 kcal. and of lactic acid 11.5 kcal., values which are essentially equivalent. Noll found no oxidation of amino acids by the enzyme. Crude preparations of the enzyme were inhibited by the following

sulfhydryl inhibitors: iodoacetate, iodoacetamide, phenylmercuric nitrate, and *p*-chloromercuribenzoate. Samples of the purified enzyme obtained from Zelitch also were sensitive to sulfhydryl inhibitors.

Tolbert & Burris (122) reported that glycolic acid dehydrogenase was activated in etiolated plants by illumination. Tolbert *et al.* (124) now have been able to demonstrate the activation of the enzyme in etiolated plants in the dark by spraying them with glycolic acid. The implication is that glycolic acid is formed photosynthetically and in the presence of its substrate the enzyme is activated. This observation is of particular interest because it constitutes the only reported instance of the adaptive formation of an enzyme in higher plants.

Organic Acids and Carbohydrate Metabolism

The glycolytic enzymes (113) and the carboxylating enyzmes (127) have been thoroughly reviewed in the last volume of this publication, so relatively little additional discussion is required.

As demonstrated by Stumpf (111) pea seeds can break down FDP[1] (fructose-1,6-diphosphate) by the usual glycolytic pathway. The formation of FDP from F-6-P[1] (fructose-6-phosphate) by phosphohexokinase and ATP has now been demonstrated in a variety of higher plants, and phosphohexokinase has been partially purified from pea seeds by Axelrod *et al.* (12).

Tewfik & Stumpf (119) were unable to demonstrate the presence of triosephosphate dehydrogenase in leaf tissues. FDP is used by such tissues and these workers presented evidence suggesting that pea leaf preparations oxidized FDP by an undefined pathway rather than by way of normal glycolysis (120). Such an explanation no longer is necessary, for Gibbs (46) was able to demonstrate two triosephosphate dehydrogenases in pea leaves. The enzyme functioning with DPN was inactive until incubated with cysteine, but the TPN[1] linked enzyme was active without such treatment. The TPN enzyme was demonstrated only in parts of the plant containing chlorophyll. Independently, Arnon & Heimbürger (8) observed triosephosphate dehydrogenase in green leaves of sugar beets, spinach, sunflower and tobacco. That it was active through TPN, and through DPN only to a lesser extent, suggested that TPN was the coenzyme active in the intact cell.

That an alternate path of hexose oxidation in leaves does exist has been shown by Axelrod & Bandurski (11). With soluble extracts of acetone powders from pea and spinach leaves they could demonstrate the reduction of TPN with glucose-6-phosphate. The extract plus 6-phosphogluconic acid and TPN yielded CO_2 and ribose-5-phosphate. F-6-P and FDP also were utilized by these preparations. Bandurski *et al.* (13) have extended their studies of ribose-5-phosphate metabolism by a fractionated spinach leaf preparation. They found that approximately one sedoheptulose phosphate and one dihydroxyacetone phosphate were formed per two pentose molecules which disappeared. The triose was further oxidized to pyruvic acid.

Stafford *et al.* (105) also have demonstrated the oxidation of 6-phosphogluconate and glucose-6-phosphate by TPN-linked dehydrogenases from plants; pentose and carbon dioxide were identified as the products of the reactions. Their work was performed chiefly with preparations from wheat germ, but preparations capable of converting 6-phosphogluconate to pentose also were made from parsley leaf, spinach, cantaloupe fruit, cucumber fruit, and turnip root. There was an indication of sedoheptulose formation by the preparation from parsley leaf. Stafford *et al.* (105) were of the opinion that the pathway through pentose was actually the predominant oxidative pathway in wheat germ.

The formation of sedoheptulose phosphate and triose phosphate should be equivalent and equal to half the pentose phosphate disappearing if a two-three split of the pentose phosphate yields triose phosphate and is accompanied by a five-two condensation to give sedoheptulose phosphate. Such stoichiometry has not yet been clearly shown. The implication of ribulose phosphate and sedoheptulose phosphate acquires particular importance because of the recent demonstration of these compounds as early products of photosynthesis (19). The metabolism of phosphogluconic acid in plants appears entirely analogous to that reported earlier for yeast by Horecker and co-workers (54, 56). Horecker & Smyrniotis (55) recently have demonstrated the reversibility of the oxidative decarboxylation of 6-phosphogluconate to ribulose-5-phosphate. The reaction was reversed by coupling it with a $TPNH^1$-generating reaction. It is probable that the three-two split in leaves is similar to that suggested by Gest & Lampen (45) for xylose fermentation by *Lactobacillus pentosus;* carbon from xylose yielded the methyl group of acetic acid. Nutting & Carson (91, 92), on the other hand, indicate that a C_2-C_2 condensation occurs in the fermentation of xylose by *E. coli,* for the molar yield of lactic acid is greater than the moles of xylose supplied.

Vickery (129) has confirmed the earlier observation that starch may be formed in excised *Bryophyllum calycinum* leaves in the dark. The increase in starch is accompanied by an extensive loss of malic acid and an initial loss of isocitric acid. Citric acid and undetermined acids increased somewhat during the experiment. It seems probable that malic acid can be converted to starch by a nonphotosynthetic process, but the route of conversion remains obscure.

Organic Acids and Photosynthesis

Several organic acids are early products of photosynthesis (28). Within a minute after exposure of organisms such as *Scenedesmus* to $C^{14}O_2$, C^{14} can be found in malic, glycolic and glyceric acids as well as in phosphoenolpyruvic, phosphoglycolic, and phosphoglyceric acids. Certain of these, such as malic and glyceric acids, may not be on the main path of photosynthesis, but the key importance of phosphoglyceric acid is now generally accepted, and glycolic acid still must be considered a possible precursor of

the two carbon compound which yields phosphoglyceric acid. Aronoff (10) presents evidence that glyceric acid may appear free and as 2- or 3-phosphoglyceric acid or 2,3-diphosphoglyceric acid in soybean leaves.

The work of Fager (40) with cell-free preparations from leaves indicated that 70 per cent of the $C^{14}O_2$ fixed in the light was in phosphoglyceric acid and pyruvic acid. There was no evidence that the pyruvic acid preceded the phosphoglyceric acid in the fixation sequence. Fager & Rosenberg (41) have reported kinetic studies which eliminate the possibility that an exchange reaction accounts for CO_2 assimilation in phosphoglyceric acid and further establish it as the first product of photosynthesis.

A number of interesting papers have appeared concerning the photoreduction of organic acids by photosynthetic bacteria. Larsen (72) has demonstrated that *Chlorobium thiosulphatophilum* can effect the carboxylation of propionic acid to succinic acid; no reaction occurs anaerobically without light. The actual path of the synthesis is not clear and, although it may go over pyruvic acid, Larsen also considers the possibility of the direct carboxylation of propionic acid to succinic acid.

Cutinelli *et al.* (34) analyzed *Rhodospirillum rubrum* grown on acetate-2 C^{14} and unlabeled bicarbonate or on labeled bicarbonate and unlabeled acetate. They found that 45 per cent of the total carbon of the bacterial protein came from the methyl group of acetate, about 23 per cent from the carboxyl, and 10 per cent from the bicarbonate. Kamen *et al.* (60) and Glover *et al.* (47) also found that *R. rubrum* incorporated the methyl group of acetate almost entirely into insoluble cellular material by photoassimilation, and that much of the carboxyl group appeared as CO_2. Aerobically in the dark, in contrast, the acetate apparently is oxidized via the tricarboxylic acid cycle, for a considerable share of the C^{14} from labeled acetate was recovered in intermediates of the cycle. Bregoff & Kamen (23) have found that *R. rubrum* releases H_2 from malate in the absence of N_2 or NH_4Cl but that no H_2 is evolved when N_2 or NH_4Cl is present. As soon as the ammonium ion is exhausted from the medium the photoevolution of H_2 begins. A number of amino acids support growth and do not suppress hydrogen production.

One of the more interesting and important recent developments in photosynthesis has been the coupling of light energy for effecting certain model carboxylation reactions which result in the synthesis of organic acids. Vishniac & Ochoa (137) first reported the reductive carboxylation of pyruvate to malate in the light by a preparation of grana plus malic enzyme from pigeon liver, TPN[1] and other necessary adjuncts. In this system light energy was used to generate reduced TPN (DPN also could be reduced) which, in turn, effected the reduction of pyruvate plus carbon dioxide to form malate. Ochoa & Vishniac (95) and Vishniac & Ochoa (138) have extended these observations to the formation of *d*-isocitrate from α-ketoglutarate, lactate from pyruvate, malate from oxalacetate, glutamate from α-ketoglutarate, succinate from fumarate, and hexosediphosphate from 1,3-diphosphoglycerate. In each instance light energy captured by grana was required to drive

the reaction. For each mole of DPN reduced photochemically 0.5 mole of O_2 was released.

Similar observations on TPN[1] reduction were reported by Tolmach (125, 126) who found that illuminated chloroplasts plus malic enzyme, TPN, manganous chloride, and pyruvate, fixed $C^{14}O_2$ much more rapidly in the light than in the dark. O_2 was liberated, but less rapidly than from crude spinach juice to which TPN was added. Arnon (7) reconstructed the same system for storing light energy but his experiments were of particular interest because malic enzyme from a plant rather than an animal source was employed. The only nonplant constitutent used was TPN, which has been found in leaves. Arnon & Heimbürger (9) have separated the malic acid formed by the extracellular process and have identified it by its movement on a paper chromatogram.

The report of Clendenning *et al.* (30) is useful in interpreting the possible significance of the various model reactions demonstrated for CO_2 fixation and reduction; all carboxylases reported from plants were tested. On the basis of various criteria, these workers excluded formic dehydrogenase and pyruvic, α-ketoglutaric, oxalacetic, oxalosuccinic, and glutamic acid decarboxylases as likely catalysts for assimilation of carbon dioxide in photosynthesis. They considered that only the malic enzyme among defined carboxylases has the requisite properties for a carboxylase active in the photosynthetic fixation of carbon dioxide. This enzyme is abundant and widely distributed and has a high capacity for fixation of carbon dioxide. Despite this evidence with model systems, which suggests the possible importance of a light driven reductive carboxylation of pyruvate to malate, tests with C^{14} do not support malate as a key photosynthetic intermediate [Bassham, *et al.* (18)]. When malate formation and utilization is blocked by malonate, photosynthesis continues with little alteration. Formation of phosphoglyceric acid apparently is effected by the primary carboxylation in photosynthesis, but such a reaction has not yet been reconstructed in a model system. This is scarcely surprising, for the precursor of phosphoglyceric acid remains undefined.

Metabolism of Succulents

Earlier tests had indicated that the concentration of isocitric acid in excised leaves of *Bryophyllum calycinum* did not show appreciable diurnal shifts. As this response was quite different from that of malic and citric acids, Vickery (128) carefully repeated the tests. The results confirm the relatively small shifts in concentration of isocitric acid from day to night. They reveal marked shifts in concentration of starch and malic acid, but Vickery cautions that the interconversion of starch and malate can scarcely result from a simple and direct process.

Fat Metabolism

The formation and oxidation of fats and fatty acids in plants has been

neglected. It is reassuring to find that these problems are now being probed with modern techniques. Stumpf & Newcomb (116) studied the synthesis of long chain fatty acids by slices of maturing peanut cotyledons. C^{14}-labeled acids were furnished as substrates to the cotyledon slices for 6 hr., and the long-chain fatty acids then were recovered from the material after saponification. Carboxyl labeled formate, acetate, butyrate, and caproate released considerable $C^{14}O_2$ during incubation. The incorporation of C^{14} into long chain fatty acids varied greatly with the carboxyl labeled, short-chain acid supplied; acetate contributed 17 per cent of its label to the higher fatty acids, butyrate 1.8 per cent, caproate, 2.7 per cent and formate 0.3 per cent. Acetate-2-C^{14} yielded less $C^{14}O_2$ but furnished more C^{14} for synthesis of the higher fatty acids than did acetate-1-C^{14}. Only 0.2 per cent of the C^{14} from valerate-3-C^{14} appeared in higher fatty acids. From uniformly labeled glucose and fructose, 6 per cent of the labels appeared in the fatty acids. Dinitrophenol at 10^{-4} *M* concentration inhibited C^{14} uptake from acetate-2-C^{14} by 43 per cent.

Newcomb & Stumpf (88) also investigated the oxidation of fatty acids by particulate preparations from homogenized cotyledons of germinating peanuts. The active particles sedimented at higher speeds than did the mitochondria and, in contrast to the mitochondria, did not have Krebs cycle activity. They oxidized palmitate-1-C^{14} and -3-C^{14} to yield $C^{14}O_2$, whereas the supernatant oxidized only palmitate-1-C^{14} to give $C^{14}O_2$. The mitochondria did not oxidize palmitate but did oxidize certain lower fatty acids.

Newly Described Organic Acids

Chromatographic separation revealed what appeared to be a new acid in several varieties of apples, peaches and plums [Hulme & Swain (57)]. The acid was particularly abundant in green apples. Its relation to citric and tricarballylic acids suggested that the new acid was a dihydroxy tricarballylic acid. Identification is tentative, but Hulme & Swain report the isolation of the pure acid and promise a characterization of it. The acid apparently is not present in all varieties of apples, for attempts of Zbinovsky in our laboratory to find it in green apples of two varieties were unsuccessful.

When Vickery *et al.* (136) investigated the organic acids of *Narcissus poeticus*, they found that about half the organic acid of the bulb was not accounted for as oxalic, succinic, malic, or citric acids. For their calculations, they assumed the unknown acid had the composition of isocitric acid. Zbinovsky (145) has been able to separate this unknown fraction on a silica gel column into two acids, neither of which is isocitric acid. The acids have been isolated in good yield from preparative silica gel columns as white, crystalline compounds with reproducible melting points, neutral equivalents, elementary analysis, and ultraviolet absorption spectra. Compound I constituted 7 per cent and compound II 35 per cent of the titratable acidity of the ether-extractable acids of narcissus bulbs. The acids are soluble in polar solvents but insoluble in benzene or petroleum ether. Potentiometric

titration indicated phenolic hydroxyls and carboxyl groups in each compound. Neither acid has elements other than carbon, hydrogen, and oxygen. The ferric chloride test is negative, but each compound gives a purple color with β-naphthol in concentrated sulfuric acid. Unsaturation is evident in compound II, but the ring appears to be partially hydrogenated; periodate will cleave the ring. Compound I serves as an active substrate for tyrosinase from potato tubers and is slowly oxidized by catecholase from the sweet potato. Catecholase oxidizes dihydric phenols as does tyrosinase but unlike tyrosinase does not attack monohydric phenols. The function of the acids is obscure and likely will remain so until the compounds are identified.

Isolation and Analysis of Organic Acids

Many descriptions of chromatographic separations of organic acids have appeared recently. These methods fall chiefly into the categories of paper chromatography, partition chromatography on columns, and ion exchange chromatography. In addition, a number of colorimetric and enzymatic methods have been described.

The usual technique for detecting organic acids on paper chromatograms is by spraying the paper with a pH indicator. Although simple in principle, the method requires care to avoid retention on the paper of interfering acids or bases from the developing solvents. As the identification of acids by R_F values alone cannot be considered reliable, the application of color tests described by Buch *et al.* (25) may prove very useful. They suggested that, after a preliminary run for orientation, chromatograms be run in quadruplicate with standards, and that each sheet then be sprayed with a different color reagent which they described.

Jermstad & Jensen (59) gave the composition of two solvents suitable for one dimensional chromatography of many plant acids. Stark *et al.* (106) have applied ascending chromatography to the separation of acids from beet sugar processing liquor which contained citric, glycolic, lactic, malic, oxalic, pyrrolidonecarboxylic, and succinic acids. Their solvent systems and techniques were quite similar to those employed earlier by Lugg & Overell (77). Govindarajan & Sreenivasaya (48) have described the separation of acids from leaves by paper chromatography. Keto acids may be converted to their 2,4-dinitrophenylhydrazones and then separated by paper chromatography (140) or column chromatography (24).

Following Isherwood's introduction (58) of column chromatography on silica gel for the separation of organic acids, the method has been adopted and somewhat modified by a number of investigators. In one of these recent reports, Frohman *et al.* (44) describe the separation and determination of fumarate, α-ketoglutarate, oxaloacetate, succinate, lactate, pyruvate, malate, *cis*-aconitate, isocitrate, and citrate. The recoveries reported were good, but in the range of operation the blank titrations constituted such a disturbingly high percentage of the total titrations that the reliability of the method remains in some question. In contrast, the method of Higuchi *et al.*

(52) for the separation of the C-4 to C-10 dicarboxylic acids on silica gel columns appears thoroughly reliable. Phares *et al.* (96) employed Celite columns for the separation of a wide variety of organic acids by partition chromatography. Certain acids which were not properly separated with acidified chloroform-butanol could be resolved with acidified ethyl ether.

Ion exchange columns have been used less than partition columns for organic acids, but the high capacity of the exchange resins makes them particularly valuable in certain applications. Busch *et al.* (26) have separated the acids of the citric acid cycle on Dowex 1 columns. The resin was used in the formate form and the organic acids were displaced with formic acid of constantly increasing concentration. Excellent separation of lactate, succinate, malate, fumarate, α-ketoglutarate, and *cis*-aconitate was achieved. Pyruvate, malonate, citrate, and isocitrate were not separated but could be resolved on a silica gel column.

Pyruvic and α-ketoglutaric acids can be analyzed in mixtures as outlined by Koepsell & Sharpe (66). The 2,4-dinitrophenylhydrazones are formed and their absorption measured at 380 mμ. The solution then is made alkaline and measured at 435 and 520 mμ. From these data the concentration of the two acids can be calculated.

A manometric determination of malate and fumarate has been described by Nossal (90), which is a modification of the earlier method of Ochoa *et al.* (93). In the presence of fumarase, a malic decarboxylase from *Lactobacillus arabinosus* releases CO_2 from both malate and fumarate but from malate only in the absence of added fumarase. Tests with the method revealed that plant tissues rarely had demonstrable levels of fumarate.

Hargreaves *et al.* (51) reported an important modification in the pentabromacetone method for determining citrate and isocitrate. They found that in the presence of metaphosphoric acid the method was no longer sensitive to a number of conditions which previously had required rigid control. In addition to greater convenience, the method offers higher recoveries and greater precision. This is particularly important in the determination of isocitrate, for it is determined by difference in citrate concentration before and after treatment of a citrate-isocitrate mixture with aconitase.

The keto acids assume such importance in the formation of amino acids that the preparation of keto acids by the method of Meister (82) should be mentioned. By enzymatic oxidation of amino acids the corresponding α-keto acids are formed, and then they can be isolated by various means. In this way 12 α-keto acids, including those from methionine, citrulline, ethionine, phenylalanine, tyrosine, and valine, were prepared and isolated.

Organic Acids and Nitrogen Metabolism

The glutamic-aspartic and glutamic-alanine transaminases have been studied in detail and their importance has been emphasized because of their extremely high activity. However, within the past few years it has become evident that numerous other transaminases are active in animals and bac-

teria. It now appears that transaminases also function in the synthesis of a wide variety of amino acids in higher plants.

After a single amino acid and α-ketoglutaric acid are allowed to react in the presence of an enzyme preparation, a sensitive test for an increase in glutamic acid can be made by paper chromatography; an increase in glutamic acid constitutes evidence for transamination. The transfer of N^{15} from a labeled amino acid to α-ketoglutarate also serves as a specific means for tracing transamination.

Stumpf (112) reported that by paper chromatography it was possible to show transamination by dialyzed aqueous extracts prepared from wheat germ, pumpkin leaves, and seedlings of lima beans, lupines, pumpkins, and peas; demonstrable transamination to α-ketoglutaric acid occurred with alanine, aspartic acid, α-aminobutyric acid, leucine, isoleucine, valine, and norvaline. Oxaloacetic and pyruvic acids were not active as amino acceptors. Tyrosine, phenylalanine, glycine, threonine, methionine, arginine, tryptophan, and histidine gave no detectable transamination to α-ketoglutarate.

Wilson & King (142) found quite a different spectrum of transaminases in young barley plants from the spectrum reported by Stumpf for seedlings. In addition to aspartic acid and alanine, they observed transamination to glutamic acid from serine, threonine, methionine, leucine, tyrosine, and glycine. Of these, tyrosine, glycine, threonine, and methionine gave no demonstrable transamination in the preparations employed by Stumpf. Actually the transaminations effected by plants are more numerous than reported in either of these investigations, for current unpublished studies by Wilson & King indicate 13 distinct transaminations in preparations from white lupine seedlings.

The changes in the glutamic-alanine and glutamic-aspartic transaminases during germination were studied by Smith & Williams (103). They found that in wax bean, pea, barley, corn, oat, squash, and pumpkin the transaminase activity per mg. protein nitrogen increased more rapidly than the total protein of the seedlings increased.

The wide distribution and wide variety of transaminases in plants indicate that probably most of the amino acids in plants are formed by transamination. This conclusion is fortified by the failure to demonstrate alternative methods of synthesis. The reductive amination of α-ketoglutarate to glutamate can be followed by transamination to form the other amino acids. One independent means for synthesis of alanine and aspartic acid has been cited by Kretovich *et al.* (69, 70), who reported that an extract of etiolated turnip sprouts plus ammonium pyruvate formed alanine, and that a preparation from pea sprouts plus ammonium oxalacetate formed aspartate. The evidence does not specifically indicate that reductive amination was the path of synthesis, and the possibility of glutamate formation followed by transamination remains.

The report of Gunsalus & Tonzetich (49) that enzymes from *E. coli* can transfer amino groups from adenine, guanine, and cytosine to α-ketoglutar

ate suggests that in higher plants also transamination may function in purine and pyrimidine synthesis.

Meister *et al.* (83) have extended their earlier work on transamination and accompanying deamidation and have found that asparagine as well as glutamine functions in this manner. They reported earlier (84) that extracts of rat liver catalyzed the transfer of the amino group of glutamine to various keto acids more rapidly than from glutamate, and that the reaction was accompanied by the liberation of ammonia from the amide group of glutamine. As with glutamine, asparagine transfers its amino group to a keto acid forming a new amino acid and oxaloacetate, only the ammonia released originating from the amide group. In most instances the transamination was more rapid from asparagine than from aspartate. Apparently neither the free dicarboxylic amino acids nor the α-keto acids corresponding to asparagine and glutamine are intermediates in the transamination-deamidation; transamination and deamidation may be closely linked or may occur simultaneously. What importance the reaction may have in plants remains to be established, but the abundance and high activity of asparagine and glutamine in plants suggests that this reaction, described for animal tissue, should be sought in plants.

Elliott (39) has extended his survey of glutamine synthesis to include seedlings of *Lupinus albus* and *L. angustifolius* as representatives of plant tissue. Extracts of ground seedlings proved more active per ml. than crude extracts from sheep brain, and the plant preparations were activated by magnesium ions and inhibited by fluoride as were the animal preparations. Glutamate, ammonia, and ATP[1] are required for the reaction, but hydroxylamine and hydrazine can substitute for ammonia as glutamyl acceptors. No comparable asparagine synthesizing system could be demonstrated. As lupine seedlings accumulate such large quantities of asparagine, the importance of asparagine in their nitrogen metabolism always has been emphasized. However, the presence of a highly active glutamine synthesizing system may indicate that its metabolic importance is as great or greater than that of asparagine in these plants.

Stumpf *et al.* (114, 115) have investigated another enzymatic reaction of glutamine. Glutamyl transphorase catalyzes the conversion of glutamine plus hydroxylamine to glutamohydroxamic acid and ammonia. The gamma-amide group of glutamine will exchange with ammonium ion as well as with hydroxylamine under the influence of the enzyme (37). The complete system requires manganous ion, arsenate or phosphate, and ATP or ADP. It has no action on asparagine. The enzyme is present in a wide variety of algae and higher plants, but pumpkin seedlings were chosen as the source of the preparation studied in detail. The actual function of the enzyme in plants remains to be established.

The reaction of fumarate with arginine to form arginosuccinate constitutes another instance of the participation of organic acids in the nitrogen metabolism of plants. Davison & Elliott (36) showed that an aqueous ex-

tract of pea meal contained an enzyme system which produced an unknown ninhydrin positive compound when incubated with fumarate and arginine. Upon heating with 6 *N* HCl, the compound yielded arginine and ornithine (identification tentative) plus a component which moved slowly on their chromatograms. The combination of arginine through its terminal-NH_2 group to an alpha-carbon of fumarate is analogous to the combination of ammonia with fumarate in the presence of aspartase. Splitting off ornithine, dehydrogenating and closing the ring would yield orotic acid, a logical precursor of pyrimidines.

Following the report by Davison & Elliott, Walker (139) has shown that *Chlorella* forms arginosuccinate from arginine and fumarate or malate. There seems little question that this compound is identical with the unknown of Davison & Elliott. Walker found that arginine was not replaced in the reaction by citrulline, ornithine, creatine, or guanidine, and only compounds which could readily yield fumarate (malate, and to a limited extent oxaloacetate) could serve in place of fumarate. Arginosuccinate is a particularly interesting metabolite, for its degradation could yield arginine, citrulline, ornithine, fumarate, malate, asparate, or a pyrimidine precursor. It also is of interest that the compound links the nitrogen metabolism of plants and animals, for Ratner & Petrack (100) observed that the condensation product formed from citrulline and aspartic acid in the urea cycle of animals "behaves, enzymatically, as a derivative or arginine and malic acid," and indicated that the linkage must be between a guanido nitrogen atom and the alpha position of the aspartate (or malate or fumarate). Again, the compound in question appears to be arginosuccinate.

Organic Acids of Tissue Cultures

Although they are not by themselves suitable sources of carbon for the growth of sunflower crown gall tissue cultures, certain organic acids will stimulate growth in the presence of sucrose [Hildebrandt & Riker (53)]. Sapp (101) separated the organic acids of sunflower galls by partition chromatography on silica gel and found that tissues grown on a sucrose medium contained malate and citrate and a surprisingly high amount of α-ketoglutarate. All the acids were decreased markedly when ammonium salts were added to the medium for tissue culture. The addition of fluoroacetate enhanced the accumulation of citrate. As further evidence for the activity of a Krebs cycle in these cultured tissues, Sapp showed that C^{14}-labeled acetate transferred its label to citrate, α-ketoglutarate and malate.

Organic Acids in Storage Organs

The acid content of apples changes markedly upon ripening and also changes during storage. Kidd *et al.* (64) have picked apples at various stages of growth and have followed the organic acids during storage. During the initial period of storage the acid level remained unchanged, but then it decreased at a rate proportional to the acid present. Krotkov *et al.* (71) ob-

served that the newly formed McIntosh apple fruit (early June) had a pH well above five, but that this dropped to about 2.8 in early July. At the time of harvest in late September the pH had risen to about 3.2 and it continued to rise during storage to 3.5. In one season the very young fruit had a higher percentage of unknown organic acids than of malic acid, but at maturity malic acid accounted for 80 to 90 per cent of the total acid. The climacteric rise in sugar content and the postclimacteric decrease was not accompanied by changes in the level of organic acids.

Potato tubers contained 0.2 per cent citric acid and 0.112 per cent malic acid on a wet weight basis after storage for six months, according to the data of Prokoshev & Petrochenko (97). At a temperature of 18–20°C. in air the total acidity remained constant, but citrate was rapidly converted to malate, about 50 per cent in three days. The acids declined in an atmosphere containing 5 per cent carbon dioxide. Barker (14) and Barker & Mapson (15) have observed that the ascorbic acid content of potato tubers rises to a maximum during growth and then decreases. The loss of ascorbic acid on storage at 10°C. is more rapid from potatoes which are harvested in immaturity than from potatoes which are mature at harvest. In tubers stored below 10°C. there is an initial rise in ascorbic acid followed by a decrease more rapid than at 10°C. They found (16) that these changes in ascorbic acid concentration were minimized when the tubers were stored in an atmosphere free of oxygen. Smith (104) observed that potato slices increased in ascorbic acid content two to three times during 48 hr. of incubation at 30°C. with aeration. Fluoride and iodoacetate inhibited the synthesis of ascorbic acid and the anaerobic evolution of carbon dioxide. The experimental observations indicated that the biosynthesis of ascorbic acid is related to glycolysis, and Smith proposed a scheme for the formation of ascorbic acid.

The chilling of many tropical fruits is followed by a darkening and improper ripening of the fruits. Miller & Heilman (85) observed that pineapples stored at 6°C. for a week and then at room temperature for two days had about 40 per cent less ascorbic acid than controls maintained at room temperature. They suggested that the ascorbic acid might keep phenolic compounds reduced and that darkening of chilled fruit might well follow the depletion of the ascorbic acid.

Ratner & Akinochkina (99) studied the influence of salt additions to the soil on organic acid production by sugar beets. Calcium chloride, sodium chloride, magnesium chloride, or potassium chloride decreased the organic acid level in the beets, whereas introduction of the cations in bound complexes increased organic acid formation.

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HEMATIN COMPOUNDS IN PLANTS[1,2]

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Introduction

In the past developments in plant physiology have contributed to general biology so that advances on the animal side owe much to many purely botanical investigations. With the study of hematin compounds in plants the debt is on the other side for our knowledge of these pigments started with the study of hemoglobin in the vertebrate blood. The elucidation of the chemistry of hematin, or iron protoporphyrin, derived from the blood pigment was a vital step in determining the structure of chlorophyll. But more important perhaps than this, the study of hemoglobin was a step which has led to the possibility of regarding hematin as a substance essential to plant life as indeed chlorophyll has long been considered in green plants.

Although these facts are now common knowledge the appreciation of the whole field requires some specialized knowledge of the biological hematin compounds themselves. In 1947 Scarisbrick contributed a review of hematin compounds in plants which is probably the only complete account of this subject (164). A comprehensive review of all hematin derivatives will be found in the book by Lemberg & Legge (120). It seems appropriate that the whole field should be considered again in view of the increasing interest which is now shown in the relation of hematin compounds to the study of plant respiration.

Distribution of Hematin Compounds

The importance of hematin compounds in the physiology of the plant first became evident with the publication of the paper by Keilin: "On cytochrome, a respiratory pigment common to animals, yeast and higher plants." (93). That hematin compounds other than hemoglobin exist in animal tissues had been proved by MacMunn, but the significance of these additional pigments was only made clear in Keilin's work. While hemoglobin in the blood of vertebrates acts as an oxygen carrier external to the cells of the rest of the tissues, cytochrome was characterized as an intracellular system of oxidation catalysts. This group of hemoprotein catalysts has been found in all types of aerobic organisms which therefore include all the plants. Although

[1] The survey of the literature pertaining to this review was concluded in October, 1952.

[2] The following abbreviations are used: AA for ascorbic acid; a.a. ox. for ascorbic acid oxidase; cyt. ox. for cytochrome oxidase; p.p.ox. for polyphenol oxidase; Hb for hemoglobin; IAA for indole-3-acetic acid; MetHb for methemoglobin; and PPD for *p*-phenylenediamine; and DDC for diethyldithiocarbamate.

it is now over a quarter of a century since the discovery of cytochrome in plants, the hematin compounds, as widely present cellular constituents, have scarcely been assimilated into plant physiology. Nevertheless it may be said that all forms of hematin compounds previously found in animals have since been found in plants including hemoglobin itself in leguminous

The position of cytochrome in the scheme of respiration is now generally accepted. Yet it is clear that there is little agreement about the application of this scheme to the physiology of plant respiration; a problem which will be considered later. One aspect, perhaps, that has not been recognized widely is that the optical properties of the cytochrome pigments within the cell give an independent physical method for study of the responses of the living plant to changes in its environment. The oxidation and reduction of cytochrome can be observed by a direct spectroscopic method (93). Only gradually, however, are such methods being developed for the study of the plant, because the concentration of these pigments in plant tissue is usually low, and thus the observation of absorption spectra is limited by the relatively large loss in transmitted light by scattering and by the presence of other pigments. In the whole living potato tuber, for example, the oxidation and reduction of cytochrome may be observed in response to changes in oxygen concentration (14). Yet the application of the inhibitor technique by Hanes & Barker to the total respiration gave no indication that cytochrome is playing any part in the respiration (78). This obvious paradox will be discussed later (p. 128).

The general distribution of the cytochrome system in plants was clearly shown by the work of Yakushiji (213). The presence of cytochrome components both in the leaves of land plants and in the green alga *Ulva lactuca* was observed after removal of the plastid pigments with acetone. Cytochrome-*c* was extracted from a variety of marine algae which belong to Phaeophyceae, Rhodophyceae, and Chlorophyceae. From the red alga *Porphyra tenera* a concentrated preparation of cytochrome-*c*, free from other pigments, was obtained.

In 1937 Mann had completed a detailed study of the distribution of the total hematin in a variety of plant tissues that were devoid of chlorophyll (133, 134). This showed that the highest concentration of hematin occurred in meristematic tissues, and it could be concluded that the higher hematin concentrations were correlated with greater metabolic activities. The results obtained in the cases of the beet root and the onion bulb are especially clear the beet actually showing rings of higher hematin concentration corresponding with the rings of cambial tissue (fig. 1B). The total hematin will include that of peroxidase and catalase, and in peroxidase-rich tissues, such as horseradish root, this enzyme may account for about one half the total hematin present (105); in most plants the fraction due to peroxidase is probably much smaller. There is no accumulated evidence yet for defining the physiological function of catalase and peroxidase. But very recently Chance (35) has obtained direct spectroscopic evidence that the catalase in *Micrococcus*

lysodeikticus is actively concerned in the respiratory mechanism of this micro-organism (see p. 145). In plants the hematin of catalase is likely to represent a small fraction of the total; much smaller in fact than that associated with components of the cytochrome system.

Hill & Bhagvat (87) showed that the cytochrome system was attached to insoluble particles which could be separated after grinding from a variety of plant tissues. This insoluble residue contained the enzymes similar to those associated with analogous preparations from animal tissues. A very important series of papers by Okunuki on the respiratory system in pollen (146) showed how similar the mechanisms involving the cytochrome in these gametophyte cells are to the mechanisms in yeast and animal tissues. Subsequent investigations over a wide field have shown that the cytochrome system in both plant and animal cells is contained in mitochondria and is thus present in the cytoplasm as part of an organized structure. An appreciation of this work cannot be attempted here; we will support our present statement with a reference to a fundamental contribution for the plant by Millerd *et al.* (141). There is now, therefore, a possibility of relating the concentration of cytochrome in a plant tissue to the density of a definite mitochondrial element. While this has not been actually carried out, observations on the distribution of cytochrome in plant tissues give an indication of a positive relationship. In any case this suggests a further method for defining the part played by the cytochrome system when the total respiration of a plant tissue is considered.

In plants we may distinguish two types of related cytoplasmic structures —the mitochondria proper and the plastids, the latter being found either as chloroplasts or as leucoplasts. The relatively high hematin content of leaves, where chloroplast material predominates, has led to the view, to be discussed later, that the plastids have a high content of hemoproteins which differ from the cytochrome components in mitochondria. Perhaps the modifications of the systems of cytochrome components observed in plant tissues may be correlated with the amount and the nature of certain cytoplasmic structures. If this be so, the part played by these hematin compounds in metabolic activity at different stages of a plant's development may soon be more clearly understood.

It is of great importance to be able to measure the total amounts of the different cytochrome components in a tissue or in a tissue preparation and to relate them to the activity in terms of a physiological response or of an *in vitro* chemical reaction. The significance of finding this relation stems from the comparative biochemical study of cytochrome, a dominant feature of Keilin's original work. This has led to a rule, briefly stated, that while normally all aerobic cells may have the capacity for synthesising hemoproteins the total hematin produced is in proportion to the respiratory activity. In short, hematin belongs to the architecture of the cell and not to the cell's lumber. Exceptions to this rule are of great interest. For example, in 1925 Keilin found that the fat body of an insect pupa contained a large amount of hema-

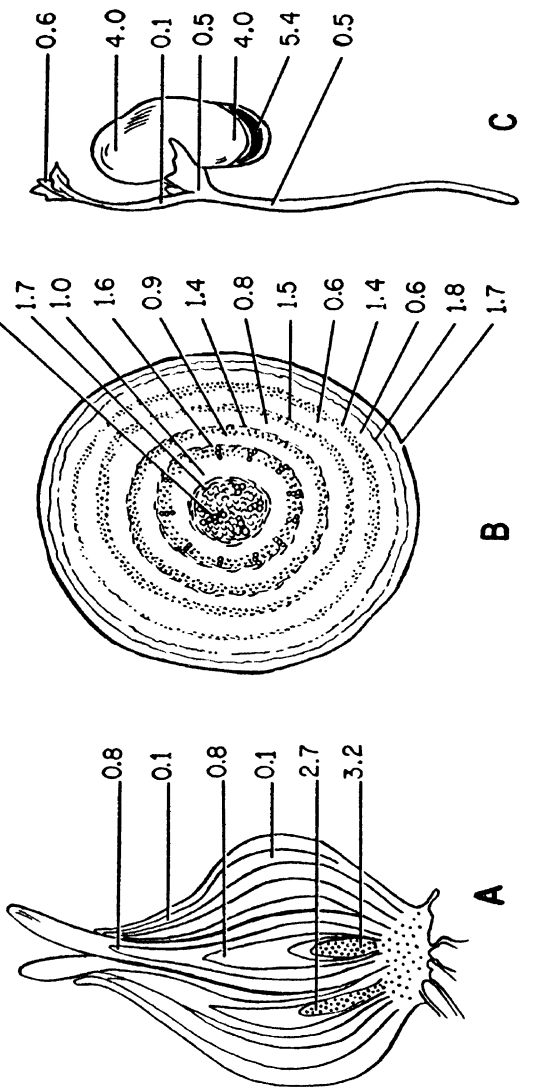


FIG. 1. Distribution of hematin in plant tissues [adapted from Mann (134)].
A: Onion bulb, B: Beet root, C: *Phaseolus* seedling. The numbers represent mg. hemin per 100 g. dry weight of tissue.

tin: this was interpreted as being the precursor of the abundant cytochrome in the wing muscles of the imago.

How then are we to interpret the high hematin content of a leaf, a tissue whose respiration is comparatively low? In order to make an attempt it is first necessary to determine the nature of the hematin compounds present and then to relate their concentrations to a characteristic constituent such as chlorophyll. The estimation of cytochrome-*c* in living yeast in relation to its respiration was described by Keilin and Hartree (96). More recently the concentrations of components *b* and *c*, in relation to the succinoxidase system, in preparations from animal tissues were measured by Slater (173). There is no doubt that similar analyses could be carried out on preparations from plant tissues. Before considering this matter in any detail, however, it is necessary to appreciate the fundamental contribution of Mann (133, 134) to our knowledge of the distribution of hematin in the parts of plants devoid of chlorophyll.

Total hematin in plants.—When protohematin or related porphyrin-iron compounds are treated with pyridine and sodium dithionite the characteristic spectrum of hemochromogen is produced. Should the hematin be bound to protein, the latter is denatured and the liberated hematin, in presence of excess of pyridine, is quantitatively converted into pyridine hemochromogen [Anson & Mirsky (7, 8), Lemberg & Legge (120)]. Thus all the hematin of peroxidase, catalase, and cytochrome-*b* will contribute to the pyridine protohemochromogen with a very strong and sharp absorption band at 557 mμ, Cytochrome-*c* will give a hemochromogen with a band at 550 mμ which, in a low dispersion spectroscope, will fuse with the protohemochromogen band. The hematin of the cytochrome components $a+a_3$ will under these conditions give rise to a hemochromogen band at 587 mμ. However, it is generally the case with plant tissues that the *a* and *c* hemochromogens formed in the above reaction are present at concentrations which are small in comparison with the protohemochromogen.

The hemochromogen reaction was put upon a quantitative basis for intact tissues by Anson & Mirsky (8) and Elliott & Keilin (55). The latter authors impregnated a slice of plant tissue of known (~1 cm.) thickness with pyridine in presence of dithionite and preserved it *in vacuo* until the hemochromogen bands attained maximal intensity. The pyridine rendered the tissue translucent and the concentration of hematin was readily determined by direct spectroscopic comparison with a standard hemochromogen (from hemin crystals) in a wedge trough. Mann was able to determine the distribution of hematin within a large section of tissue by illuminating small areas of the tissue on a microscope stage and matching the resulting spectrum against a standard. His results for onion bulb (A) and for beet root (B) are shown in figure 1. The concentrations are expressed as mg. hemin ($C_{34}H_{32}O_4N_4FeCl$) per 100 gm. dry weight. It was found that expressing the results on a fresh weight basis did not appreciably alter the character of the results. Sections of a branch from a tree showed the presence of hematin in both wood and

bark, but with a far higher concentration in the bark. In a given plant the greatest concentrations of hematin were found in the most actively respiring tissues. It must be pointed out, however, that there is no absolute relationship between the activity of metabolism and total heme content when passing from one species to another. For example, a horse-radish root contained 10 mg. per cent. hemin, while the developing bulb of an onion contained 3.2 mg. per cent. Again in a germinating bean seedling (Fig. 1C) the cotyledons contained 4 mg. per cent and the tip of the shoot 0.6 mg. per cent. Like the previously mentioned case of the fatbody of an insect pupa, these are exceptions to the general rule and their interpretation may be possible when the nature of the actual hematin compounds has been more completely worked out. Thus in the germinating bean there is more cytochrome in relation to total hematin in the shoot than in the cotyledons. In this connection Mann drew attention to the fact that a high hematin content was characteristic of developing leaves previous to the appearance of chlorophyll. He discussed this in relation to the iron nutrition of the plant and gave the first definite biochemical interpretation of a chlorosis long known to be the result of iron deficiency, although chlorophyll itself is not an iron compound.

That protoporphyrin may be a common precursor for both the hematins and the chlorophyll in plant cells is supported by the experimental facts so far obtained. This subject has been reviewed by Granick (75) who, on the basis of his experiments with *Chlorella* mutants, suggested that while the iron compound of protoporphyrin (protohematin) gives rise to intracellular oxidation catalysts, the magnesium compound may be a stage in the development of chlorophyll.

When now we consider the hematin present in leaves, where respiration is low, would it be possible to decide whether the hematin compounds are related to the maintenance of the chlorophyll or whether they take part in the active process of photosynthesis? At present there is but little knowledge of any of the partial reactions of photosynthesis, and thus there is no direct biochemical information. We may only argue, from the existing data on the nature of the hematin compounds present, by analogy with the intracellular hematins whose function has been clearly defined.

Hill & Scarisbrick (88) made a study of the hematin compounds present in the leaves of higher plants. Owing to the presence of much higher concentrations of chlorophyll, the direct spectroscopic estimation of hematin compounds in preparations from leaves is impossible unless the chlorophyll and other plastid pigments are extracted by acetone. Mann (133), however, has found that with certain plant tissues treatment with acetone results in a definite loss of hemitin. This is likely to be greater the more acid is the plant tissue for many hemoproteins are split by acid acetone. Hill & Scarisbrick found that with bakers' yeast there was no loss of hematin on treatment with acetone and considered that for many plants where the preparations are not too acid (in contrast to the horse-radish root) the acetone method could safely be used on the green parts for the removal of chlorophyll. Observations

on normal green leaves showed that the concentrations of hematin were high relatively to the other parts of the plant. They were, in fact, of the same magnitude as that found by Mann for the developing axis: of the order of 4 mg. per 100 g. dry weight of tissue. Direct examination of acetone-treated leaf preparations in a thick aqueous suspension showed the presence of a non-autoxidizable cytochrome component which later was obtained in soluble form and designated cytochrome-*f* (α-band at 555 mμ).

Cytochrome components of leaves.—Hill & Scarisbrick found that aqueous extraction media did not separate any detectable amount of cytochrome-*f* from the green chloroplast material in any of the leaves tested. However, by ammonium sulfate fractionation of the press-juice from macerated leaves, two cytochrome components were obtained in solution. One of these components was autoxidizable, α-band at 559.7 mμ in the reduced form, and was designated cytochrome-b_3 on account of certain resemblances to cytochrome-b_2, α-band at 556.3 mμ, which is associated with the lactic dehydrogenase of yeast [Bach, Dixon & Keilin (10); Dixon & Zerfas (48)]. The component b_3 appears to be characteristic of plant tissues and is not limited to the green parts. The second soluble component, α-band at 550 mμ, was nonautoxidizable and was identified as cytochrome-*c*. Goddard's isolation of this pigment from wheat germ (70) and his demonstration that *c* from a plant source had the same catalytic properties as *c* from heart muscle focussed attention upon the essential role of the cytochromes in plant respiration. It was concluded that in the leaf the concentrations of cytochromes-*c* and b_3 were together not more than one tenth that of cytochrome-*f*.

It was found impossible to extract cytochrome-*f* in an unmodified form from acetone preparations of leaves. If, however, fresh leaves were ground with ethanol containing ammonia, so that a concentration of 50 per cent ethanol was rapidly attained, cytochrome-*f* was extracted. The alcoholic solution was precipitated by cold acetone and part of the cytochrome-*f* could then be extracted from the precipitate in aqueous solution. The yield of the soluble pigment varied greatly with the species of plant. The best plant so far found is garden parsley which was used by Davenport & Hill (45) in their investigation of this hemoprotein.

Cytochrome-*f* has not so far been detected in yeast or in animal tissues, and there is no evidence for its presence in the plant apart from the chloroplasts where it may be a structural component. A similar pigment, however, was shown to occur in a *Euglena* and in three algae. The reduced form is unaffected by oxygen and there is no reaction with carbon monoxide. Oxidation with ferricyanide gives rise to a parahematin type of absorption spectrum while excess of ferrocyanide at once reduces the pigment. This enabled Davenport & Hill to measure the oxidation-reduction potential by the spectroscopic method, and it was found that in the range pH 6 to 7.7 E_0' (30°) was constant at +0.365 V. In the more alkaline range $E_0'/pH = -0.06$ V indicating the presence in ferricytochrome-*f* of a basic dissociating group with pK 8.5. Cytochrome-*f* at pH 7 is more oxidizing than cytochrome-*c* by about

0.1 V and thus has a more oxidizing potential than any hemoprotein so far examined.

The absorption spectrum of ferrocytochrome-*f* is similar to that of cytochrome-*c* but the bands are more sharply defined and lie 5 mμ nearer the red end of the spectrum. The α-band is asymmetric and in solutions above pH 10 it can be observed to split into two components, a strong narrow band at 556 mμ and a narrow but weaker band at 551 mμ; this effect is reversible up to about pH 11 where denaturation of the protein occurs. As the prosthetic group and also its mode of attachment seem to be identical in cytochromes-*c* and *f*, the observed differences must arise from differences in the proteins. This would account for the fact that cytochrome-*f*, unlike *c*, is precipitated by ammonium sulfate below one half saturation and that it is more easily denatured than cytochrome-*c*. Further, the behavior of cytochrome-*f* in the ultracentrifuge indicates that it has a molecular weight of 110,000 and that more than one hematin group is present. The existence of cytochrome-*f*, as such, in the chloroplasts might be doubted on the grounds that it has not been directly observed before treatment of the plant tissues with organic solvents. Davenport, however, has recently examined chloroplast preparations from growing etiolated leaves (44). Yellow suspensions from such material, when examined with a small dispersion spectroscope, clearly showed the absorption bands of cytochrome-*f* in the visible region. In addition, when the preparations were reduced by dithionite, a strong band corresponding with that of cytochrome-*b* in yeast was observed, together with a very faint

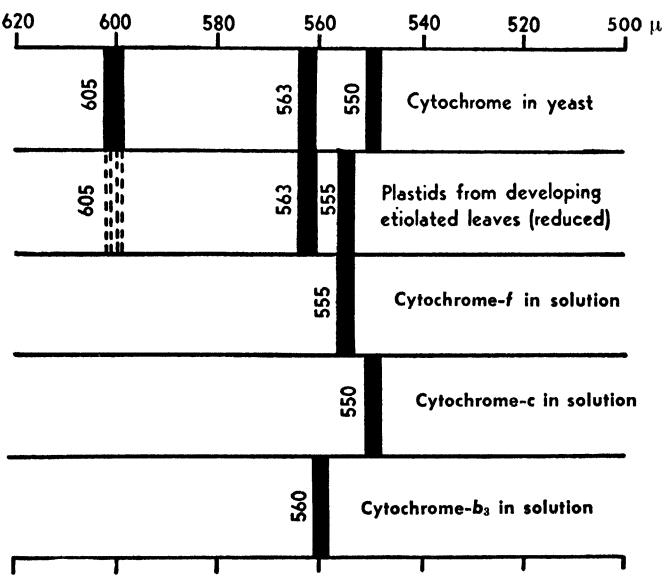


FIG. 2. Hematin compounds from leaves compared with cytochrome in bakers' yeast; α bands only.

band of cytochrome-*a*. This spectrum is shown diagrammatically in Figure 2. It can be concluded from results up to the present that the high hematin content of unexpanded leaves observed by Mann (133, 134) is maintained throughout their subsequent development and also that the chloroplasts, which contain the greater part of the hematin compounds, have hemoproteins similar to the normal cytochrome components but not identical with them. Chloroplast preparations from spinach leaves have been found by Rosenberg & Ducet (160) to show cytochrome oxidase activity. It is not possible yet to decide whether this activity is characteristic only of the chloroplasts or whether it becomes associated with them during the disintegration of the leaf. The latter view could be supported by the fact that the cytochrome oxidase activity is relatively low. McClendon (130) studied the differential centrigugation of chloroplast preparations from tobacco leaves. He found that no sharp separation of the different intracellular components could be achieved. As Hill & Scarisbrick (88) point out, the conclusion that certain hematin compounds are actually located in the chloroplast is derived only from indirect evidence.

When a comparison is made between the hematin compounds present in leaves and in an aerobic yeast it could be legitimately concluded that there is enough of the cytochrome components in the leaf to support a high rate of metabolic activity if the situation were truly analogous. However, while the estimation of total hematin in a tissue preparation offers no serious difficulties the determination of the individual cytochrome components is subject to appreciable error.

Some possible errors in spectroscopic determination of heme compounds.— The direct observation of absorption band intensities in a tissue or in a cell suspension may give results which are too high. This may arise from the influence of cell structures and from differences of refractive index. This effect has been described in detail by Keilin & Hartree (101, 102) in connection with the great increase in intensity of absorption bands which occurs in the presence of ice crystals in preparations examined by transmitted light at low temperatures. Under these conditions the intensity of absorption bands relative to the background spectrum may be increased up to 20 times when a solution of a colored substance is frozen. In addition the absorption bands may become much more sharply defined at very low temperatures. This gives a method for the detection of pigments which would escape observation at ordinary temperatures. In the scattering caused by living cells at ordinary temperatures the intensifying effect is probably very much less. The effect becomes negligible in the determination of total hematin because pyridine renders the tissue preparation more transparent and completely destroys the intracellular structure. It is, however, possible that the spectrum of peroxidase in living horse-radish root is intensified by this structure effect. Keilin & Mann (105) found that while the measured activity of the root corresponded to a Purpurogallin number (P.Z.) of 0.5, calculation from direct observation of the absorption spectrum of peroxidase in the root gave

a value of 1.5. Here the factor must be less than 3 because of the difficulty of ensuring that all the enzyme originally present in the root is directly available in the P.Z. determination.

Possible role of hematin compounds in photosynthesis.—From Keilin & Hartree's experiments with bakers' yeast (96) it can be calculated that the concentration of cytochrome-*c* in their solid fresh yeast was about 3.6×10^{-5} *M*, while the Q_{O_2} was 200. On the basis of extraction the concentration of *c* would only have been about $0.4\times10^{-5}M$. Table I, from Davenport & Hill (45), shows the values obtained for the molar concentrations of cytochrome-*f*

TABLE I

THE CHLOROPHYLL AND CYTOCHROME-*f* CONTENTS OF PLANTS

Plant	Total Chlorophyll*	Total *f** in acetone powder	*f** in ethanol extract	Chlorophyll/Total *f*
Parsley	1.8×10^{-3}	0.48×10^{-5}	0.17×10^{-5}	380
Elder	4.0×10^{-3}	0.92×10^{-5}	0.24×10^{-5}	430

* Molar concentration referred to fresh weight of leaves.

in fresh leaves, derived from measurements on the acetone-treated leaf (total *f*), and for the concentration in an ethanol extract of fresh leaves. A parallelism between the amount of chlorophyll and the amount of cytochrome-*f* is indicated in the two species of plant.

The Q_{O_2} of photosynthesis of leaves varies from 40 to 100 under optimum conditions. Thus the ratio Q_{O_2} (respiration)/cytochrome-*c* in yeast approximates to the ratio Q_{O_2} (photosynthesis)/cytochrome-*f* in leaves. Now the ratio photosynthesis/respiration is at least 10 for these leaves as is also the ratio cytochrome-*f*/cytochrome-b_3+*c*. It could thus be inferred that *f* is present in proportion to the activity of photosynthesis rather than to the respiratory activity in which *c* is involved. The molecular ratio chlorophyll/*f* lies around 400 (Table I) and this figure could be related to the experiments of Emerson & Arnold (56) on photosynthesis in flashes of light of short duration. Their work leads to the conclusion that at light saturation one molecule of oxygen per flash (=4 atoms hydrogen) is liberated for about 2500 chlorophyll molecules. However, this refers to *Chlorella*, an organism for which no quantitative data relating to hematin compounds have been obtained.[3]

While it may legitimately be concluded that the chloroplasts contain a cytochrome component resembling *c* but with modified properties, it is now suggested that, by analogy with cytochrome-*c*, the amount present is in proportion not with the respiratory activity of the green tissue but with the photosynthetic activity. Thus we are tempted to suppose that certain he-

[3] Recent observations by Hill indicate a chlorophyll-*f* ratio in this organism of the same order as the examples in Table 1.

matin compounds take part in the process of photosynthesis. This supposition, as will be seen later, could derive support from an important analysis by Tamiya (192) of the effect of certain inhibitors of catalase on photosynthesis.

The Cytochrome System as Terminal Oxidase in Plant Respiration

The problem of the nature of the terminal oxidases of plant tissues continues to attract considerable attention. The very useful reviews of James (90) and Goddard & Meeuse (72) illustrate a general trend towards the acceptance of cytochrome oxidase (cyt. ox.[2]), rather than polyphenol oxidase (p.p.ox.[2]) or ascorbic acid oxidase (a.a.ox.[2]), as the general intermediary system between oxygen and the dehydrogenase systems of plant cells. On superficial examination the problem appears simple: by examining the effects of specific substrates and inhibitors and of cytochrome-*c* on the O_2 uptakes of suitably prepared tissue slices or homogenates, it should be possible to assess the activity of the various oxidase systems and their contribution to normal respiratory processes. Unfortunately, some of the published work in this field reveals an inadequate appreciation both of the limitations of the method and of the difficulties in interpretation of the results.

The most specific test for cytochrome oxidase is the ability to oxidise ferrocytochrome-*c*. However, since the products of oxidation of polyphenols can also oxidise cytochrome-*c*, this test becomes unequivocal only for tissue preparations which are devoid of p.p.ox. substrates. The reaction can be followed spectrophotometrically (1) with highly active oxidase preparations but in practice is normally studied manometrically in presence of a "cytochrome oxidase substrate," i.e., a substance which can reduce cytochrome-*c* and so provide a constant source of the real substrate. The more thoroughly investigated animal tissue preparations provide good models for an approach to the same problems in plant biochemistry (14). Slater (172) has discussed the limitations of previously used reducing agents for cytochrome-*c* [ascorbic acid (AA[2]), hydroquinone, catechol, epinephrine and *p*-phenylenediamine (PPD[2])] and recommends ascorbic acid. The other reducers suffer from the great disadvantage that they are oxidized by ferricytochrome-*c* to highly reactive quinones (or imines) which may undergo further nonenzymic oxidation or which may inhibit the enzymes under investigation. Thus heart muscle cytochrome oxidase will oxidize catechol or hydroquinone in presence of added cytochrome-*c*, but the reaction is soon inhibited by the resulting quinones. A similar but slower inactivation may occur with PPD (80). Ascorbic acid on the other hand undergoes a one-step oxidation involving loss of 2H and has a suitable redox potential (172).

For differentiation between possible oxidases however, AA is of limited value since, besides being oxidized by a.a.ox.[2] it is also oxidized, in presence of a suitable phenolic carrier such as are present in plants, by p.p.ox.[2] (106). Furthermore, it is rapidly oxidized by cyt. ox.[2] and, although with some types

of colloidal animal tissue preparations AA is only oxidized if cytochrome-*c* is added (96), it does not follow that oxidation by plant preparations would invariably require the addition of cytochrome-*c*. Plant biochemists have generally used PPD with or without extra cytochrome-*c* in testing for cyt. ox. (14, 17, 50, 136) and here a stimulation of O_2 uptake by cytochrome-*c* is a certain indication of the oxidase. Similarly, since the only known mechanism for the oxidation of succinate is via the cytochrome system, the ability of a tissue preparation to oxidise this substrate is good evidence for the presence of cyt.ox. (14, 182, 201, 209). Barrón *et al.* (11) endeavoured to distinguish between p.p.ox. and cyt.ox. activities in the potato by the use of catechol, hydroquinone, and AA. Since all three substances can reduce cytochrome-*c* and since AA (and probably hydroquinone) can be oxidized by p.p.ox. in the presence of phenolic carriers no clear picture emerges. In fact, the use of any of the substrates normally used in testing for p.p.ox. is liable to yield ambiguous results. Not only do they reduce cytochrome-*c* but they give rise to products which inhibit endogenous respiration as well as p.p.ox. (106, 115, 138, 156, 165). In estimating cyt-ox. this difficulty can be overcome by using ascorbic acid (above), hydroquinone in presence of 1-phenylsemicarbazide (152), or very low concentrations of a leuco-indophenol yielding a highly coloured oxidation product (176).

The inability to extract cytochrome-*c* from plant tissues by methods worked out for animal tissues has been brought forward as evidence for the absence of this pigment and, by implication, of cytochrome oxidase, in certain plant tissues (11, 77, 180). Goddard (70), however, has pointed out that although cytochrome-*c* can be obtained in a purified form from wheat germ it cannot be extracted by the standard trichloracetic acid method (97) for heart muscle. Nielands (144) was able to extract cytochrome-*c* from the fungus *Ustilago sphaerogena* with alkali and to purify it. The pigment has a higher mol.wt. (18 to 20,000) than heart cytochrome-*c* and a much lower isoelectric point (~ 7). Its catalytic activity in a rat liver succinoxidase system, however, is equal to that of heart cytochrome-*c*.

The work of Okunuki (146) drew attention to the parallelism between the respiratory systems of plant and animal cells. This author studied several species of pollen and was able to detect cytochromes-*a*, *b*, and *c* and to extract *c* in a crude form. The respiratory activity of pollen was found to be sensitive to cyanide and to CO; the latter inhibition being largely reversed by light. Furthermore the high affinity of the respiratory system for O_2 and the stimulation by cytochrome-*c* of the oxidations of both PPD[2] and succinate strongly indicated that cyt-ox.[2] was involved. A widespread occurrence of cytochromes-*a*, *b*, and *c* in plants has been reported by Bhagvat & Hill (14). These authors examined a variety of plant tissues devoid of plastid pigments and anthocyanins and invariably detected the three cytochromes. In certain cases (shoots from newly germinated seeds, apple, potato) the pigment could be observed spectroscopically in intact tissue, but in all cases disintegration of the tissues followed by differential centrifugation gave pre-

parations in which the cytochrome spectrum was clearly visible. Plants which showed marked p.p.ox. activities yielded preparations with comparatively feeble activities in respect to cytochrome and succinic oxidase activities. This may well be due to a destruction of the latter enzymes by p.p.ox. systems during disintegration of the tissues. The oxidation and reduction of cytochromes as a result of intermittent aeration was observed with intact tissues and with homogenates containing succinate. Homogenates oxidized ferrocytochrome-*c* instantaneously in air. Bhagvat & Hill conclude that the cytochrome system in plants has the same function as in yeast and animal tissues. The visual observation of spectrum bands of cytochrome in plant tissues where they are present only at low concentrations [$\sim$ 5 per cent of that in yeast (14)] requires no little experience; it is thus probable that earlier reports that cytochrome is absent from certain plants are erroneous. The considerable intensification of absorption bands at low temperatures (101) should be of assistance in this field. Hill & Scarisbrick (88) have isolated from green leaves cytochrome-*c* (α-band 550 mμ), similar to that isolated from other sources, and two new components b_3 (α-band 559.7 mμ) and *f* (α-band 555 mμ) which are apparently characteristic of leaves. The two latter have been discussed earlier in this review. By examining bundles of wheat and corn roots in water and in HCN with a microspectrophotometer, Lundegårdh (127) was able to detect the α and γ-bands of cytochromes-*a*, *b* and *c*.

The experiments of Bhagvat & Hill (14), Stafford (182), Waygood (209), and Millerd (140) have shown that the succinic oxidase system in plants is attached to particles sedimenting at 15,000 g. In carrot root slices succinate gives increasing stimulation of respiration as the pH is lowered [Turner & Hanly (201)] presumably because only the undissociated acid can diffuse into the cells. Similarly, it is only at low pH that small concentrations of malonate will inhibit the respiration of carrot root [Hanly, Rowan & Turner (79)]. The presence of the succinic oxidase system in plants raises the question of the operation of a tricarboxylic acid cycle. Bonner (17) and Laties (117), using oat coleoptiles and barley roots respectively, found that the malonate inhibition of pyruvate oxidation was reversed by Krebs intermediates. This work has been criticised on the grounds that the stimulations are very slight (52) as are also those observed with rhubarb leaves (143). Although no evidence for the cycle could be obtained from the metabolism of carrot root (201), Barrón *et al.* (11) have demonstrated a synthesis of citrate in potato tissue, and the work of Pucher & Vickery (149) on the metabolism of organic acids in tobacco leaves has yielded positive evidence for a cycle of the Krebs type. In addition an isocitric dehydrogenase has been demonstrated in several plants (46, 211). [See also Steward & Thompson (187) and the review of Vennesland & Conn (202)].

Attempts to inhibit oxidases specifically have in many cases led to an uncritical choice of inhibitors. In spite of the work of Albert *et al.* (2, 3) and of the warning given by Stenlid (185) it is still widely accepted that sodium diethyldithiocarbamate is a specific chelating agent for copper, both in ion-

ised and protein-bound forms, under physiological conditions. Furthermore diethyldithiocarbamate is readily oxidised through the cytochrome system (96) to Bis (diethylthiocarbamyl) disulfide (antabuse) which is a potent inhibitor of at least two enzymes: succinic dehydrogenase (96) and aldehyde oxidase (155).

Cyanide and azide are inhibitors of the three main possible terminal oxidases (cyt. ox., p.p.ox. and a.a.ox.[2]). Hence the well-established fact that the respiration of young plant tissue (carrot leaves, barley seedlings) became less sensitive to HCN and HN_3 as they matured led earlier workers to assume that the cytochrome system was progressively replaced, as terminal oxidase, by other HCN- and HN_3-insensitive systems. These were considered, largely on negative evidence, to involve autoxidizable flavoproteins (see ref. 90). There is, however, considerable evidence that the lack of inhibition was due to causes other than a change in the nature of the respiratory system. Keilin (94) showed that the respiration of bakers' yeast was insensitive to HN_3 at pH 7.3 but became increasingly sensitive at lower pH. Beevers & Simon (13) demonstrated a similar pH dependence with iodoacetate, fluoride, and malonate. Stenlid (183) found that Keilin's observations could be closely paralleled with barley roots and that it was only unionized HN_3 (pK 4.7) that was effective as inhibitor. The same author (184, 185) found that while young carrot leaves exhibited a strong and pH-independent azide inhibition the respiration of older leaves was inhibited only at low pH (4.5) and in fact was stimulated at higher pH (7.0) [see discussion of work of Marsh & Goddard (90)]. Since these results could also be obtained with dinitrophenol in place of azide, it is assumed that they result from "uncoupling" of phosphorylation. The effect is similar to that observed by Tissières (200) in respiratory studies on *Aerobacter*. Simon (170) and Simon & Beevers (171) studied the effect of weak acids (iodoacetate, HN_3, HCN, HF) on a range of cells and organisms and deduced that the full inhibitory effect could as a general rule only be expected at two pH units below the pK; in other words the cells were freely permeable only to the unionised acids. The difficulties of maintaining a fixed HCN concentration in manometric experiments when CO_2 is being absorbed by KOH-KCN mixtures are stressed by Laties (118).

Allen's (4) experiments on the effect of inhibitors on the oxidation of glucose by sporidia of *U. sphaerogena* emphasise the importance of the order of addition of substrate and inhibitor. Prior addition of HCN, HN_3 or iodoacetate leads to inhibition of glucose oxidation, but if glucose is already present the inhibitors have a much less marked effect. It is assumed that the pathway of glucose assimilation is modified by the inhibitors.

It is now generally accepted that a light-sensitive CO inhibition of respiration is a definite indication of the functioning of cytochrome oxidase. The contrary argument, i.e., lack of photosensitivity or only a very slight inhibiting action by CO in the dark, cannot, however, be used as a strong argument against cytochrome oxidase function. Lack of photosensitivity can obviously arise when a tissue or preparation is pigmented or when, on account of low

respiratory activity, the quantity required is too opaque. However, even when the material does not suffer from these drawbacks a clear photosensitive inhibition may still be unobtainable even though there is independent evidence that cytochrome oxidase is functional (see below). For this reason attempts to assess, from the effect of light on the CO inhibition, the relative contribution of p.p.ox.[2] (photo-insensitive) and cyt.ox.[2] to the respiration of plant tissue (166) must be accepted with reserve. The participation of cyt. ox. in the system which provides energy for movement of bracken spermatozoids has been ingeniously demonstrated by Rothschild (162). Movement in an atmosphere containing CO was photographed in green light and in white light. Since only the latter was able to bring about photodissociation of CO from cyt.ox. the difference in motility was clear evidence that the oxidase is functional.

A fundamental difference between cyt.-ox. and other oxidases is that the former can function optimally at very low O_2 pressures (86). This property was utilized by James & Beevers (91) in their study of the respiration of the *Arum* spadix. This tissue exhibits a very high respiratory rate; $Q_{O_2} = 32$ compared with ~ 1 for the rest of the plant. Spadix respiration is insensitive to HCN, DDC, or CO but shows a marked dependence upon O_2 pressure. There is good evidence in this case that a flavoprotein is involved in the respiratory chain. Arguments in favour of various terminal oxidase systems have been discussed by Whatley (211).

A major obstacle to the study of respiratory mechanisms in plants is the fact that cellular disintegration initiates reactions which are quite different both in magnitude and in their nature from those of intact tissues. With most animal tissues this difficulty does not arise (100), but the plant biochemist is faced with the alternative of intact or sliced tissue where the degree of penetration of substrate or inhibitor is always in doubt or homogenized material where, especially in the case of p.p.ox.-containing plants. a respiratory activity far greater than that of the intact tissue may be realized. In fact even slicing may bring about as much as a four-fold increase in respiratory activity. Attempts to influence the respiration of intact leaf by addition of cytochrome-*c* have been made (143), and the absence of any effect has been considered noteworthy (161) in spite of the fact that the rate of diffusion of a molecule of this size into plant cells is negligible. Figures given by Hackney (77) show that the Q_{O_2} of cut apple tissue is about 400 while that of the whole fruit is <1, the difference being due to a high p.p.ox.[2] activity. That the low figure for the intact fruit is not due to a low internal O_2 tension is apparent from the results of Smith (179). The polemics of Sreerangachar & Roberts on the "fermentation" reaction of tea have now been resolved by the demonstration by the latter author of the presence of cytochrome oxidase in tea leaf (47) while the former's claim that the "fermentation" reaction of the dried leaf is due to p.p.ox. brought into action when the leaf is damaged is amply established (181).

A further difficulty in the interpretation of inhibitor experiments is the

following. When an enzyme is studied in isolation in the presence of a concentration of substrate sufficient to saturate it a characteristic relationship between inhibitor concentration and per cent inhibition can be observed. On the other hand, the enzyme will *in vivo* be a unit in a catalytic chain, and only if its turnover rate is the limiting one in that chain, i.e., if it catalyses the over-all rate determining step, will its apparent sensitivity to inhibitors be as high as when it is reacting in isolation. If it is not involved in the rate determining step, it will not be working at full capacity, and a partial inhibition of the enzyme may not be reflected in the activity of the complete catalytic chain. The following experiments can be cited to illustrate this point. A heart muscle preparation containing the complete succinic dehydrogenase-cytochrome oxidase system (succinic oxidase) will oxidize rapidly both PPD[2] and succinic acid (96). Whereas the former oxidation, which involves cyt.ox. and cytochrome-*c*, is very sensitive to CO the latter, involving a more complex catalytic chain, is only slightly sensitive (80). At the same time the oxidation of succinate by a similar preparation from etiolated wheat seedlings (209) shows a marked photoreversible inhibition by CO. The respiration of *Aerobacter aerogenes* cultured in presence of sufficient iron for optimum growth is comparatively insensitive to CO and HCN. On the other hand metal-deficient cells which show a much feebler cytochrome spectrum show the expected high sensitivity to these inhibitors [Tissières (200)]. Nevertheless, it is clear from Tissières' spectroscopic observations that the cytochrome system is functional in both cases. Furthermore, the degree of inhibition by CO observed, for instance, in the classical studies on *Acetobacter* and *Torulopsis* from Warburg's laboratory (116) can seldom be obtained with commercial samples of bakers' yeast yet there is good spectroscopic evidence that cytochrome oxidase is the terminal oxidase.

An important contribution to this question are Darby & Goddard's studies on the respiration of the fungus *Myrothecium verrucaria* (43). Whereas the intact mycelium is insensitive to CO and HCN, oxidase preparations obtained by grinding the mycelium with glass powder or alumina show normal inhibitions. Azide, however, inhibits both intact and ground mycelia. In discussing possible explanations of the results these authors suggest that the cytochrome oxidase is not saturated *in vivo* with ferrocytochrome-*c*, i.e., there is an excess of cyt.ox.[2] in relation to cytochrome-*c*. Similar arguments can be put forward to account for the HCN-insensitive respiration of *Neurospora* ascospores (73) and *B. subtilis* spores (99) where the much higher respiration after germination is HCN-sensitive. Alternative explanations put forward by Darby & Goddard are (*a*) intracellular protection of cyt.ox. due to orientation of the enzyme in a structural unit (see 100) or to competing reactions, and (*b*) the functioning of an alternative terminal oxidase when cyt.ox. is inhibited. Unfortunately, no experimental approach to these alternatives seems feasible at present. The fungus is devoid of p.p.ox.[2]

Failure to obtain clear-cut results in the case of intact leaf tissue treated with cyt.ox. inhibitors (e.g., ref. 47) is due to limitations in diffusion since

chloroplast suspensions give positive tests when examined manometrically in presence of CO or with PPD[2] and cytochrome-*c* [Rosenberg & Ducet (51, 161)]. In their studies on leaves from a wide range of plants these authors find that cyt-ox. is located in chloroplast preparations while p.p.ox. is in the cytoplasmic fluid (50).

In plants at the embryonic or immediate post-embryonic stage there is general agreement that cyt-ox. functions as terminal oxidase. Studies with inhibitors and on the effect of PPD+cytochrome-*c* have given unequivocal evidence in the cases of corn-embryos [Maxwell (136)] and etiolated oat seedlings [Bonner (17)]. The latter author finds that *o*-dihydroxy phenols do not influence the O_2 uptakes of these seedlings but argues that as such substances stimulate the O_2 uptake of potato tissue and spinach leaves that p.p.ox.[2] must act terminally. In view of the difficulties in interpreting results with so-called specific substrates (see above) this conclusion is not fully acceptable. In any case, proof of the presence of p.p.ox. is not sound evidence that it functions as terminal oxidase. Although Waygood (209) isolated an active succinic oxidase from wheat seedlings he proposes that a.a.ox.[2] may replace cyt.ox. as terminal oxidase. A red-violet pigment which appears in seedling extracts and which is reducible to a leuco form is considered by him to act as a redox link between dehydrogenases and the ascorbic acid oxidase system. Bhagvat & Hill (14) extracted a similar chromogen from sea-kale which, in the leuco form, rapidly reduced cytochrome-*c*. These authors, however, discount the possibility that such pigments are respiratory intermediates. On the other hand Mapson & Goddard (135) found in ungerminated pea seeds an enzyme which can bring about the reduction of oxidized glutathione by reduced triphosphopyridine nucleotide and demonstrated a pathway for hydrogen transfer from certain substrates (isocitrate, malate) to dehydroascorbic acid. In conjunction with a.a.ox. this system would perhaps provide a respiratory mechanism alternative to the cytochrome system.

The use of *p*-nitrophenol as a specific inhibitor of p.p.ox. (19) is criticised by Stenlid (184). On the other hand thiourea (71) and phenylthiourea (136) inhibit p.p.ox.[2] strongly but have little or no effect on cyt-ox.[2] Using the former reagent, Goddard & Holden (71) carried out some decisive experiments which showed that although potatoes contain a very active tyrosinase (p.p.ox.), their thiourea-resistant respiration is photoreversibly inhibited by CO and that furthermore the cyt.ox. content is adequate to account for their entire respiration.

At the present time there remains little substantial evidence against the view that the general terminal oxidase in plants is cytochrome oxidase. Much of the opposing evidence is either ambiguous or of a negative nature. The above considerations call for a re-examination of claims that the cytochrome system functional as terminal oxidase at the early stages of a plant's development is replaced by other systems as the plant matures. Failure to take such considerations into account has led, for example, to the conclusion that the terminal system in barley seedlings consists of iron-enzymes at 6 days, cop-

per-enzymes at 8 days, and flavoproteins at 10 days (113). Faced with such revolutionary turmoil the enzymologist may well lose heart.

Anion Respiration and the Cytochrome System

Lundegårdh (124, 125) has reviewed in detail his theory of the coupling of anion absorption and accumulation by plant tissues and the activity of the cytochrome system, while wider aspects of the mechanism of ion absorption by roots have been covered by Overstreet & Jacobson (147). The respiration of many plant tissues is regarded by Lundegårdh as consisting of two components: ground respiration and anion or salt respiration. The latter component is strictly paralleled by the rate of anion absorption; it shows high sensitivity to HN_3 and HCN and, in wheat roots, is independent of O_2 tension within the range 1 to 100 per cent O_2. These facts form the basis of Lundegårdh's view that the cytochrome system provides the motive power for adsorption against a concentration gradient. The complex of cytochrome components is pictured as an organized structure (cf. 100) forming an "electron ladder" orientated statistically at right angles to the membrane separating two levels of salt accumulation (medium and bulk of protoplasm or protoplasm and cell-sap). The anions move in the opposite direction to the electron transfer from dehydrogenases to molecular O_2 via the cytochrome system.

Using a very sensitive microspectrophotometer Lundergårdh was able to detect the α- and γ-bands of cytochromes-*a*, *b* and *c* in bundles of wheat roots (126, 128). A curve was obtained in the form of a difference spectrum of the roots immersed in water (cytochrome partly oxidized) and in 1 m*M* HCN (cytochrome reduced). Substitution of water by salts of organic or inorganic acids caused the cytochromes to become completely oxidized. The partial reduction at low salt concentration would, according to theory, be due to limitation of electron transfer through the cytochrome system. In presence of movable anions this restriction would be removed.

Valuable support for Lundegårdh's hypothesis has come from Robertson's laboratory (142, 157, 158) where investigations have been carried out mainly on carrot root slices. Since each O_2 molecule requires 4 electrons plus $4H^+$ to form water, the maximum molar ratio for salt accumulation/salt respiration should be 4. Experimental values rising with increasing KCl concentration to 3.4 have been obtained. The salt respiration was taken as the difference between respiratory rates in water and in salt. Failure to reach the theoretical figure could be due to (*a*) back diffusion; (*b*) effect of cutting the tissue; (*c*) withdrawal of water by osmosis at high salt concentration; and (*d*) "leaky" cells, i.e., a change of permeability arising from experimental treatment. Lundegårdh regards this leakage as an explanation of the fact that respiration of wheat roots in water is always greater than the level to be expected from extrapolation to zero salt concentration of the respiration observed in salt solutions.

A fact difficult to reconcile with the Lundegårdh hypothesis is that the stimulation arising from the presence of salt persists after removal of salt

from the medium (157). Similar observations have been made with beetroot tissue [Sutcliffe (189)]. The latter author dismisses the only reasonable explanation, viz, an increase of back diffusion (leakage) as protoplast salt concentration increases. It would thus appear that the salt stimulation is not a direct result of the quantity of salt transported but is due to some effect on the respiratory system; in fact the maximum rate of salt respiration can be attained at salt concentrations below those necessary for maximum accumulation (157). In strong support of the anion transport theory, however, are reports of simultaneous photoreversible CO inhibitions of salt respiration and salt accumulation in carrot slices [Weeks & Robertson (210)] and in wheat roots [Sutter (190)]. In a discussion of Lundegårdh's hypothesis Arens (9) pictures the salt absorption process as one of electro-osmosis.

It has been found by Robertson, Wilkins & Weeks (159) that 2:4-dinitrophenol inhibits salt accumulation in carrot disks but increases the respiratory activity and furthermore that this increase can be considered as salt respiration on the basis of its high sensitivity to CO and HCN. These experiments suggest that salt absorption involves utilization of phosphate bond energy.

The very rapid inhibitory action of HCN and the fact that intact roots can slowly oxidize ferrocytochrome-*c* (though at a rate of only ~1 per cent. of that which could be expected on the basis of the cyt.ox.^{2} content) indicate that cyt.ox. is localized near the cell surface (129), i.e., at the external end of the "electron ladder." On the other hand, in most tissues the cytochrome system is located in the mitochondria, and it is, therefore, pertinent to inquire whether this slow oxidation is not due to slight damage to the roots which may bring the ferrocytochrome-*c* in contact either with the cyt.ox. or with a phenol oxidase system (see p. 125). Assuming that the molar extinction coefficients of the 3 cytochromes are equal, the ratio of their concentrations in wheat roots is $a:c:b:=1:2:3$–4. On inhibition of succinic dehydrogenase by malonate or fluoride, the roots still show respiratory activity above the "ground" level. Under these conditions only *a* and *c* are functioning, presumably in conjunction with some other dehydrogenases. However, salt accumulation can only take place if the complete cytochrome system is functional. Thus malonate and fluoride exert the same disturbing effect on the coupling of respiration and accumulation as does 2:4-dinitrophenol (159).

Mention has been made (p. 127) of the difficulties in interpreting the effect of inhibitors on respiratory processes. In this connection it would appear that the differentiation between "ground" and salt respiration on the assumption that only the latter is sensitive to respiratory inhibitors, may be highly artificial. A more reasonable assumption would be that both are sensitive to degrees dependent upon experimental conditions and that no sharp distinction is justifiable.

Adaptation of the Cytochrome System in Resting Yeast Cultures

It has been known for some time that marked changes in the cytochrome

and cyt.ox.[2] contents of bakers' yeast can occur under certain conditions of culture (188) or can arise by mutation (154, 174). The products show a general resemblance to brewers' yeasts inasmuch as they have high fermentative ability but exhibit a very slow O_2 uptake in presence of glucose.

Recent experiments have shown that the reverse process, i.e. the development of a typical bakers' yeast from a brewers' yeast can occur in resting yeast cultures where the possibilities of cell division and selection have been eliminated; in other words the individual yeast cell can adapt its respiratory systems to environmental conditions. Chin (38) aerated a suspension of washed brewers' yeast in a phosphate-glucose medium. The original culture showed diffuse bands a_1 (585–590 mμ) and b_1 (550–566 mμ) but already after 2 hr. aeration b_1 had split into the normal b and c bands. Further aeration caused a replacement of a_1 by a followed by a general increase in intensity of all 3 bands until they showed striking resemblance to the bands of bakers' yeast (see fig. 2). Parallel to the spectroscopic changes there were observed increase in Q_{O_2}, a decrease in $Q^{N_2}_{CO_2}$, and increased sensitivity of the respiration to CO. These changes could not be reversed under anaerobiosis unless aeration was interrupted as soon as b_1 had split into 2 bands. While glucose could be replaced by sucrose in the aeration medium other substrates (succinate, lactate, glycerol, pyruvate, ethanol, and acetate) were less effective. In later experiments Chin (39) has found that aeration of non-proliferating brewers' yeast leads to (*a*) a more pronounced Pasteur effect; (*b*) a higher catalase acivity, (*c*) a decrease in the activity of several dehydrogenases; and (*d*) a ten-fold increase in cytochrome oxidase activity which runs parallel to the increase in the intensity of the cytochrome-*a* band. The rate of cytochrome transformation was strongly inhibited by 2:4-dinitrophenol and by acriflavine.

Ephrussi & Slonimski (58) attempted to bring about the reverse process (bakers' yeast→brewers' yeast) in nitrogen-free media but, like Chin, they were unable to do so. They did, however, find that after 30 hr. proliferation of *Saccharomyces cerevisiae* under anaerobic conditions (corresponding to 6 generations), the typical *a b c* spectrum was replaced by the diffuse a_1 and b_1 bands and the yeast was now highly fermentative. Aeration of this product brought about changes very similar to those obtained by Chin. There was a rapid synthesis of protohematin, i.e., from 0.7 to 2.1 μg. per mg. nitrogen during 12 hr., together with an increase in the cytochrome-*c* content from 0.012 μg. Fe at 2 hr. to 0.055 μg. Fe at 12 hr. During the above-mentioned anaerobic proliferation there was no hematin synthesis, the decrease in hematin content being largely a dilution effect.

In a second communication these authors give further details of their experiments (57). They find that, contrary to Chin, the b_1 band of their anaerobically cultured yeast is not split into more than one component at liquid air temperature. It is possible, therefore, that their yeast was at the fully anaerobic stage. However, their criticism of Chin's work, viz., that he did not take adequate precautions to ensure anaerobiosis, is out of order

since the latter author did not grow his anaerobic yeast but obtained it commercially. In a discussion of the mechanism of adaptation of cytochrome, Ephrussi & Slonimski (57) consider that, since the kinetics of cytochrome formation are first order and not those of an autocatalytic reaction, the Yudkin hypothesis (132, 214) is to be favored in this instance. According to Yudkin's views, the addition of a substrate displaces the equilibrium between enzyme and precursor, according to the mass action law. Since the protohematin content even of the anaerobic form of the yeast is sufficient to provide the quantity of cytochrome observed in the aerobic form, the O_2 lack must either prevent synthesis of the specific proteins or prevent the formation of the hematin-protein linkage. In common with Chin, Ephrussi & Slonimski regard b_1 as a precursor in aeration experiments of *b* and *c*. Replacement of O_2 by other H-acceptors (e.g., methylene blue) failed to induce the spectral change from the anaerobic to the aerobic pattern.

Previously reported variations in terms of cytochrome bands among different strains of *B. subtilis* (99) have been re-examined by Chaix & Roncoli (24, 25) and have been shown to depend upon duration of aeration of growing cultures. All strains examined showed $a+b_1$ when very young but eventually showed the classical *a b c* spectrum. In fact, even well-aerated proliferating *S. cerevisiae* cultures contained only b_1 at the initial stages. Schaeffer (167) reports that anaerobic culturing of *B. cereus* leads to virtual disappearance of cytochromes-*a* and *c* and to a marked diminution in the protohematin content. Nevertheless, the anaerobic cultures are able to bring about a more rapid aerobic oxidation of glucose than the original aerobic cultures. This apparently conflicting result may possibly arise from increased dehydrogenase activity under anaerobic conditions as was observed by Chin (39) in the case of brewers' yeast. If the cytochrome system is present in excess, then any increase in dehydrogenase activities will be reflected in the over-all rate of respiration.

The dramatic changes in the enzymic make-up of respiring, nonproliferating cells, described in this section, raises a problem which the plant physiologist cannot ignore. Although such experiments, mainly for reasons of technical expediency, have been limited to unicellular organisms, it is now relevant to enquire whether similar enzymic changes might not occur in higher organisms. Can it be assumed that plants away from their normal environment, or plant slices exposed, for example, to atmospheres other than air, retain their normal intracellular catalytic mechanisms? Thus, a change in the relative activities of dehydrogenases on the one hand and the cytochromes on the other might result in a component in the respiratory system becoming limiting although it was not so *in vivo*. Such an alteration of balance could result in a marked change in the reaction of such tissues to enzyme inhibitors (see p. 129).

Function of Hemoglobin in Root Nodules of Leguminous Plants

The reviews of Scarisbrick (164) and Virtanen (203) have dealt ade-

quately with the period up to 1947. However, some reference to work carried out prior to this date will be helpful to an appreciation of the present position. General reviews on symbiosis of nodule bacteria (16, 163, 199) and on nitrogen metabolism (131) also refer to the nodule hemoglobin problem.

Kubo's (114) view that the red nodular pigment was a hemoprotein allied to hemoglobin (Hb[2]) was finally established by Keilin & Wang (108) who showed that it was a true Hb reacting reversibly with O_2 and CO and that it resembled myogolobin in undergoing a fairly rapid autoxidation in air to methemoglobin (MetHb[2]). These authors discuss the possible alternative roles of O_2 carrier and oxido-reduction catalyst and point out that a clear distinction is difficult in view of the wide variation in properties of Hb's (109). The invariable association in nodules of Hb with "effective" strains of *Rhizobium* and the inhibition of fixation by low concentrations of CO clearly establish that the pigment is directly involved in fixation (107).

Virtanen (203, 207) proposed a redox function for the pigment coupled with a reduction of N_2 to hydroxylamine ($N_2 + MetHb \rightleftarrows NH_2OH + Hb$) which could then be utilized to convert oxalacetic to aspartic acid. Attempts by Keilin & Smith (107) to confirm this view failed and these authors were unable to detect MetHb in active intact nodules. Nevertheless, the possibility of a redox function cannot be ignored since Colter & Quastel (40) have shown that hydroxylamine can effect both oxidation and reduction of blood Hb ($NH_2OH + Hb \rightarrow NH_3 + MetHb$; $NH_2OH + MetHb \rightarrow N_2 + Hb$). The Helsinki school has studied other nitrogen-fixing micro-organisms in an attempt to throw light on the nodule problem (204, 205, 206). Thus in the fixation of N_2 by *Clostridium butyricum* NH_2OH is produced only under aerobic conditions and the proposed mechanism is reduction of N_2 to NH_3 and subsequent oxidation to hydroxylamine. With *Azotobacter*, however, hydroxylamine is produced aerobically much more readily from N_2 or nitrate than from NH_3. Here a mechanism similar to that proposed for *Rhizobium* (207) is favored: $N_2 \rightarrow N_2O \rightarrow (NOH)_2 \rightarrow NH_2OH$. Zelitch (215) has shown that *Cl. pasteurianum* that are fixing N_2 will accept NH_3 as nitrogen source without previous adaptation whereas cells grown on ammonia media lack the ability to fix N_2. These experiments argue strongly in favor of NH_3 as an intermediate in N_2 fixation. Some preliminary studies of the inhibitory effect of hydroxylamine on respiration of, and N_2 fixation by, *Azotobacter* [Pethica, Roberts & Winter (148)] are considered to argue against the view that hydroxylamine is an intermediate in the fixation process. Zelitch, Wilson & Burris (216) allowed soy bean nodules to fix N_2^{15} and determined the isotope concentrations in the amino acids of the nodule proteins. Its preponderance in glutamic rather than aspartic acid is also considered to oppose the Virtanen theory since hydroxylamine reacts more readily with oxalacetic acid than with α-ketoglutaric acid. This argument, however, presupposes the absence of transamination reactions. The

use of N_2^{15} in such studies is described at length by Wilson (212) in a review of biochemical aspects of N_2 fixation.

Soy bean MetHb contains 2 components with isoelectric points 4.4 and 4.7 (53). Ultracentrifugal and osmometric studies of similar material containing 0.26–0.27 per cent iron [Ellfolk & Virtanen (54)] indicated a mean M of 20,000. The faster moving component (M 17,000; 1 atom iron) is regarded as the pure pigment. The green pigment formed at the cessation of N_2 fixation yields biliverdin on treatment with acid (208) and is thus allied to the bile pigments produced in catabolism of blood pigment. The formation of bile pigment in nodules has also been studied by Heumann (83) who found that as the nodules aged Hb[2] was broken down from the nodule-base outwards, parallel to the destruction of the *Rhizobium*-containing bacteroids. A similar irreversible change was observed on transferring the roots to a H_2/O_2 atmosphere. Contrary to the general belief that *Rhizobia* can only fix N_2 when associated with root nodules (177), fixation on blood-agar media has recently been reported [Heumann (84)].

Smith (177) has studied the distribution of Hb in nodules of *Phaseolus vulgaris* and soy beans. The pigment is present solely in the bacteroids where its concentration ranges from 0.1–0.5 m*M*. Nitrogen fixation in the nodules is inhibited by CO at concentrations much lower than those required to inhibit fixation by *Nostoc* or *Azotobacter* in which processes Hb plays no part. Pigment-free nodules are produced by "ineffective" strains of *Rhizobium* while failure to fix N_2, even with effective strains may be caused by (*a*) boron deficiency; (*b*) plant nearing end of growth; (*c*) one week in darkness. In the last instance failure is associated with cessation of carbohydrate metabolism (83); plants grown in darkness in a glucose medium produce red nodules. On the other hand effective variants have been produced by X-ray irradiation of ineffective strains (92).

Since the Hb-bound O_2 in nodules would suffice for <4 min. of nodule respiration, there is no evidence that the pigment can function as an O_2 store, and furthermore since it is in a fixed position within the nodule it cannot function as O_2 transporter (178). The possibility of a more specific effect of the Hb upon the reaction rate of respiratory enzymes with O_2 was suggested by Kubo (114) who found that nodule Hb increased the respiratory rate of *Rhizobium* suspensions at low O_2 pressures. However, the findings that pig Hb (123) and nodule CO−Hb (178) are as effective as nodule oxyhemoglobin argue against this view. In any case Smith has shown that in air containing 5 per cent CO (which ensures complete formation of CO−Hb) the respiration of detached nodules proceeds at the same rate as in air. There is apparently an extra-nodular factor entering into the fixation process since this ceases when the nodules are detached from the roots. On the other hand even when they are attached their respiration is not influenced by 5 per cent CO.

The effects of mammalian Hb on the respiration of *Rhizobium in vitro*

have been further studied by Burris & Wilson (23) who criticise Smith's view that the increased rate of respiration is a result of change from resting to proliferating metabolism. They point out that the effect is immediate and that there is no indication of logarithmic growth: in any case other proteins are ineffective. These authors favor a specific dynamic action of the protein but several experimental observations are put forward in opposition: (*a*) Heat denatured Hb[2] cannot replace the native pigment; (*b*) at low O_2 pressures (0.01 atm.) the effect of Hb is enhanced; (*c*) in 20 per cent O_2 the presence of 5 per cent CO does not reduce the stimulation by Hb but in 1 per cent O_2 this concentration of CO is inhibitory. It is not, however, clear that these are serious objections since denatured Hb would be insoluble while the effect of low O_2 pressure is difficult to interpret when the nature of the action of the protein is unknown. The protein must presumably be broken down before it can enter the cell and influence the respiratory systems, and since the proteolytic activity of *Rhizobium* is weak this process may become limiting in 20 per cent O_2 when the O_2 consumption is higher by a factor of three than it is in 1 per cent O_2. Again, in 20 per cent O_2 the $CO:O_2$ ratio was 0.25 which would not affect cytochrome oxidase, but in 1 per cent O_2 the ratio was 5 and under such circumstances both the Hb and cytochrome oxidase of the bacteria would have been inhibited and a greater reduction of the overall respiratory activity would be expected.

The present position of the nodule pigment problem is thus very unsatisfactory. Although the work of Keilin and of Smith has thrown considerable doubt upon the Virtanen hypothesis, neither their work nor later publications have given any fresh indications of the function of Hb[2] in N_2 fixation. The alternative viewpoints, viz.: that hemoglobin may function either as a specific stimulator or as nutrient material for bacterial growth, arising as they do from considerations of the influence of mammalian Hb on *Rhizobium* suspensions, cannot justifiably be applied to the intact nodule. The marked differences between nodule and mammalian hemoglobins in terms of affinity for CO and O_2 and of autoxidizability, together with the fact that both can apparently stimulate cultures of nodule bacteria, strongly oppose the theory of a so-called specific stimulator.

Catalase and Peroxidase

It is convenient to consider these hemoprotein enzymes jointly in view of their close similarity, now very clearly established, in terms of chemical constitution, spectroscopic relationships, and the mechanism and kinetics of their reactions with peroxides. The peroxidatic activity of catalase (95, 98) is now widely accepted as a more likely function of the enzyme than the classical reaction $2H_2O_2 \rightarrow 2H_2O + O_2$ while it is also possible to demonstrate a catalase-like (*catalatic*) reaction of peroxidase. Although many tissues show both catalase and peroxidase activities it cannot be assumed that both enzymes are present since all hemoproteins can act as feeble catalases and peroxidases and since each enzyme can exhibit the charac-

teristic activity of the other, albeit at a much lower level (103). In general, peroxidase is a plant enzyme while catalase is present at high concentration in yeast, bacteria, and animal tissues. There are exceptions to this rule such as the peroxidases of milk and leucocytes (196) and, in spite of its low concentration in plants, catalase in a high state of purity can be isolated from green leaves (67).

In the present review the term peroxidase signifies the enzyme from horse-radish root. The cytochrome-*c* peroxidase of yeast has not been detected in higher plants. This is not surprising since Chance (32) has shown that the horse-radish enzyme can efficiently catalyse the oxidation of ferrocytochrome-*c* by H_2O_2. Knowledge of plant catalases has been greatly limited by the difficulties of isolation: a problem only recently solved (67). Since the plant enzyme is similar to the extensively studied animal catalases some reference to the latter may be useful in considering the possible role of catalase in plants.

Isolation and properties.—A new method of purification of peroxidase [Keilin & Hartree (103)] yields a pure enzyme of activity (Purpurogallin Number) = 1,220 and mol. wt. = 39,800. The discrepancy between these figures and those of Theorell (195) for crystalline enzyme (930 and 44,100 respectively) is apparently due to different carbohydrate contents. The former authors give a detailed comparison of peroxidase and liver catalase with methemoglobin in terms of reactions with inhibitors and with H_2O_2. It is concluded that "paraperoxidase" is an artefact.

An extension of earlier work (197) from Theorell's laboratory led to the preparation of further artificial peroxidases from the peroxidase protein and various hematins. Such products showed up to 63 per cent of the activity of the original enzyme (198). The regeneration of peroxidase activity in plant tissue extracts after heat inactivation (82) is now considered to be related to their cytochrome-*c* content (151). A marked increase in peroxidase activity can also be observed when aqueous extracts of horse-radish roots are preserved at 5° for several weeks (80).

The extensive purification of catalase from spinach leaves has been achieved by Galston, Bonnichsen & Arnon (67). The product (Kat.f = 23,600) shows a close similarity, both spectroscopically and in its relation to inhibitors, to liver and blood catalases. The hematin content (0.56 per cent) is about one half that of other catalases whereas the catalase activity is considerably less than one half that of purified animal and bacterial catalases. If the molecular weight is of the same order as that of other catalases ($\sim$240,000), these data are parallel with those of Bonnichsen (20) who found that liver catalases in which 2 hematins of a 4-hematin molecule were converted to bile pigment had disproportionately low activities; this gives a strong indication that more than one hematin per molecule is necessary for catalase activity.

Distribution and function.—Galston (63) has compared the catalase contents of vegetable tissues grown in culture. Slow growing crown-gall

tissue contains much less catalase than actively growing normal tissues, a relationship analogous to the decreased catalase activity of animal tumour tissue (22). Cultivation in presence of indole-3-acetic acid (IAA[2]) and especially of 2,4-dichloro-phenoxyacetic acid markedly decreased catalatic activity. Although several authors (see ref. 67) have reported that on germination the catalase content of seeds rises and subsequently falls, germination of cotton seeds apparently leads to a decrease in catalase and an increase in peroxidase activities [Altschul, Karon & Kyame (5)]. The catalase activity of seed preparations, detectable on adding H_2O_2, is suppressed by addition of a peroxidase substrate (H-donor), a phenomenon also observed in the case of wheat germ and of mixtures of catalase and peroxidase (186). Altschul *et al.* discount the possibility of a peroxidatic function for catalase, but in assessing their evidence it must be borne in mind that their experiments were carried out on acetone-dried material subjected to autolysis under toluene. Eyster has studied the catalase contents of maize mutants deficient in chloroplast pigments (60). Homogenates of albino seedlings show a much lower catalase activity than similar preparations of green seedlings but, when grown in the dark, both types develop a catalase activity markedly higher than that shown by either under normal illumination. This change is readily reversed on exposure of dark-grown plants to light. Gylling & von Euler (76) found that although leaves of green barley contained more catalase than did those of white mutants, the inverse relationship held for the seeds of the two types. A considerable increase in catalase content and general metabolic activity has been observed in orchid flowers after pollination (89). Catalase and peroxidase in "koksagiz" have been investigated by Doman (49) and Mikhlin & Pshenova (139). During the period of accumulation of caoutchouc there is a steady fall in peroxidase and catalase activities. At the root centre where caoutchouc is formed peroxidase is weaker, and polyphenol oxidase stronger than at the periphery.

The function of catalase in plants remains uncertain. The very high concentration of the enzyme in animal tissues, particularly those containing enzyme systems which *in vitro* are known to reduce O_2 to H_2O_2, supports the view that catalase plays a peroxidatic role. Similarly, the very high catalase content (2 per cent) of *Micrococcus lysodeikticus* is considered by Herbert & Pinsent (81) to favor the same view. Anan (6), using liver catalase, has been able to demonstrate the peroxidatic activity of catalase plus H_2O_2 (added as solution) towards ethanol and nitrite more effectively than Keilin & Hartree (98) were able to do with the former substrate. Working at high dilutions and a molar ratio hematin Fe/H_2O_2 added per sec. $\sim$1, Anan found that 6–7 per cent of added H_2O_2 was utilized peroxidatically. In plants, however, even assuming that H_2O_2 is formed, there is no evidence that the much lower concentrations of catalase can function in this way. A purely catalatic role is suggested by Tewfik & Stumpf (194) who have obtained from pea-leaf a fructose diphosphate oxidizing system which involves flavine adenine dinucleotide, adenosine triphosphate, and ascorbic acid.

Catalase apparently serves here to decompose H_2O_2 arising from autoxidation of reduced dinucleotide.

The possible role of catalase in photosynthesis has been discussed by Rabinowitch (153) and more recently by Tamiya (192). In a valuable analysis of the influence of inhibitors on photosynthesis Tamiya finally rejects the view that catalase can be concerned in the liberation of oxygen. In the writers' opinion, however, the question of whether catalase is involved is not the most important part of this author's work. Although photosynthesis appears to be sensitive to all inhibitors which affect catalase, it seems clear that cyanide and hydroxylamine must be inhibiting different parts of the photosynthetic mechanism. The work of Gaffron (62) has shown that the final stage, the liberation of oxygen, is sensitive to hydroxylamine but scarcely affected by cyanide. It should perhaps be pointed out that most hemoproteins react with catalase poisons, and our present knowledge of photosynthesis would not be incompatible with the assumption that more than one hemoprotein is involved. However, a consideration of nonlimiting rates might well defeat attempts at quantitative analysis.

Although several indications of possible functions of peroxidase in plants have been put forward, these are at present uncoordinated. Plant tissues generally show either peroxidase activity or polyphenol oxidase activity. The latter types (e.g., apple, potato) show a very rapid O_2 uptake when bruised and darken rapidly as phenolic H-donors are oxidised. The former (e.g., horse-radish and turnip roots), show no such marked changes. Since similar H-donors are utilized by both enzymes it is conceivable that they play similar roles.

Galston & Baker (65) propose that the IAA[2] oxidizing system isolated from etiolated pea seedlings consists of a flavoprotein which gives rise to H_2O_2 when its leuco-form undergoes autoxidation, and peroxidase which catalyses the oxidation of IAA by H_2O_2. They find that the activity of crude tissue preparations is increased by light and that the action spectrum is reminiscent of the absorption spectrum of riboflavine. The same authors have shown that riboflavine can act as photoreceptor in the destruction of IAA by visible light (64). The inhibition of oxidation of IAA by catalase and other substances which decompose H_2O_2 [(65), see also Goldacre (74)] supports such a mechanism as do also the preparation of an artificial IAA oxidase by addition of peroxidase to the xanthin-oxidase system (66) and the finding that IAA and H_2O_2 do not react in absence of peroxidase (168). A study of the same oxidizing system in intact plants [Tang & Bonner (193)] has shown that it is absent in green leaves. At the same time the process of light inactivation of IAA is rapid in green leaves but absent in etiolated seedlings. It thus appears that the oxidase exerts a protective action in maintaining IAA[2] at a nontoxic level. The oxidation of reduced triphosphopyridine nucleotide by O_2 as catalysed by wheat germ extracts (41) is also considered to involve a peroxidase. The inhibition of this reaction by catalase gives indication of peroxide formation. The extent to which re-

sults obtained with disintegrated tissues can be applied to any discussion of the metabolism of intact plants does, of course, raise a problem of considerable difficulty (see also p. 129).

The crude IAA oxidase of Galston & Baker is activated by low concentrations of Mn^{++}. Similarly the growth of *Avena* coleoptile sections in a medium containing IAA[2] is increased by Mn^{++} (18). Of great interest, therefore, is the demonstration of a catalytic function of peroxidase in the oxidation of Mn^{++} to Mn^{+++} by Kenten & Mann (110, 111). It was shown that horse-radish and other roots contain a system, subsequently identified as peroxidase plus a substrate (H-donor), which can catalyse the oxidation of Mn^{++} by H_2O_2. These authors suggest a mechanism whereby the oxidized substrate brings about an oxidation of Mn^{++} which may then act as oxidizing agent for plant metabolites:

$$AH_2 \text{ (peroxidase substrate)} \xrightarrow[+\,H_2O_2]{\text{peroxidase}} A$$

$$A + Mn^{++} \longrightarrow Mn^{+++} \text{ (or } Mn^{++++}) + AH_2$$

$$Mn^{++} + O_2 \xleftarrow{H_2O_2} Mn^{+++} \xrightarrow{\text{metabolites}} Mn^{++} + \text{oxidized metabolites}$$

This scheme does provide for an explanation of the activating effect of Mn^{++} on plant respiration (85, 147). Experiments with purified peroxidase preparations showed that phenol, *p*-cresol, *o*-cresol, and resorcinol were active substrates for Mn^{++} oxidation while hydroquinone, catechol, pyrogallol, and caffeic acid were not. This classification is unexpected since compounds of the latter group are the more active in the classical peroxidase reaction. However, Kenten & Mann have now shown that in presence of the amine oxidase system, as a source of H_2O_2, and peroxidase, Mn^{++} will act as a redox carrier in the oxidation of oxalic acid (112). Simakov (169) has shown that pyrogallol and other phenols can act as carriers in the oxidation of indigo carmine by peroxidase. The idea that an enzyme's substrate specificity may vary according to whether the substrate is being oxidized in bulk or as a carrier at a very low concentration could be put forward to meet one of the main criticisms of the hypothesis of the peroxidatic role of catalase, viz.: that no "biological substrate of major importance" (121) appears to undergo a peroxidatic oxidation.

Little advance has been made in the elucidation of the mechanism of oxidation of dioxymaleic acid (DM) by O_2 in presence of peroxidase since it was reviewed by Lemberg & Legge (120). The inhibition of this reaction by catalase (191) suggests the intermediate formation of H_2O_2 and all theories of reaction mechanism assume that this is formed by an initial autoxidation of DM. By rigorous purification of DM and buffer solutions it is possible to abolish autoxidation virtually completely without affecting the rate of the enzyme-catalysed reaction (80). On the other hand, aeration of a concentrated solution of peroxidase containing DM, rapidly gives rise to red derivatives (80) which can be identified spectroscopically with known peroxidase-peroxide compounds (105). However, at the high dilution of

enzyme used in manometric experiments it is possible that the initial stage in the reaction is the formation of a peroxidase-DM compound.

Estimation of Peroxidase.—The chief drawbacks to the classical purpurogallin method of Willstätter & Stoll are the fluctuations in apparent activity of an enzyme preparation. These difficulties were investigated by Ettori (59) who was, however, unable to trace their cause. A careful purification of pyrogallol appears to make the test more reproducible (103) as does also its adaptation to a manometric method which measures CO_2 evolved in the oxidation of pyrogallol (59). Replacement of pyrogallol by leuco 2:6-dichlor-benzenoneindo-3′-chlorophenol is claimed to increase sensitivity and reliability (175). Purr (150) uses ascorbic acid as substrate with a trace of *o*-tolidine as a carrier while Boiarkin (15) proposes the use of benzidine. The last three methods have the advantage that there is no accumulation of oxidized phenolic substrate which may influence the reaction either by inhibiting the enzyme or by acting as a carrier in a nonenzymic oxidation of substrate. Mikhlin & Bronovitsknin have developed an iodometric method in which p.p.ox.[2] does not interfere (137).

Estimation of catalase.—Bonnichsen, Chance & Theorell (21) modified the classical "Katalase Fähigkeit" determination by using a higher concentration of enzyme and measuring the decrease in H_2O_2 concentration over shorter periods. Under these conditions it is shown that a more reliable extrapolation of the velocity constant to zero time can be performed. In order to avoid inactivation of enzyme by contact with H_2O_2 Feinstein (61) used perborate to provide a permanent low concentration of peroxide. A simple method giving a continuous record of peroxide concentration is that of Beers and Sizer (12) who estimated H_2O_2 in terms of its absorption at 210–240 mμ. In the two latter methods a proportionality was observed between enzyme concentration and rate of decomposition of substrate Although the above methods are all dependable when pure catalase is used, their reliability becomes doubtful when crude tissue preparations are to be tested on account of (*a*) utilization of H_2O_2 in side reactions; (*b*) extra reduction of permanganate by impurities during titration of excess H_2O_2; (*c*) lack of transparency in the ultra violet region. A manometric method should be free from these drawbacks and it is desirable that a method such as that described by Altschul *et al.* (5), which could be quantitatively related to the Kat.f. determination, should find general acceptance.

Reaction mechanisms.—Full consideration of the numerous papers on this subject from Chance's laboratory on the reaction kinetics of peroxidase and catalase or of the new theories of catalase action proposed by Tamiya and co-workers (145) does not come into the scope of the present article. Reviews by Chance himself (26, 31, 33, 37), by Lemberg & Legge (122), and by George (68) cover all but the latest contributions to the subject. However, several points arising from Chance's work will be considered in the light of subsequent developments.

Chance's inclusion of ascorbic acid (AA) as H donor for the peroxidatic

reactions of peroxidase and catalase (26) calls for reconsideration. Although the secondary peroxidase-H_2O_2 compound (Per-H_2O_2-II) appears to react with AA (28), this may well be the result of a reaction of AA with free H_2O_2 and a resultant shift in equilibrium leading to a disappearance of compound II. In support of this contention is the finding that AA undergoes a coupled oxidation in the notatin-glucose system, under the conditions described by Keilin & Hartree (98), both in presence and in absence of peroxidase (80). Similarly, the accelerated disappearance of the primary catalase compound (Cat-H_2O_2-I) on addition of AA is due to a more rapid transition to the inactive Cat-H_2O_2-II rather than to a peroxidatic oxidation of AA (30). This view is confirmed by the observation that catalase protects AA from oxidation by the notatin-glucose system (80).

By using the notatin system to provide a steady but very low concentration of H_2O_2 Chance (29) was able to stabilize the compound Cat-H_2O_2-I and, by demonstrating its close similarity to the primary peroxidase compound, to further the analogy between the two enzymes (103). The reaction of Cat-H_2O_2-I with H_2O_2 and with ethanol (the catalatic and peroxidatic reactions of catalase) are precisely analogous though the rate constants are very different, the former proceeding 40,000 times faster than the latter.

Experiments by George (68, 69) throw new light on the constitution of the H_2O_2 compounds of peroxidase. Although it was known that reducing agents accelerate the change of Per-H_2O_2-I to the secondary compound (27) this was not attributed to Fe valency changes. George has shown that Per-H_2O_2-II requires only one equivalent of ferrocyanide to reduce it to peroxidase instead of the two equivalents that would be necessary to reduce the H_2O_2 of a peroxidase-H_2O_2 adduct. This work suggests that while Per-H_2O_2-I may well be a ferric iron-H_2O_2 complex, or an equivalent structure in which the oxidation state of the compound is $+5$, the secondary compound contains iron with an effective oxidation number of $+4$. The difference of one oxidizing equivalent would explain the effect of reducing agents in accelerating the I$\rightarrow$II reaction. George discusses various possible structures for Per-H_2O_2-II. Using the rapid-flow technique, Chance (34) has confirmed George's findings.

Since the decomposition of H_2O_2 into O_2 is essentially a dehydrogenation, Lemberg & Foulkes (119) proposed that an essential factor in the catalase reaction is a reducible group in the protein which could accept hydrogen from an intermediate Fe-peroxide complex. A recent and more detailed mathematical analysis of Chance's kinetic findings on the catalase-H_2O_2 reaction suggests that this type of mechanism provides the most reasonable explanation of the available data (36).

Of the six bonds in the octahedral Fe complex of peroxidase, four are linked to porphyrin nitrogen atoms and one to the protein. The sixth linkage, following the work of Theorell and of Chance (196), has been considered as Fe-OH. In the case of the analogous acid form of methemoglobin, Coryell, Stitt & Pauling (42) proposed a water molecule instead of

the OH group. Keilin & Hartree's (104) spectroscopic study of desiccated methemoglobin lends support to this conclusion while their spectroscopic investigations of peroxidase at liquid air temperatures favor a similar Fe-H_2O linkage in peroxidase (101).

A serious handicap to discussion of catalase and peroxidase for the present review has been the paucity and uncertainty of our knowledge of their contributions to cellular metabolism. It is thus a matter of great satisfaction that a recent investigation by Chance (35) has demonstrated the reversible formation of a catalase-peroxide complex as a normal component reaction of cellular respiration. Chance found that the catalase in a suspension of *Micrococcus lysodeikticus* is in the form Cat-H_2O_2-I under aerobic conditions and that it rapidly reverts to the free enzyme when the oxygen supply is exhausted. The complex reacts with donors (nitrite, formate) exactly as it does *in vitro* and from a study of the influence of formate concentration on the concentration of the complex it was deduced that the steady-state concentration of H_2O_2 was as low as $10^{-8}M$ in aerated suspensions. This result eliminates one of the long standing difficulties in assessing the role of catalase, i.e., the fact that except in very few instances there has been no evidence for H_2O_2 formation within strictly aerobic cells which are respiring normally (98). At a concentration of $10^{-8}M$ only Chance's methods can detect and estimate H_2O_2 with certainty and at this level the peroxidatic activity of catalase would be of the same order as the catalatic activity. Thus although the cytochrome system is shown to be functional as terminal oxidase in this organism, there appears to be an alternative respiratory pathway involving oxidase systems which reduce O_2 to H_2O_2 and possible utilization of the latter for peroxidatic reactions mediated by catalase. A decision on whether this alternative system can make an appreciable contribution to the total respiration must await further experiments.

While such observations are at present limited to *M. lysodeikticus* on account of its exceptionally high catalase content, the possibility of similar reactions in other organisms must nevertheless be given serious attention.

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THE RELATION OF CHEMICAL STRUCTURE TO BIOLOGICAL ACTIVITY IN GROWTH SUBSTANCES[1,2]

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Introduction

A survey of the scientific contributions to the development of a particular domain of research may be a sorting of the material under appropriate chapters without further comment, thus providing a useful stocktaking over a given period of time. The analysis, then, is left entirely to the reader.

We learn now, that more and more requests are made for reviews presenting critical analyses of the work referred to, leading, as far as is feasible, to an appraisal of the present status of the subject. Though realizing that this kind of review may introduce controversy, since the personal opinions of the author come to the fore to a greater extent, this latter way has been chosen here. In the author's opinion the risk of bias is counterbalanced by a more vivid picture of the situation and by the consideration that at the present stage it seemed possible, indeed, to arrive at a certain co-ordination of the material when treated from a central point of view. Moreover, if the interpretations become too personal in character now and then, this will be corrected when another reviewer analyzes the material from his point of view. Thus, in the course of time the greatest common divisor serves as a guide for future research.

Our discussion of the structure-activity relationships of growth substances mainly pertains to their growth promoting activities. Inhibitory actions are considered in certain cases when this supplementary evidence clarifies the picture. Growth inhibitors proper (e.g., unsaturated lactones and other compounds of a rather heterogeneous type) are left out of consideration because they require a separate discussion.

This review is not strictly limited to the recent literature although this has been emphasized. Older papers in many respects had to form the basis of a critical analysis, therefore an effort has been made to cover the development of this part of growth substance research more or less from the beginning.

The Importance of the Test Methods

In analyzing structure-activity relationships of growth substances, one has to decide first of all about the type of action that will serve as a test,

[1] The survey of the literature pertaining to this review was concluded in September, 1952.

[2] The following abbreviations will be used: 2,4-D, 2,4-dichlorophenoxyacetic acid; DNAA, di-*n*-amylacetic acid; IAA, indoleacetic acid; MCPA, 2-methyl-4-chlorophenoxyacetic acid; NAA, naphthalene-2-acetic acid; PAA, phenylacetic acid; POAA, phenoxyacetic acid; TIBA, 2,3,5-triiodobenzoic acid.

since the effect of a certain scheme of structural variations may be studied with respect to quite a number of responses, each in its own right. For a fundamental study of the problem, a type of action is preferred which, as far as can be judged, expresses most directly or simply the function of the active compound.

It is for this reason that of all the possible responses such as stimulation of cell elongation, initiation of roots (60, 190), induction of parthenocarpy (92, 186), modification of organs [formative effects (131, 184, 187)], control of abscission (171) or of bud development (124, 146, 147), inhibition of root growth (1, 16, 26, 27, 78, 101, 102, 115, 130) or of seed germination (38) [Norman & Weintraub (106)], the stimulation of cell elongation has been used most extensively.

Our discussion will center around the results obtained with this type of response also because of the fact that the most important conclusions can be drawn only from a homogeneous material. Very often discussions in this field have become confused by mixing results obtained with different test methods.

This consideration leads to a further limitation, since the methods used for detecting stimulation of cell elongation are not equivalent with respect to the ideal test, i.e., the direct exposure of the "primary active sites" involved in the response to the compounds investigated. In every practicable test available until now, secondary factors (penetrability, polar transport, inactivation of the compounds) obscure the ideal picture and one has to select the test method which approximates the ideal one as far as possible.

Therefore the *Avena* straight growth [11, 14, 67, 69, 117, 118, 141, 143 (cf. 150)] and the pea test [119, 144, 149, 173, 175 (cf. 103, 145)] are preferred to the *Avena* curvature test [(cf. 85), 172], since in the former two (immersion tests) secondary factors are excluded to a larger extent. It is interesting in this connection that in the straight growth and the pea test the response is proportionate to the logarithm of the concentration, whereas in the standard *Avena* test the curvature is directly proportionate to the concentration. It would seem that in the immersion tests the influence of a surface (to which the active compound is adsorbed?) comes to the fore.

From a purely chemical point of view the pea test has the advantage of being the test resulting in the highest relative activities. Nevertheless, the response is probably more complex than that of normal cell elongation (67, 107) and the straight growth test approximates better the physiological conditions present in the intact plant.

Since, in the main, the results of both tests parallel each other, both will be used as the basis for the measure of activity. Sometimes other tests will be included in the discussion if evident parallels with the straight growth and pea tests are present and extra information may thereby be gained [especially root growth inhibition, roughly proportionate to log C (78)].

This rather extensive limitation of the scope is preferred because in the author's opinion, it is most important to arrive at a common basis in this

probably most fundamental part of the work. Thereafter, a successive extension to the whole scale of responses to growth substances will contribute much more rapidly to our understanding of the relation between structure and activity than if an attempt is made to incorporate all the responses at once.

Norman & Weintraub (106) clearly outlined the limitations of existing data as determined by the variations in the test objects (changes in sensitivity), different experimental conditions and techniques, etc.; thus many of the data available possess a semi-quantitative character only.

When trying to express results of a certain test method in more or less absolute terms, one has to remember, however, that the response to a stimulating agent, whose action in higher concentrations always becomes an inhibitory one, is described by an activity curve with a maximum. As Steiner (127) indicated, two compounds may differ in that their maximal responses, attained at the same concentration, are different, or in that their equal maximal effects come about at different concentrations (with all possible transitions). Numbers expressing activity in one way or another, even if they are identical, therefore may have a different meaning. Thus, it is necessary to determine activities for a series of concentrations to obtain an idea of the course of the activity curve. However in studying structure-activity relationships, relative activities are often more important than absolute ones and, therefore, in critical experiments, activities of the compounds should be determined simultaneously under identical conditions.

Structure and Activity

The analysis of the structural features of a physiologically or pharmacologically active compound essential for its specific activity has always been fascinating because it may yield a double gain: insight into the mode of action, and a more deductive trend in synthetic work aiming at new compounds of comparable or enhanced activity. Often, however, the complexity of the structure of the active agent or of the test methods used in assessing its activity, or both, have been barriers in attaining that end. Growth substances seem to be very attractive for this kind of analysis because both their structures and the test methods are relatively simple. Moreover, rather early in growth substance research a large number of active compounds became available, making it possible to indicate the structural elements shared by all of them while noting those partly absent in inactive substances.

In this way [after more or less introductory investigations by Kögl & Kostermans (76), Thimann (136, 137), and Haagen-Smit & Went (51)], Koepfli, Thimann & Went (74), applying the pea test, for the first time formulated the five, now well-known structural requirements for cell elongation activity: (*a*) a ring system nucleus; (*b*) a double bond in this ring; (*c*) a side chain; (*d*) a carboxyl group (or a structure readily converted to a carboxyl) on this side chain, at least one carbon atom removed from the ring;

and (*e*) a particular space relationship between the ring and the carboxyl group.

In subsequent years a large number of compounds were screened for activity, and the results obtained have led to restatements of these requirements in different forms, a development which has been reviewed in a number of papers [Audus (9); Linser (88); Norman & Weintraub (106); Thimann (138)]. While referring to these sources in general, some fundamental points will be repeated here, in order to analyze the newer material against that background.

As more compounds became known these five requirements appeared to fall short of describing exactly the essential features in two ways: some compounds met the requirements although they were inactive; others did not meet them, although they were active. Apparently in this form the requirements were too descriptive and not sufficiently functional. Therefore, attempts were made to establish their real purport and, subsequently, to reformulate them in such a way as to be more limiting, since only then might they serve as a guide for the synthesis of active compounds.

Veldstra (153) condensed the five requirements into two: (*I*) a basal ring system with high surface activity (*a*, *b*); and (*II*) a carboxyl group (or its dipole) in a very definite spatial position with respect to this ring system [out of its plane (*c*, *d*, *e*)].

Later on they were formulated in more detail [Veldstra & Booij (161)] as: (*A*) a basal ring system (nonpolar part) with high interface activity; and (*B*) a carboxyl group (polar part), in general a group of acidic character, in such a spatial position with respect to the ring system, that on adsorption of the active molecule to a boundary (the nonpolar part playing the most important role), this functional group will be situated as peripherally as possible.

Requirement *I*(*A*), in which the original (*a*) and (*b*) have been given a measurable form, was deduced from the behavior of the most active compounds upon polarographic assay which indicated that the nuclear double bond most probably could not play a part in a reversible oxidation-reduction process and that all highly active growth substances possess a high surface activity as measured by their suppression of the so-called oxygen maximum. Though in principle offering a possibility of indicating a quantitative lower limit, *A* does not yet give complete satisfaction since it fails to provide an upper limit.

Because high interfacial activity appeared to be present in highly active growth substances, it was considered probable that the ring system functions largely in the adsorption of the active molecule to the site of action. This view was based in addition, on the structure of the known growth substances consisting of a nonpolar (lipophilic) skeleton (*L*), bearing a polar (hydrophilic) carboxyl group (*H*). For such a compound, with asymmetric distribution of *H*- and *L*-parts [amphipatic structure (54)] and, moreover, of a low chemical reactivity, some action at a boundary between lipophilic

and hydrophilic phases, e.g., at a protein (enzyme) surface, is considered most plausible. Of course, this consideration is no proof of this type of action, but it provides a logical starting point for a further analysis, in which its exact value has to be determined.

Indications that in growth substance actions an important role is played by adsorption phenomena, may be deduced from the fact that in all immersion tests, where secondary factors are excluded to a larger extent than, for example, in the *Avena* curvature test, the effects are proportional to the logarithm of the concentration, whereas in the *Avena* test activity is directly proportional to the concentration.

Linser (89) paid close attention to this question by analyzing the concentration-activity curves of growth promoting and growth inhibiting compounds and of their mixtures [e.g., indoleacetic acid (IAA)[2] and eosin] in cell elongation in *Avena* coleoptiles. Linser concluded that the eosin curve suggested an adsorption to be governing the inhibitory effect, and that the more complicated form of the IAA curve might result from a dual adsorption process (one promoting, one inhibiting). By mathematical treatment of this material, Kaindl (68) deduced, from a target theory model, concentration-activity curves agreeing satisfactorily with the experimental results.

More recently Hellström (57) treated Åberg's results from experiments with growth substances and antagonists in root growth from a comparable point of view. Here also it proved possible to describe the inhibitory effects as an adsorption process. Furthermore, from a highly interesting analysis of the interaction between growth substances and antagonists, using different adsorption patterns, theoretical curves similar to the experimental ones were deduced.

The boundary between the mercury droplet (rather nonpolar) and the aqueous solution surrounding it in the polarographic technique is an attractive model in studying accumulation of *L/H* compounds at an interface; with respect to the primary active sites in the cell, it must remain, of course, an extremely rough model. Therefore, it is not surprising that the degree of suppression of the oxygen maximum is not, in general, a measure of growth substance activity [Paleg & Muir (113)].

The polarographic technique cannot distinguish faultlessly between physiologically active and inactive compounds, nor does a direct comparison of compounds with different ring systems yield a parallel to the biological test. But in series of structurally related compounds with different growth substance activities, it is found without exception that the substance with the highest growth activity possesses the highest interfacial activity. Evidently this model differs so much from the biological interface that it is useful only under the last mentioned circumstances where the critical factor is, insofar as possible, surface activity.

In a further stage of the investigations on the importance of interfacial activity, Booij & Veldstra (20) looked for a closer model of biological structures. They replaced the polarographic test, to a large extent, with the inter-

action of growth substances with oleate micelles in an oleate coacervate, as a model for protoplasmic membranes.

The results obtained, particularly the finding that the effects on the coacervate completely parallel the action on beet membranes in the beet test (161), led to the idea that in the most active growth substances the surface activity is such that a definite balance between the hydrophilic carboxyl group and the lipophilic ring system (H/L balance) is attained. It was thought that when H is too high the compounds prefer the aqueous phase and, if L is overweighted, other sites compete with the primary active ones resulting in either case, in a suboptimal occupation of the primary active sites and consequently in a lowered activity or in inactivity. When the "high surface activity" of requirement A is limited in this way, though not yet sharply enough, requirement A together with B, in fact, embodies a twofold relation between the ring system and the carboxyl group, viz., a physicochemical and a spatial one.

By comparing the effect on the oleate coacervate of the series of normal fatty acids [Booij & Bungenberg de Jong (19)] with that of growth substances (R—COOH) it is possible to estimate to a certain extent the "aliphatic lipophily equivalent" of the different R-residues. The effect of γ-indole-3-butyric acid, naphthalene-1-acetic acid, and 2,4-dichlorophenoxyacetic acid as well, is found to be equivalent to that of octanoic acid, i.e., the indole-3-propyl-, naphthalene-1-methyl-, and 2,4-dichlorophenoxymethyl-residues corresponding to an aliphatic chain of 7 C atoms [Veldstra (156)]. This means also that the indole ring system is less hydrophobic than that of naphthalene, as was established directly by Booij (17).

That this interesting equivalence of H/L balance in some highly active growth substances must not be interpreted in too absolute a sense is clear from the fact that with the highly active IAA[2] the value for R is intermediate between those of a C_5 and a C_6 chain, which is the same as that found for R in 2,3,6-trichlorobenzoic acid [Veldstra (160)].

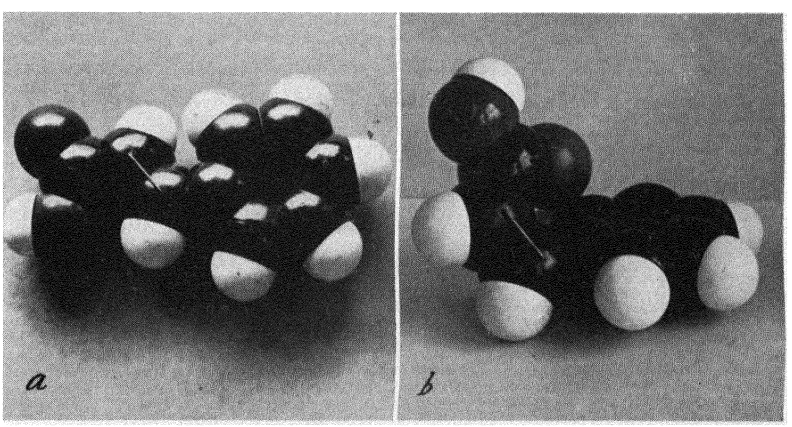
Nevertheless, it appears that if surface activity is measured by the oleate coacervate test and checked with the beet test, a closer analogy to physiological conditions is obtained than heretofore and leads to a better characterization of the growth substances with respect to the role of the ring system. Thus, of the compounds so far investigated in the oleate coacervate, the residue, R, of the active ones has a lipophily equivalent of C_5-C_8, with a maximum at C_7.

Consistent with the view that the function of the ring system implies a contribution to a reversible fixation of the growth substance at the receptor involved in the growth response, are the facts that the presence of a more hydrophilic ring system (e.g., pyridine) and the introduction of hydrophilic substituents into the lipophilic nucleus result in inactive or weakly active compounds. In both cases one obtains more symmetrically hydrophilic substances, differing fundamentally from the amphipatic structure of the highly active compounds.

To arrive at a final judgement of these questions it is highly desirable that model systems be developed, more and more resembling the biological structure in which growth substance action takes place. For that purpose protein components very probably will have to be introduced (56) and a study of the localization of the action inside the cell may provide important information.

Instructive results were obtained recently by Brian & Rideal (22) in a study of the effects of 2-methyl-4-chlorophenoxyacetic acid (MCPA)[2] on monolayers of lipoid, protein, lipoprotein, and material from plant tissues. Adsorption of the growth substance to these monolayers is largely dependent on the nature of the "substrate" (e.g., protein or lipoprotein) and on the actual state of its surface (varying with pH).

Requirement *II(B)* was formulated (153) because of the spectacular difference in physiological activity between the stereoisomeric *cis*, and *trans*-cinnamic acids (the former only being active). It appeared by a study of molecular models that, as contrasted with the *trans* acid, the *cis* acid cannot assume a flat form, because rotation of the side chain is hindered by the hydrogen atoms at the *ortho* positions. *Trans*-cinnamic acid will tend to occur in a flat form because of conjugation phenomena (178). The difference in structure in a three-dimensional sense is evident from Figure 1.



a. *trans*-cinnamic acid b. *cis*-cinnamic acid

FIG. 1. Molecular Models

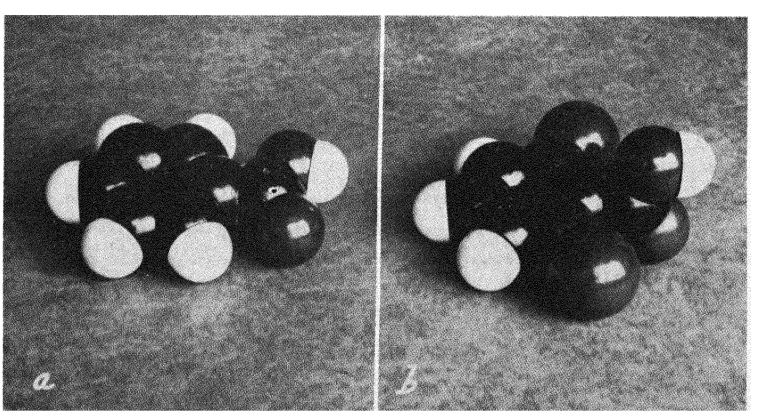
The *cis* acid, possessing a somewhat weaker activity in the oleate coacervate than the *trans* isomer, and being the stronger acid of the two as to these properities, would not be expected to excel the *trans* form as a growth substance. In the author's opinion, one had to conclude from these facts that the particular spatial structure of *cis*-cinnamic acid is the decisive factor in its physiological activity. With other pairs of *cis-trans* acids, viz., tetralideneacetic acid and naphthalene-1-acrylic acid, the same relations were found:

exclusively the *cis* form is physiologically active (153, 157). Havinga & Nivard (55) provided independent evidence for a nonflat form of the *cis* acids by means of ultraviolet absorption spectra, demonstrating that with the *cis* forms hindrance of conjugation occurs, viz., that the carboxyl group (or its dipole) is out of the plane of the ring system.

Thimann (138, p. 30) criticized the reasoning as applied to the tetralideneacetic acids. In his opinion, because the valencies at the exo-cyclic double bond are co-planar, the carboxyl group would be expected to lie in the plane of the ring in both compounds. Apart from the fact that the spectra reveal that in the *cis* acid the carboxyl group is not co-planar with the benzene nucleus, this objection is based on a misunderstanding. The plane of the ethylene grouping which coincides with that of the benzene ring in the *trans* acid was considered to be at an angle with it in the *cis* form.

The three-dimensional amphipatic structure deemed essential for a growth substance, was put to the test by using it as a guide for the synthesis of active compounds. This led to establishing activity for 1,2,3,4-tetrahydro-1-naphthoic acid (157, 163) and *cis*-2-phenylcyclopropane-1-carboxylic acid (158, 164).

The activity of 2,3,6-trichlorobenzoic acid [Bentley (12)], which lacks at first sight the peculiar spatial relation between carboxyl group and ring system as indicated above, could be explained along similar lines. It was proved by means of ultraviolet spectra that di-*ortho* substitution causes a nonflat form of the molecule, the carboxyl group being rotated in such a way that its plane is more or less perpendicular to that of the nucleus (160). The same factor operating in 2,6-dichlorobenzoic acid proved this acid to be active also (Fig. 2).



a. Benzoic acid b. 2,6-Dichlorobenzoic acid

FIG. 2. Molecular Models

The same very probably holds true for 1-naphthoic acid to a certain extent, resulting in a weak activity of this compound, which can be enhanced

by chlorine substitution in the 2-position (160). Thus, in our opinion, the spatial relationship between the carboxyl group and ring system, as defined in requirement *II(B)* is fundamentally the same in all the different types of growth substances known, although realized in different ways. This requirement, having been met without exception until now by the active compounds, thus seems to define an essential feature in a usable form.

Thimann (138) has doubted its value because, in his opinion, a compound like *cis*-cinnamic acid, in which the peculiar spatial form is fixed, should be more active than IAA[2] for example, in which it is only one of many positions attained by free rotation. In our opinion, this conclusion is not justified, however, because one may assume that in the interaction of the receptor and IAA (generally a compound with a nonfixed side chain which can be present in the ideal form) the frequency of the ideal form will be high; in fact, the carboxyl group will be "attracted" to its counterpart in the biological structure, resulting in a limitation of free rotation in the adsorbed state as compared with that of the free molecule in the solution. One would be able to judge the importance of this factor only by comparing IAA with an indole-derivative with a fixed side chain. In a comparison with *cis*-cinnamic acid which has a lower adsorption affinity than IAA, variation of two properties simultaneously cannot result in an evaluation of the exact value of one of them separately. It is possible, moreover, that a previous fixation of the side chain in the desired position in itself does not lead to highly enhanced activity when compared to a close analogue with a free side chain and, that in the latter, the absence of hindrance to attain the exact (active three-dimensional amphipatic) structure is decisive for activity.

Such a hindrance is present in the *trans* forms of cinnamic acid, tetralideneacetic acid, and 2-phenylcyclopropane-1-carboxylic acid, where the side chain is fixed more or less in the "wrong" position (by conjugation, by structural influences, or both). Probably it is present also in one of the optically active forms of certain α-substituted propionic acids and in isobutyric acid derivatives, caused by the α-substitution and by one of the α-methyl groups, respectively.

Questions in connection with the formulation of requirement *II(B)* arise if it is considered why compounds, in which (as far as can be judged) no such hindrances occur, and which, still meeting requirement *I(A)*, possess a much lower activity than isomers to which they are equivalent with respect to *I(A)* and *II(B)*, e.g., naphthoxy-1-acetic acid and naphthalene-2-acetic acid as compared to their -2-and- 1- isomers. Such effects of the position of the side chain are as yet incompletely understood. By determining concentration-surface tension curves for the isomeric naphthaleneacetic acids, indications were obtained that in solution the physiologically more active naphthalene-1-acetic acid is more three-dimensionally asymmetric than the -2- isomer [Veldstra (156, 157)].

Again, one has to consider whether such a difference in solution would not disappear to a large extent in the adsorbed state if the possibility of

fitting on the receptor were the same for both acids. It has to be presumed possible that different relative dimensions of the isomers (and different relative "positions" of binding forces therein) when present in the required spatial forms, cause a different degree of matching of the primary active sites and consequently a different receptor-growth substance dissociation constant. It is interesting that, of the three acids, phenylacetic, phenylpropionic, and phenylbutyric acid, phenylpropionic is the strongest inhibitor for α-chymotrypsin, possessing an enzyme-inhibitor dissociation constant much lower than that of phenylacetic and phenylbutyric acid [Neurath & Gladner (104)]. As to the total adsorption affinity, differences caused in this way are not made sufficiently clear by the oleate coacervate because it lacks the specificity of the biological structure.

It is here that we see the shortcomings of both requirements *I(A)* and *II(B)* in the present form, though we consider them to have included the features essential for an active compound in such a way that they possess a usable selective character. They may take the desired and still more limiting form if, by means of a model system approximating biological conditions to a greater extent, "surface activity" comes to stand more and more for the specific adsorption affinity for the sites involved in the growth response. A comparative study of positional isomers may contribute to a judgement of the possible importance of dimensional factors in more detail.

For this purpose the investigations by Klotz *et al.* (71, 72), and Teresi & Luck (91, 134, 135) on the interaction of organic anions with proteins may be of importance because methods are used to obtain information about the topography of the protein surface and protein sites involved in the combination with a consideration of the different types of binding forces (electrostatic, van der Waals', hydrogen bond).

Several investigators have formulated requirements for activity most pertinent to a special group of growth substances. Accordingly they will be discussed with the respective types of compounds.

The ring system.—Although the most active compounds possess an indole, naphthalene, or substituted benzene nucleus, quite different ring systems (mostly containing a benzene nucleus fused to one of another type) provide active compounds when bearing the appropriate side chain [Norman & Weintraub (106].) The material as a whole is still too heterogeneous to permit a systematic comparison, but in some cases one can get an idea of the relative importance of structural details.

When comparing naphthalene-1-, indole-3-, indene-3-, and coumarone-3-acetic acid (I–IV), for example, it is interesting to observe the effects of naphthalene, indole, etc., in the oleate coacervate. Booij (17) established that the condensing effect of naphthalene is the strongest, that of indole the weakest. The values for indene and coumarone are intermediate and practically identical. Although usually the condensing action of a ring system cannot be compared directly with the opening effect of the growth substances obtained by introduction of the acetic acid side chain (the localization

in the micelles may be different), here one may conclude that with respect to the lipophilic character there is the series: naphthalene > indene = coumarone > indole, which does not parallel the relative physiological activities. Indene- and coumarone-acetic acid possess a relative activity of 20 and 15, respectively, in relation to IAA[2] (= 100, pea test).

Apparently, the —NH— group differs from the —O— and —CH_2— groups in some way essential for activity (hydrogen bond donor?). It is interesting that the growth substance activity of indene- and coumarone-acetic acid (with a similar lipophily of the ring system) is of the same order.

Pyridine has a swelling effect on the coacervate, viz., an action fundamentally different from that of naphthalene, indole, etc., to which very probably the inactivity of pyridyl-3-acetic acid will be related. A study of the influence of lipophilic substituents in the pyridine nucleus would be significant. It is also in this way that information about the importance of structural details in the ring can be obtained.

The area of the ring system of the naphthalene or indole nucleus seems to be most favorable. This is equalled to some extent by certain substituted benzene nuclei, e.g., 2,4-dichlorophenol. Reduction or enlargement of this size, generally results in decreasing activity. This may be explained by a deviation from the proper H/L balance and may be connected with dimensional factors pertaining to the site of action.

Until now no active compound has been found lacking a double bond in the ring system; when only one is present (as in cyclohexene) a position of the side chain next to the double bond is required for activity (176). Erdtman & Nilsson (36, 66a) pointed to the possible equivalence of this unsaturation to the lone pair of electrons on the oxygen atom in the phenoxyacetic acid derivatives, also adjacent to the acetic acid residue. In the sulphur analogues of 2-methyl-4-chloro- and 2,4-dichlorophenoxyacetic acid (two lone pairs of electrons on the S), the activity of which is already lower than that of the phenoxyacetic acids, oxidation to the corresponding sulphoxides (one lone pair) and sulphones (no lone pair) results in inactivation (181). Thus, no unambiguous conclusion is possible and more material is required for comparison before the meaning of this unsaturation adjacent to —CH_2COOH can be exactly analyzed.

For the same reason, it is not possible to include auxin *a* and *b* in the discussion because too few ring-substituted cyclopentene derivatives are available [1-cyclopentene-1- and 2-cyclopentene-1-acetic acid both are inactive in the pea test (138, cf. 46)], and no proper evaluation of the different structural elements is possible. On the other hand, increased knowledge about the topography of the sites decisive for growth, very probably will be indispensable for a satisfactory answer to these questions. Only in that way can differences in affinity of aromatic, hydro-aromatic, and aliphatic parts of the compound for those sites be understood.

The polar group in the side chain.—The carboxyl group is unsurpassed for activity as an acidic group in the side chain. Replacement of COOH by

SO_3H in naphthalene-1-acetic acid results in an inactive compound [pea test, up to 10^{-3} *M* (153)]. Indole-3-methanesulphonic acid (VII) was reported to be inactive in the *Avena* curvature test [Erdtman & Pettersson (37)] and to be antagonistic to IAA[2] [Wieland, Fischer, & Moewus (179)]. These latter results could not be corroborated by Veldstra *et al.* (166); on the contrary, in the cress root test indications of some activity as a growth substance were obtained. For this reason compound VII was assayed in the pea test and found to be active [activity at 5×10^{-4} *M* corresponding to that of NAA[2] at 10^{-5} *M* (166)].

Indolylsulphuric acid (VIII) was earlier reported active (153). Its naphthalene-1- analogue (VI) also was assayed (166). This naphthalene derivative was also found to be inactive in concentrations up to 10^{-3} *M*. Such a difference between compounds with SO_3H in the side chain of a naphthalene and an indole ring system is rather incomprehensible. In view of the low phytotoxicity in the beet test, the naphthalene derivatives were assayed at higher concentrations than 10^{-3} *M*. Over the range of 10^{-3} to 10^{-2} *M*, V and VI were found to be clearly active from 5×10^{-3} *M* upwards, V being the least active of the two (166). Evidently, the limit of 10^{-3} *M* used formerly (practically all of the types of growth substances being toxic for the pea tissue beyond that limit or possessing too low a solubility) was too low. It may be that such a high concentration is required in the medium so that the critical concentration for activity is reached inside the cell; but the compounds will have to be investigated more closely in relation to their carboxyl analogues before anything definite can be said.

At any rate, in the author's opinion, physiological activity of a sulphonic acid, in many physico-chemical respects equivalent to the corresponding carboxylic acid (among others, its action in the oleate coacervate), may be considered as an argument for a more physico-chemical type of growth substance action. Moreover, it is important to note that such sulphonic acids, whose pK values are very low, will be completely dissociated at physiological pH-values, which means that the action is exerted by the anions!

Naphthalene-1-nitromethane (aci-form) is active (153) and its growth substance character has been confirmed recently in the root test by Åberg (5), and also by its interaction with a growth substance antagonist. The activity of non-acidic compounds is discussed separately in a later section.

The side chain.—Since the high activity of 2,3,6-trichlorobenzoic acid has become known, and since 2,6-dichlorobenzoic acid, 1-naphthoic acid, and its 2-chloro-derivative are active compounds, it is clear that a side chain in the form originally considered is no longer a "conditio sine qua non." Instead, compounds can be active if the carboxyl group is bound either directly to the ring system or by means of a shorter or longer chain of a varying character. The latter must enable the carboxyl group to attain the physiologically active position and not cause a significant shift in reactivity and solubility (distribution) properties from that of a simple, normal carbon chain (with the corresponding number of atoms) as a standard. For this

reason, hydrophilic substituents (other than the carboxyl group) have to be avoided (except in the auxins *a* and *b*).

The length of the aliphatic chain influences the activity in a different way, dependent on the ring system and the test methods. Of phenylacetic acid and its homologues, only the former is active in the pea test. In the indoleacetic acid series, Thimann & Bonner (140) observed an oscillation in the *Avena* curvature test, the acids with an odd number of C atoms in the side chain (e.g., propionic acid) being less active than those with an even number. In the pea test the differences are less pronounced (74). Grace (49, 50) noted a comparable oscillation for the naphthalene- and indoleacetic acid series in the rooting of cuttings. With 2,4-dichlorophenoxyacetic acid, naphthoxy-2-acetic acid, and a number of their homologues the differences were even more pronounced in their effects on growing tomato plants [Synerholm & Zimmerman (132)]; the acetic and butyric acids were active, the propionic acids inactive. As an explanation, the latter authors suggested that the higher homologues would not be active per se but, being subject to a β-oxidation, would produce the active 2,4-D only when possessing an even number of C atoms in the side chain.[3] As long as we are not informed exactly about the framework of the site of action, a periodicity of groups with which the carboxyl group interacts might be considered possible too.

In acetic acid derivatives, introduction of a methyl group in the α-position is relatively harmless (for different activities of the antipodes from these racemates see a later section), while a phenyl group in that position often annihilates the activity, as generally is done by di-α-substitution. In our opinion, it is most probable that a decrease of activity or inactivation is caused in these substances by interference of the substituent (too large or in the wrong spatial position) with the realization of the configuration required for activity.

Substituted indole-3-acetic acids.—In the first publication on the structural specificity of IAA,[2] Kögl & Kostermans (76) established that alkyl substitution in the pyrrole nucleus strongly reduces the activity in the *Avena* curvature test or causes it to disappear, whereas the physiological activity is less sensitive to substitution in the benzene ring. Thereafter, substitution effects were studied by Koepfli *et al.* (74) with β-indole-3-propionic acid and γ-indole-3-butyric acid. It was found that the high activity of these acids in the pea test was reduced to zero in their 2-carboxy-derivatives and in the 5-, 6-, and 7-methoxy-derivatives of the propionic acid. A comparable introduction of methoxy groups in IAA has no such inactivating influence; 5-, 6-, and 7-methoxy-IAA [synthesis (39)] are quite as active as the parent acid [Findlay & Dougherty (40)].

Chlorine substitution was studied by Stevens & Fox (128, 129) with 2-methyl-IAA (pea test), and more extensively (halogen and alkyl-substitu-

[3] Quite recently, Fawcett, Ingram & Wain (*Nature*, **170**, 887, 1952) found evidence for β-oxidation in the series of ω-phenoxyalkylcarboxylic acids in the flax plant.

tion) by Hoffmann, Fox, & Bullock (61) in tests on tomato plants and in the pea test with IAA. When referring to the results obtained with the latter test, it is clear again that substitution in the pyrrole ring diminishes the activity markedly. The action decreases only slightly or is even enhanced with substitution in the benzene ring, as with 4-, 5-, and 6-chloro- or 5-fluoro-IAA[2] (61). (For synthesis of the compounds see 25, 41, 129.) Introduction of a substituent in the 7 position of IAA or of IAA already substituted somewhere else, generally causes a notably lower activity and sometimes (2-methyl-7-chloro-IAA, 2-methyl-7-nitro-IAA) inactivity.

The deduction that both the 2 and the 7 positions, i.e., those neighboring the —NH— group preferably should be free, recalls the indications obtained in comparing IAA and indene- and coumarone-acetic acids, that the —NH— group possibly has some function in bond-formation (cf., the very low activity of 1-methyl-IAA). A 2-substituent, especially when larger than a methyl group, might also interfere with the position required for the side chain.

Hoffmann *et al.*, suggest that the effect of benzenoid chlorine substituents has to be attributed to an inhibition of the catabolism of the respective compound. This possibility should be investigated with the aid of enzyme systems known to inactivate IAA (133). The favorable effect of chlorine substitution in the 4-, 5-, or 6-positions also might be connected with an enhanced lipophilic character, which possibly is not yet maximal in IAA (cf., the stronger lipophilic character of the highly active indolebutyric acid, whereas such a lengthening of the side chain in the naphthalene series causes a decreased activity). The complete inactivity of all 2-carboxy derivatives can be viewed in the same light and an investigation of the compounds in the oleate coacervate might give information on this point.

Phenylacetic acid, cinnamic acid, and related compounds.—The activity of phenylacetic acid (PAA)[2] in the pea test, practically uninfluenced by introduction of one methyl group in the —CH_2—, or by replacement of both hydrogen atoms by a methylene group, is lost by substitution with two methyl groups (74). The view, already expressed by Thimann (138), that this effect very probably is caused by steric factors, is supported by results obtained with DL α-*n*-propyl- (IX), α-isopropyl- (X), and α-allyl-PAA (XI) [Veldstra & van de Westeringh (163)]. IX and XI are more active than the parent acid and X, with the branched chain, is almost inactive. As the activity of DL allyl-PAA[2] is practically wholly due to the (+) form, the other racemates will have to be resolved too, before an exact comparison can be made. With the introduction of an alkyl-substituent in the fairly water-soluble PAA, the lipophily of the molecule increases, which in itself may be responsible for the enhanced activity of the *n*-propyl and allyl-derivatives. The fact that the effects in the oleate coacervate [*n*-propyl > isopropyl > allyl, Booij & Veldstra (21)] do not parallel the physiological activities proves that other factors are dominant, however.

Because 4-nitro- and 2,4-dinitrophenylacetic acid are inactive (139), it

has been concluded that the introduction of a nitro group abolishes activity (106) or that this substituent is highly deactivating to the benzene ring (139), resulting in lowered binding capacity. Neither of these generalizations appears justified because 2-nitro-PAA proved to be as active as PAA and the 3-nitro isomer even more active [Veldstra *et al.* (166)]. Thus, NO_2 is inactivating only in the 4-position here.

That this effect is more connected with the character of the substituent than with its 4-position follows from the fact that 4-bromo-PAA is more active than PAA in concentrations up to 10^{-4} *M* (166). This difference between NO_2 and Br is also apparent in the oleate coacervate (21). 4-Br-PAA is much more active than PAA, as is observed generally with lipophilic substituents in that position, whereas the action of the 4-nitro-derivative is similar to that of PAA. Apparently the NO_2 group behaves here as a relatively hydrophilic substituent, especially in the 4-position [cf. Booij (18)].

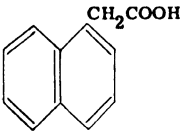
These interesting relations of the nitro-phenylacetic acids are not yet fully understood. Evidently, as with derivatives of phenoxyacetic acid and benzoic acid, NO_2 in the 3-position is compatible with activity, while the derivatives with 4-NO_2 are inactive without exception. The effect of 2-NO_2 varies. The differences between 3, and 2-, 4-positions are comprehensible for phenoxyacetic and benzoic acids because of electromeric effects. With PAA, other factors must play a role.

Nivard (105) investigated some substituted *cis*-cinnamic acids. In the pea test 4-methyl-*cis*-cinnamic acid has about the same activity as the parent acid; the introduction of chlorine in the 2- and 4- positions enhances the activity. The activity of both types of substances approaches that of NAA[2] (at 4×10^{-5} *M*). At higher concentrations, the 4-chloro-derivative is more toxic than its 2-isomer.

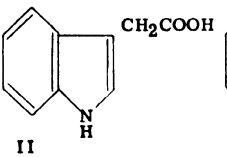
Both 2- and 4-nitro-*cis*-cinnamic acid are inactive in the same range of concentrations. It might be that NO_2-substitution in 2- and 4-positions enhances the degree of resonance between the carboxyl group and the benzene nucleus (which is low in the *cis* acids because of steric hindrance as compared to their *trans*-isomers). The substitution may thus affect the spatial structure of the *cis* forms. However, this cannot fully be judged at the moment. Further analysis should include a study of 3-nitro-*cis*-cinnamic acid which might be active.

Trans- and *cis*-1-phenylcyclopropane-2-carboxylic acid (XII, XIII) were investigated because, in a spatial sense, they are analogous to *trans*- and *cis*-cinnamic acid. Just as with the latter, only the *cis* form proved to be active [Veldstra & van de Westeringh (164)]. Another analogue of *cis*-cinnamic acid (XIV), 2-phenyl-cyclohexene-1-carboxylic acid (XV), where the "hunch" is enlarged, is practically inactive [Veer (152)], possibly because of steric hindrance. This might be investigated further with the aid of α- and β, mono- and dialkyl-*cis*-cinnamic acids.

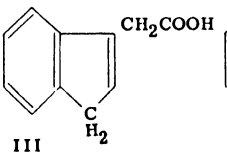
Naphthalene and phenanthrene derivatives and related compounds.— Experiments have been made to determine whether introduction of a second



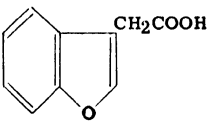
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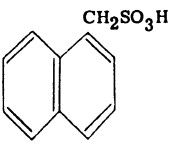
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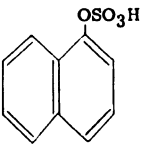
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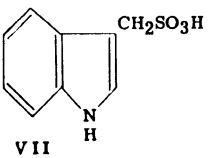
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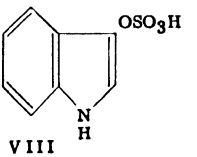
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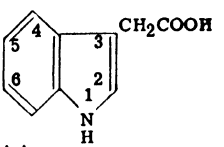
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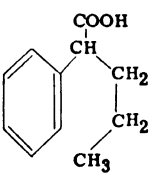
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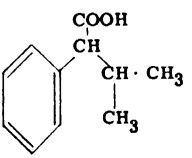
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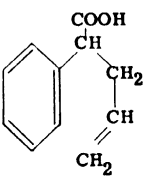
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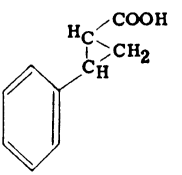
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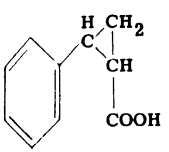
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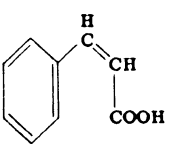
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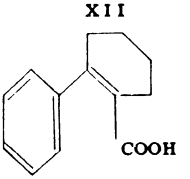
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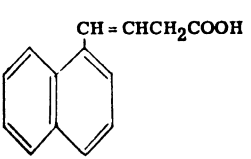
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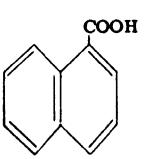
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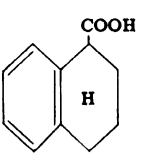
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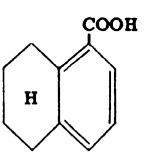
XVI



XVII



XVIII



XIX

carboxyl group in the side chain of certain naphthalenepropionic and -butyric acids, the low activity or inactivity of which might be connected with too lipophilic a character, would result in more active compounds (in the pea test). No such effects were observed with (naphthalene-1-methyl) malonic acid, (naphthalene-1-methyl) bromomalonic acid, and α-(naphthalene-2-methyl) succinic acid, because all of them were inactive [Veldstra *et al.* (166)].

Enlarging the ring system beyond the scope of that of naphthalene mostly reduces the activity, as was confirmed in the case of the isomeric phenanthrene-2-, -3- and -9- acetic acids. At 10^{-4} *M*, the former is inactive, its -3- isomer is very slightly active (both are derivatives of naphthalene-2-acetic acid) and the -9- isomer (to be considered as derived from naphthalene-1-acetic acid) somewhat more active (corresponding to 10^{-6} *M* NAA[2]). At higher concentrations toxicity readily interferes. Of the other tricyclic ring systems tested, fluorene-9-acetic acid is inactive and the corresponding carbazole-9-acetic acid is very weakly active (at 10^{-4} *M* corresponding to 4×10^{-6} *M* NAA) (166).

Introduction of a double bond in the side chain of the weakly active γ-naphthalene-1-butyric acid in conjugation with the nucleus, leads to γ-naphthalene-1-isocrotonic acid (XVI), in which the free rotation of the side chain is restricted and the activity abolished [Mentzer (95)].

1-Naphthoic acid (XVII) was found to be active [Veldstra (157)]. It is discussed with the substituted benzoic acids. Its 1,2,3,4-tetrahydro-derivative (XVIII) was investigated because of its three-dimensional amphipatic structure, considered to be essential for activity. High activity came up to expectations in this respect [(157); Veldstra & van de Westeringh (163)]. Hydrogenation in the 5,6,7,8-positions (XIX), which does not affect the spatial position of the carboxyl group, does not enhance the activity.[4]

Phenoxy and naphthoxy compounds.—The practical importance of 2,4-D[2] has catalyzed the synthesis of a large number of related compounds [Thompson *et al.* (148), Weintraub *et al.* (171)], especially of phenoxyacetic acid (POAA[2]) derivatives, with substituents in the nucleus, in the side chain or in both. This fact has made possible comparative investigations using several tests. Thus, structural details essential for activity could be recognized.

Muir, Hansch & Gallup (100) investigated systematically the effects of nuclear substitution in POAA on cell elongation (straight growth test). Whereas 2-, 3- and 4-chloro or bromo-POAA proved to be more active than POAA (the effect being most pronounced in the 3- and 4-positions), trihalogen substitution involving simultaneous occupation of 2- and 6-positions invariably resulted in inactive compounds [established earlier by Zimmerman

[4] From reprints kindly sent to us by the authors, we learned that T. Mitsui and A. Tamura (*J. Agr. Chem. Soc. Japan*, **25**, 17, 1951), also had investigated hydrogenated 1-naphthoic acids. The 1,4-dihydro- and 1,2,3,4-tetrahydro-derivatives were found to be strongly active.

et al. (131, 184) in other tests; also Linser (86, 87). The authors explained this lack of activity by assuming that in a "two point reaction" of the growth substance with a substrate the carboxyl group and a position *ortho* to the attachment of the side chain are involved and therefore at least one has to be free. The very low activity of 2,4,6-trimethyl-phenylacetic acid was considered to substantiate this interpretation. 2,4,6-Trimethyl-POAA, however, possesses the same activity as POAA, and the assumption had to be made that the reaction with the substrate may take place through a methyl group or by removal of the methyl group. In the writer's opinion this seems to be rather improbable.

Subsequently, Hansch & Muir (52), analyzing different possibilities by which substitution in the 2-position might prevent the growth substance—substrate reaction (steric hindrance of a reaction in which the side chain plays a role or masking of the first atom thereof so that a reaction involving this atom is prevented), concluded again that growth substances are involved in a chemical reaction with a plant substrate through one of the *ortho* positions. The inactivity of some other 2,6-substituted POAA's and of 2-methyl-4,7-dichloro-IAA are considered to give further support to this view. Since 2-methyl-7-chloro-IAA is inactive (see substituted IAA's) the latter example is of no value in the present discussion, for which 2,4-disubstituted IAA's would be required. Further, a 2-methyl-indolyl-3- derivative is already di-*ortho* substituted with respect to the side chain, and the 4-position here is not a direct equivalent of the 2- or 6- position in POAA.[2]

That a true chemical reaction forms the basis of growth substance action is very improbable because an interaction causing a specific physiological response requires many weak bonds [a few strong ones not conferring specificity (154)]. In the author's opinion, the arguments supporting the chemical reaction concept are rather poor. Thimann (139) also has pointed this out in his discussion of the activities of different mono- and di-substituted POAA's. 2,6-Dichloro-POAA was found to be weakly active (pea test). The discussion of this *ortho* effect (chemical reaction hypothesis) will be concluded when the action of benzoic acid derivatives is analyzed.

Leaper & Bishop (84) compared the effect of all mono-, di-, and trichloro-POAA's on tomato plants and as inhibitors of the growth of *Lupinus albus* seedlings. Their results can be included in our discussion since they parallel those of Muir *et al.* (52, 100), Thimann (139), who obtained comparable activities for a number of the compounds, and Wain (170), who found about the same results when assessing all of them in the straight growth and pea tests. Leaper & Bishop summarized their results in a table (Table I) in which the most efficient compound is rated 100. It is clear that the activity, maximal for the 2,5-derivative and very high for the 2,4-derivative too, is less for both the mono-substituted and the tri-substituted POAA's. This, on the whole, may be connected again with a most favorable H/L balance for the di-substituted POAA's [as indicated by the effects of a number of them in the oleate coacervate (161)]. However, no explanation can be found along

these lines for the rather specific position effects and other factors must be dominant.

TABLE I

RELATIVE "HORMONE" EFFICIENCY OF CHLOROPHENOXYACETIC ACIDS IN TOMATO TEST (84)

Position of chlorine groups in nucleus	In cell elongation (epinasty, etc.)	In rooting on intact stems
2	2.0	10.0
3	10.0	12.5
4	10.0	25.0
2,3	1.0	5.0
2,4	66.6	77.5
2,5	100.0	100.0
2,6	0	0
3,4	20.0	10.0
3,5	0	0
2,3,4	1.0	0
2,3,5	0	0
2,3,6	0	0
2,4,5	40.0	250.0
2,4,6	0	0
3,4,5	0	0

The authors point to the fact that all active compounds (i.e., the highest active, XX–XXIII) possess free 2-6- and 5-3-positions (positions *para* to each other), and logically this is considered to be a requirement for activity in this series. As to its meaning, they speculate that a possibility exists for an oxidation to a quinonoid structure which might take part in an oxidation-reduction system required for growth. Further degradative oxidation is considered to connect the phytocidal properties of the phenoxy acids with that of maleic acid derivatives. The high importance of detailed investigations of growth substance metabolism in plant cells must be admitted, and such speculations may stimulate it. Nevertheless, at the moment they can hardly contribute to an analysis of structure-activity relationships pertaining to growth promoting activity. This is true because of the fact that for most other types of growth substances no such reactions can be envisaged, and because there are no coercive reasons to assume that the growth-promoting action of 2,4-D[2], etc., proceeds along lines fundamentally different from those of the other ones.

A pair of free 2- and 5-positions is a condition for activity in this group although it is not possible to define exactly the meaning of this observation. In the author's opinion, Thimann's explanation (139) is descriptive only. When trying to localize foci in a substituted benzene nucleus, partaking in (weak) bond formation during interaction with the sites involved in growth,

comparative investigations by physical methods are indispensable. Moreover, as long as no more is known about the pattern of these sites (as a matter of fact, working with only half of the acting system—the growth substances), a complete answer to these questions cannot be anticipated. Free positions may take part in bond formation, but they may possibly have to remain free for steric reasons, bonding occurring elsewhere in the nucleus.

Of the three mono-nitro-phenoxyacetic acids, only the 3-nitro derivative is active in the pea test [Veldstra (156, 157)]. Mesomerism, occurring exclusively in the 2- and 4-derivatives, will restrict free rotation of the side chain because the O—CH_2 bond will tend to lie in the plane of the benzene nucleus. By use of molecular models it was found that with the O—CH_2 bond in such a position the most probable conformation of the molecule would be a rather flat one. The frequency of the special spatial (nonflat) form, considered essential for activity, would then be higher in the (active) 3-nitro-POAA,[2] in which rotation of the side chain is totally free. But other factors (cf., the nitro-phenylacetic acids) may be of importance as well.

As to substitution in the side chain, Osborne & Wain (109 to 112), investigating a series of aryloxyacetic acids in different tests (including straight growth and pea test), found that substitution of an alkyl group in the —OCH_2COOH side chain (e.g., aryl-$OCH(CH_3)COOH$) had little effect upon activity generally, However, α,α-di-substituted compounds (aryl-$OC(CH_3)_2COOH$) were inactive in straight growth, but some of them were slightly active in the pea test. Because of these facts it was considered possible that a chemical reaction involving a hydrogen atom on the carbon adjacent to the carboxyl group operates in the growth response (cellelongation), so that at least one such hydrogen atom would be required for activity. Wain (169) discussed the activity of naphthalene-1-nitromethane and of 1,2,3,4-tetrahydronaphthoic acid also, from the point of view of this α-hydrogen hypothesis.

An α-hydrogen atom taking part in a chemical reaction generally cannot be a prerequisite for activity because α-methylene-phenylacetic acid is quite as active as the parent acid and 2,3,6-trichlorobenzoic acid is highly active. All of these lack the α-hydrogen.

The objections raised to the hypothesis of Muir *et al.*, apply equally well to the α-hydrogen concept. In addition, it may well be that α,α-di-substitution does not imply the absence of a reactive atom, but the presence of a steric hindrance, preventing the molecule from fitting the receptor in the optimal active way. In our opinion, the fact that certain aryloxy-isobutyric acids are slightly active in the pea test (109) also points in this direction (cf. later sections regarding a comparison of isobutyric acids and one of the optically active forms of propionic acids).

Substituted benzoic acids.—Zimmerman & Hitchcock (185) introduced substituted benzoic acids into growth substance research when reporting mild activity for cell elongation with 2-bromo-3-nitrobenzoic acid [also very

weakly active in the pea test (161)] and formative effects with 2-chloro-5-nitro- and 2,3,5-triiodobenzoic acid; thereafter attention was mainly paid to the formative effects. Compounds of this type gained renewed interest as growth promotors when Bentley (12) found 2,3,6-trichlorobenzoic acid (XXIX) to be highly active in straight growth, an observation soon corroborated in other tests [pea test: Thimann (139); Veldstra (160); tomato plants: Zimmerman & Hitchcock (188)]. This led to the investigation of several other substituted benzoic acids [Muir & Hansch (99); Veldstra & van de Westeringh (165); Zimmerman, Hitchcock & Prill (189)].

From a survey of the results obtained, it can be concluded that hydrophilic substituents (OH, NH_2) do not confer activity on the resulting benzoic acid derivative while lipophilic ones (Cl, Br, I, CH_3) may do so. The nitro group, having a dual character depending on the position, is effective mainly in the 3-position. The activities indicated schematically below the formulas indicate that *ortho* substitution starts to "activate" benzoic acid (mainly in straight growth; in the pea test the effect is less clear). Activation becomes more pronounced with di-*ortho* substitution (provided the substituent is not more bulky than chlorine or methyl) and is maximal for the 2,3,6-derivatives. Apparently the 4-position has to remain free. These results were considered highly interesting because the very active 2,3,6-trichlorobenzoic acid and similar compounds did not seem to meet the requirements for high activity as formulated by Koepfli *et al.* (74), Muir *et al.* (100), and Veldstra (153).

Muir & Hansch (99) broadened the concept of growth substance action being dependent on a chemical reaction at the *ortho* position of the side chain involving a displacement of hydrogen (phenoxyacetic acid series), to include the benzoic acid derivatives by assuming that in the latter the electronegative *ortho* substituent (e.g., chlorine) takes part in the reaction with an electron-rich substrate. The reasoning was based on an analysis of the electronic characteristics of the active benzoic acids and phenoxyacetic acid.

Hansch, Muir & Metzenberg (53) claimed to have produced further evidence for this view by establishing that the increased elongation of *Avena* coleoptile sections caused by 2,6-dichlorobenzoic acid, is accompanied by the release of chloride ion. According to Veldstra & van de Westeringh (165), the data of Hansch *et al.* (from three experiments) do not permit the conclusion that the release of chloride ion is essentially connected with the physiological activity of the compounds. Apart from the fact that a far larger number of experiments would be required to decide whether the differences observed between the water controls and the solutions are statistically significant, it would have to be proved that such an increase of Cl^- is absent when inactive chlorobenzoic acids are used. Actually, a release of Cl^- is observed, with 2,4-dichlorobenzoic acid (inactive). It would also have to show that the amount of Cl^- released is proportionate to the increase in length of the coleoptile sections.

Furthermore, 2,6-dibromobenzoic acid (XXVI), which should be active on the basis of the *ortho* reaction concept, was found to be inactive (165). On the other hand, with 2,6-dimethylbenzoic acid (XXVII), very slightly active, 3-nitro-2,6-dimethylbenzoic acid (XXXI), active, and 3-chloro-, 3-bromo- and 3-iodo-2,6-dimethylbenzoic acid (XXXII), highly active, a reaction with a plant substrate involving displacement of the methyl group or another form of chemical reaction with the inert *ortho* substituent can be ruled out. Because of these facts the *ortho* reaction hypothesis was considered to be no longer tenable (165).

It also conflicts with a more general consideration. The hypothesis assumes that a physiological response will occur once the molecules of the growth substance have become irreversibly fixed to the receptors [Hansch *et al.* (53)], implying that inactivity = no reaction. If true, then the inactivity of 2,4-dichlorobenzoic acid, which should be capable of the *ortho* reaction [comparing the higher reactivity of chlorine in 2,4-dichloronitrobenzene with that in its 2,6-isomer, quoted by Hansch *et al.* (53)], is quite incomprehensible.

Highly questionable also must be the tacit assumption of the *ortho* reaction hypothesis that the distance between the *ortho* position and the carboxyl (the other reacting group) would be unimportant to the interaction with the substrate. The *ortho* positions in phenoxyacetic acids, benzoic acids, and others are by no means equivalent in this respect. In our opinion, the activity of certain substituted benzoic acids must be connected with a much more specific property of the molecules which is not shared by their inactive isomers.

Such a characteristic for the active acids was found by comparing their ultraviolet absorption spectra with that of the inactive acids [Veldstra (160)]. The spectra indicated that in all active compounds with substituents in the 2- and 6-positions, this di-*ortho* substitution suppressed the conjugation between the carboxyl group and the nucleus. This means that the carboxyl group, tending in benzoic acid to be coplanar with the ring because of the conjugation, is rotated out of the plane of the benzene nucleus (Fig. 2). In this way the active molecules attain a spatial form fundamentally equivalent to that considered essential for activity. Thus, all the different types of growth substances share this peculiar spatial feature (in a more or less fixed form or having the potentiality of assuming it) which, however, is attained by different means. From this point of view, the activity of 1-naphthoic acid may be understood; according to expectation, its activity is enhanced by chlorine substitution in the 2-position [XXXIII (160)].

The inactivity of 2,6-dibromobenzoic acid, in which the same steric factors are operating as in its dichloro-analogue, needs further explanation. Veldstra & van de Westeringh (165) thought it possible that the carboxyl group is masked by the larger bromine atoms and therefore cannot perform its function with respect to the primary active sites. The inactivity of 2,6-dimethoxybenzoic acid (XXVIII) would be caused then, in the same

way. In this connection it is interesting that 2-chloro-6-bromobenzoic acid was found to be very weakly active (<2,6-dichlorobenzoic acid), and that no acylation of 2,6-dibromoanaline takes place (166) under the conditions in which acylation [according to Löfgren (90)] of 2,6-dichloroanaline occurs.

These facts indicate that active, substituted benzoic acids are obtained by introduction of lipophilic substituents not larger than chlorine or methyl in the two *ortho* positions, activity being enhanced by introduction of a third substituent in the 3(5) position. It would be interesting to investigate the maximum size of substituents larger than chlorine that could occupy this position. By the third substituent the molecule becomes lipophilic in character, comparable to that of IAA, while in the much weaker active 2,6-dichlorobenzoic acid the *H/L* balance is shifted to the hydrophilic side. Veldstra (160) considered this to be essential for the enhanced activity of the 2,3,6-substituted acids. Thimann (139) favored the view that in the latter cases the 5-position is maximally "activated," but its importance cannot be judged at the moment.

No explanation can as yet be given for the fact that in benzoic acids a free 4-position is required for activity. 2,4,6-Trichlorobenzoic acid (XXX) would be expected to be active if the factor indicated by Veldstra (160) is dominant. Apparently, however, other unknowns interfere. Very probably, more knowledge about the pattern of the primary active sites in the cell will clear up such details [cf., the antagonistic activity of 3-nitro-4-halogen benzoic acids (98)].

In this survey of the attempts to formulate limiting requirements for activity, we have considered the arguments for our conclusion that the free α-hydrogen and *ortho* reaction concepts are either simply descriptive or are not acceptable because the facts which cannot be explained by them fundamentally impair the hypotheses. As far as we can see such difficulties are not encountered with the concept that activity depends on a dual relation between the ring system and the carboxyl group, as indicated by the *H/L* balance and the three-dimensional amphipatic structure.

Although we cannot synthesize growth substances "tailor made" we have achieved a fair idea of the outlines of the active structure. By using measurable properties as limiting factors (i.e., *H/L* balance) and physical methods [surface tension, ultraviolet spectra, dipole moments (105), etc.] for the analysis of the spatial form of the active molecules, it has been possible to formulate requirements of more than a descriptive character. These have guided the synthesis of active compounds such as 1,2,3,4-tetrahydro-1-naphthoic acid, *cis*-2-phenyl-cyclopropane-1-carboxylic acid, and 2-chloro-1-naphthoic acid. Thus, while all active compounds can more or less be put on a common basis, we do not know, sufficiently, the restrictions which must be observed. Thus, we cannot explain the lack of activity of a number of compounds which, most probably, is connected with the fine structure of the biological interfaces (enzyme surfaces). Therefore, in our opinion, the problem of structure-activity will not be completely solved as long as

our knowledge of the nature of the action is so much less than that of the structure.

Bonner & Bandurski (15) recently have given a clear picture of the present understanding of the action. They emphasized the fact that there is evidence which indicates a functional connection between a metabolically controlled and dependent water uptake, in which adenosinetriphosphate may play a role, and growth substance action. It appears to us to be of the utmost importance for the progress of growth substance research, that the question be studied whether or not this, indeed, constitutes the "primary reaction." Particularly in relation to water uptake, the results of physiological, biochemical, and physico-chemical investigations distinctly show a converging trend [cf., also von Guttenberg *et al.* (167, 168)]; the problem is clearly outlined in this domain and decisive experiments may be designed.

Optical Isomerism and Activity

Interest in the problem of optical isomerism and activity was initiated when Kögl & Verkaaik (75, 77) found (+)α-indole-3-propionic acid (XXXIV) to be 30 times as active in the *Avena* test as the (−) isomer. In the straight growth test the (+) and (−) acids were equally active, however, and it was proved that the differences in the *Avena* curvature test were caused by retention of the (−) acid in the apex by selective adsorption, viz., by differences in basipetal transport. Arguments for the view, originally held, that the (+) acid "fitted" an asymmetric cell constituent essential for the primary activity, while the (−) acid did not, were no longer valid.

Recently, differences in activity between optical antipodes were found in the straight growth and pea tests. Wain (125, 169) reported the (+) form of α-naphthoxy-2-propionic acid to be highly active in six different types of test, including straight growth and the pea test, whereas the (−) forms showed only negligible activity. Veldstra & van de Westeringh (163) resolved the physiologically active racemates of 1,2,3,4-tetrahydro-1-naphthoic acid (XVIII)) and α-allyl-phenylacetic acid (XI) and found the (−) form of the former and the (+) form of the latter highly active in the pea test. The (+) and (−) forms were weakly active and practically inactive, respectively.[5]

Wain *et al.* (126) reported the activity of the racemates of 2,4-dichlorophenoxypropionic acid (XXXV) [cf., also Thimann (138)], and 2,4,5-trichloro-phenoxypropionic acid (XXXVII) for the greater part to be due to the (+) acids (straight growth and pea test). Åberg (3) established differences in growth regulating activities between the enantiomorphs of α-naphthoxy-1-propionic acid (XXXVIII) and of α-naphthoxy-2-propionic acid (XXXIX), using inhibition of flat root growth as a test. With medium concentrations of the first substance the (−) acid is a stronger inhibitor than

[5] See also Mitsui, T., *J. Agr. Chem. Soc. Japan*, **25**, 186 (1951); **25**, 526 (1952): Kato, J., *Mem. Coll. Sci., Univ. Kyoto*, [B]**20**, 33 (1951).

its (+) isomer, whereas, with the second one the difference is far more pronounced, the (+) form being about 3000 times as active as the (−) form. It even behaves as an auxin antagonist.

Similarly, Åberg (5) investigated the (+) and (−) enantiomorphs of α-2,4-dichlorphenoxy- and α-3,4-dichlorophenoxy-propionic acid (XXXV, XXXVI), α-1-chloronaphthoxy-2-propionic acid (XL), α-naphthoxy-2-butyric acid (XLI), α-naphthalene-2-methylpropionic acid (XLII), and α-naphthalene-mercapto-2-propionic acid (XLIII). Without exception the (+) forms showed the strongest growth substance action while the (−) acids of the first two were weakly active and all the others antagonistic to different degrees (cf. Table I).

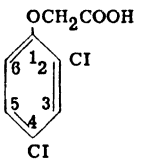
These observed differences in activity can hardly be attributed to translocation effects because the straight growth, pea, and root tests are all immersion tests in which transport problems are essentially eliminated. The obvious conclusion is that, except for indole-3-propionic acid, the most active enantiomorph owes its "selection" to an interaction with some asymmetric cell component which plays an essential role in the growth response to active compounds. Wain *et al.* strongly favor this view. Indeed, starting from their hypothesis that an unsaturated ring system, a carboxyl group and α-hydrogen atom are essential for growth regulating activity, a specific "three-point contact" easily explains the superiority of one of the enantiomorphs of an optically active acid.

Nevertheless, such a comparatively simple explanation is not quite satisfactory because one would expect inactivity of one of the enantiomorphs if a specific three-point contact were generally governing the fitting of the molecules to the primary active site. Actually (−) 1,2,3,4,-tetrahydro-1-naphthoic acid is weakly active in the pea test, whereas with α-indole-3 propionic acid the asymmetry of the molecule does not influence activity in the straight growth text.

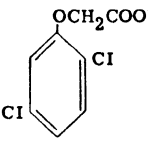
The realization that the most active growth substances, indole-, naphthalene- and 2,4-dichlorophenoxyacetic acid, are not optically active, suggests that an α-substitution (leading to such activity) in certain compounds interferes by steric hindrance with the interaction of the growth substance and its counterpart in the cell, even if, for example, a two-point contact were decisive for activity.

When investigating the (+) and (−) forms of α-2,4-dichlorophenoxy-, α-3,4-dichlorophenoxypropionic acids, and α-naphthoxy-2-*n*-butyric acid[6] in the pea test, the (+) acids were found to be the most active ones (166). Whereas (−) α-naphthoxy-2-*n*-butyric acid is practically inactive, the (−) forms of the chlorinated phenoxypropionic acids are still active, particularly the 2,4-dichloro-derivative, offering a good parallel with Åberg's results. Thus, α-substitution in active acetic acids appears to interfere in one of the resulting enantiomorphic forms more easily if, for other reasons,

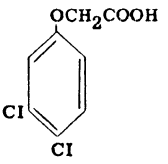
[6] Kindly put at our disposal by Dr. Matell, Uppsala, Sweden.



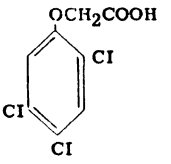
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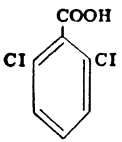
XXI



XXII

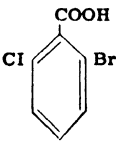


XXIII



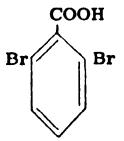
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XXIV



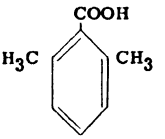
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XXV



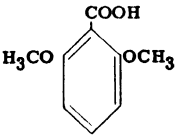
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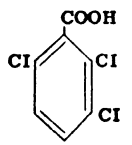
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XXVII



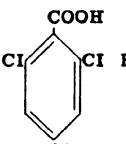
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XXVIII



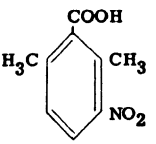
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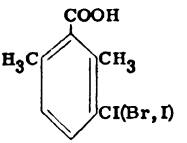
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XXX



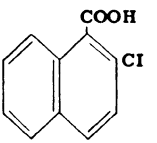
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XXXI



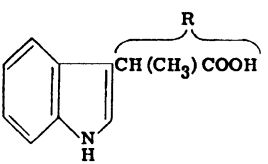
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XXXII

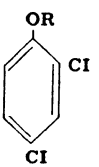


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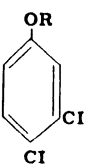
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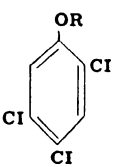
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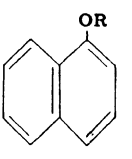
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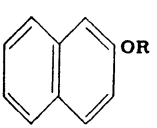
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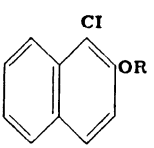
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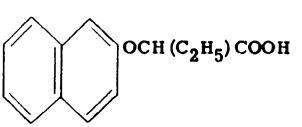
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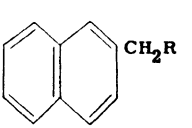
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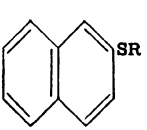
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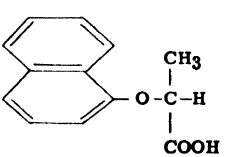
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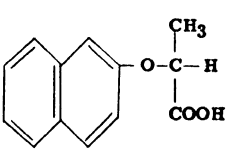
XLII



XLIII



XXXVIIIa



XXXVIIIb

the activity of the parent acid is already lower than that of IAA,[2] NAA, or 2,4-D, or if the α-substituent is enlarged. Di-α-substitution, as in α-isobutyric acid derivatives, would then, in the same way, cause the lower activity or inactivity actually found (109) because the substitution types of (+) and (−) α-propionic acid derivatives are present simultaneously.

It is quite possible that the investigations by Neurath *et al.* concerning structural requirements for specific inhibitors of carboxypeptidase (35) and α-chymotrypsin (104) may contribute to the analysis of specific structural requirements for growth substances. Whereas, D-phenylalanine (α-amino-β-phenylpropionic acid), for example, is strongly inhibitory for carboxypeptidase, the L-isomer is not, but β-phenylpropionic acid (optically inactive) is about 32 times more effective than D-phenylalanine! β-Indole-3 propionic acid is a still more active inhibitor of α-chymotrypsin than β-phenylpropionic acid. Here too, apparently, α-substitution in only one of the resulting enantiomorphs interferes with inhibitory activity.

The study of the influence of optical specificity on growth substance activity implies a refinement in the analysis of structure-activity relationships. In our opinion, the results obtained so far stress the eminent importance of the spatial form of the active molecule, as discussed in the section on structure and activity, rather than give information about the type of contact between the growth substance and its receptor.

As was observed in the case of 1,2,3,4-tetrahydro-1-naphthoic acid, α-allyl-phenylacetic acid, and α-naphthoxy-1- and α-naphthoxy-2-propionic acids, the most active enantiomorphs of racemates related in a certain sense, may have opposite signs of rotation. If the growth response depends on the degree of fitting of the active molecule on the primary active site, one would expect the most active antipodes to belong to the same steric series, irrespective of their sign of rotation. Veldstra & van de Westeringh (163) obtained evidence of this with (−) 1,2,3,4-tetrahydro-1-naphthoic acid and (+) α-allyl-phenylacetic acid from the solubility properties of their salts with (−) menthylamine.

Far more conclusive evidence was presented by Fredga & Matell (43) with (−) α-naphthoxy-1- and (+) α-naphthoxy-2-propionic acid. The same configuration could be ascribed to the acids with different signs of rotation because the acids with the same direction of rotation furnish a quasi-racemic compound and also by synthesizing the (−) and (+) acids from sodium naphtholate and (−) α-bromopropionic acid.

Taking into account the configuration of (−) α-bromopropionic acid (45) and the inversion of the configuration during the reaction between naphtholate ion and bromopropionate ion, the acids very probably have to be represented by XXXVIII *a* and XXXVIII *b*.

In the same elegant way it has been shown that (+) α-phenoxypropionic acid is sterically related to (+) α-naphthoxy-2-propionic acid. The most active forms of phenoxy-, naphthoxy-1- and naphthoxy-2-propionic acids thus very probably can be considered as derivatives of *d*(−) lactic acid (44).

Meanwhile, Matell (93) has extended this method of establishing steric relations to the enantiomorphic acids investigated by Åberg (5) in the flax root test. The configuration of the acids with the highest growth substance activity could be connected again with *d* (−) lactic acid. The results obtained with all of the compounds examined in this way until now are summarized in Table II, along with their activities in the flax root test.

TABLE II

OPTICAL ISOMERISM AND ACTIVITY
[ACCORDING TO MATELL (93, 94) AND ÅBERG (5)]

Compound (α-)	*d*-form			*l*-form		
	Sign of rotation	Activity in flax root test (growth substance activity)		Sign of rotation	Activity in flax root test (growth) substance activity)	
		schematic	LC_{50}		schematic	LC_{50}
Phenoxypropionic acid	(+)	++	5.89	(−)	±	
Naphthoxy (1) propionic acid	(−)	++		(+)	PA	
Naphthoxy (2) propionic acid	(+)	++++	7.68	(−)	A	
2,4-Dichlorophenoxy-propionic acid	(+)	++++	7.48	(−)	+	4.89
3,4-Dichlorophenoxy-propionic acid	(+)	++++	7.57	(−)	++	5.46
2,4,5-trichlorophenoxy-propionic acid	(+)			(−)		
1-chloronaphthoxy (2) propionic acid	(+)	+	4.96	(−)	PA	
Phenoxybutyric acid	(+)	++		(−)	−	
Naphthoxy (1) butyric acid	(−)			(+)		
Naphthoxy (2) butyric acid	(+)	+++	7	(−)	A	
Naphthalene (2) methyl-propionic acid	(+)	++		(−)	PA	
Naphthalenemercapto (2) propionic acid	(+)	+++	6.77	(−)	A	

− inactive
\+ very weakly active
++ weakly active
+++ active
++++ highly active

LC_{50} negative logarithm of molar concentration causing 50 per cent inhibition
PA probably antagonistic
A antagonistic

The steric relations of the last two compounds in Table II are not yet definitely known. Matell (94) found indications, however, that the (+) forms which are more active growth substances than their antipodes, possess the same configuration. These important experiments by the Swedish investigators seem to rather clearly establish stereochemical specificity of the highest active growth substances in this series. It may be expected that before long the configuration of similar compounds may be deduced from studies of their proportionate effects as growth substances, a possibility indicated already by Fredga (42).

The effects of the (+) and (−) forms of α-allyl-phenylacetic acid and α-2,4-dichlorophenoxy-propionic acid in the beet test, where completely identical curves were obtained for the antipodes, indicate that optical isomerism does not influence the interactions with protoplasmic membranes (166).

Activity of Non-Acidic Compounds

Esters, amides.—Often, esters and amides of active acids have been found active, too, though generally to a less extent. Kögl & Kostermans (76) were the first to raise the question whether the activity of esters of IAA[2] in the *Avena* test is due to the compounds proper, or to IAA formed upon hydrolysis *in vivo*. Because the activity decreased in the series of homologous esters, it seemed probable that a partial hydrolysis caused the activity.

Because the polar transport of auxins within the plant is associated with a free carboxyl group, it has been rather generally considered plausible that a conversion of the derivative into the parent acid is a prerequisite for activity. One must admit, however, that in most cases there is no direct evidence for this view.

Thimann (138) questioned an explanation of the activity of naphthalene-1-acetamide along these lines because he found its concentration-activity curve to be quite different from that of the acid. If hydrolysis were involved, the two curves were expected to run parallel. This reasoning is valid, however, only if the degree of conversion of amide to acid is independent of the concentration in the medium. Since the conversion does not occur in the solution but inside the cell and transport has to take place, it is quite conceivable that the amount of acid formed would vary with the concentration of the amide. Then, a curve different from that of the acid would result. The second argument, that no trace of ammonia could be detected in the solution, can also be questioned because the ammonia formed in the cell is not necessarily excreted into the solution. On the other hand, Åberg (4, 5) established that naphthalene-1-acetamide has a weak antiauxin activity in his test.

The interesting results of Wood & Fontaine (182) also indicate that most probably a conversion of amides into acids precedes the action. They found that of the 2,4-dichlorophenoxyacetyl derivatives of DL-, L-, and D-amino acids only the first two types were active plant growth regulators (formative

changes in tomato plants), suggesting that the plants are unable to split readily the amide linkage of 2,4-dichlorophenoxyacetyl-D-amino acids.

Aldehydes.—After Went & Thimann (177) considered the possibility that indoleacetaldehyde might be an intermediate in an enzymatic conversion of tryptamine to indoleacetic acid, seven years elapsed before Larsen (79) obtained evidence for the occurrence of indoleacetaldehyde in ethereal and chloroform extracts from plants (*Pisum, Vicia Faba, Helianthus, Brassica*). The neutral compound could be converted by an enzymatic oxidation into an acid active in the *Avena* test. The acid showed properties resembling those of IAA.

Comparable results were presented by Hemberg (58) and Gordon & Nieva (47) for potato tubers and pineapple leaves, respectively. In the latter the aldehyde character of the neutral compound was deduced from its reaction with dimedone and sodium bisulfite. At that time the pure indoleacetaldehyde was not available and the investigations were carried out with preparations obtained by reacting tryptophan with isatin or ninhydrin (79).

The α-aldehyde was active in the *Avena* test without any lag period, therefore, the compound was thought at first to be active per se. When Larsen (81) found, however, that coleoptile tissue is capable of converting the aldehyde very rapidly into the acid by an enzymatic process and that the activity displayed by the aldehyde could be accounted for by the acid formed in this way, the aldehyde was regarded then as a precursor to IAA. [For an extensive review, see Larsen (82).] The same capacity to oxidize the aldehyde enzymatically was found to be present in pineapple leaves (Gordon & Nieva, 48) and in *Artemisia* roots [Ashby, (7)].

No information, therefore, was obtained as to the activity of indoleacetaldehyde itself. Pohl (114) concluded that the aldehyde had to be designated as an inhibitor because in Moewus' (101, 102) cress root test, the aldehyde (from tryptophan), in contrast to the acid, does not show any stimulation at low concentrations. When pure synthetic indoleacetaldehyde became available by an elegant synthesis from indole [Brown, Henbest & Jones (23, 24)] this question could be investigated more precisely. In the *Avena* straight growth test the activity of the aldehyde was found to be equal to or smaller than that of the amount of acidic compound produced in the solution in the presence of coleoptile sections. This means that the aldehyde must be either inactive or inhibitory.

Naphthalene-1-acetaldehyde (for synthesis see 62, 63, 96) was reported by Larsen (80) to be very weakly active in the *Avena* test, while Veldstra & Booij (161) found this aldehyde to be almost as active as the acid in the pea test. Ashby (7) compared naphthalene- and indoleacetaldehyde as to root inhibition (*Artemisia*); the latter proved to be 10 times as active as the former. More recently, Larsen (83) made a detailed study of the activity of the aldehyde in the *Avena* test and found it to be about 11 per cent of that of the corresponding acid. An enzyme system, present in coleoptile

juice, could form one mole of acid from two moles of the aldehyde, indicating that the conversion may imply a dismutation to acid and alcohol, a type of reaction deemed probable, too, for the enzymatic transformation of indoleacetaldehyde. In this connection it was shown that indole-3-ethanol (tryptophol) does not interfere with the determination of the activity of the corresponding aldehyde (83). Ashby (7) established the formation of an acid, presumably NAA[2], by incubating naphthaleneacetaldehyde with *Artemisia* root juice. Since no time lag occurs in the response to the aldehyde, the conversion to acid in the living tissue must proceed rapidly if this conversion is required for activity. In root inhibition the relative activity with respect to NAA proved to be of the same order (10 per cent) as that found by Larsen (83) in the *Avena* test.

Phenylacetaldehyde, in comparison with phenylacetic acid, possesses a relative activity of 7.3 per cent in the *Avena* test (83). 2,4-Dichlorophenoxyacetaldehyde [for synthesis see (34)] gives rise to an active acid when incubated with oat coleoptile extract [Atkinson (8)].

2,3,6-Trichlorobenzaldehyde was reported by Bentley (12) to be more active in the *Avena* straight growth test than the corresponding acid within a small range of concentrations (about 1 mg. per l.). At higher and at lower concentrations the proportions were the reverse. In the pea test, however, the activity was but a very weak one, and much lower than that of the acid [Veldstra (160); Thimann (139)]. Thimann tentatively attributed the activity to acid formed in the plant tissues. Zimmerman & Hitchcock (188), studying various responses of tomato plants, found the acid and the aldehyde, on the average to be equivalent. 2,6-Dichlorobenzaldehyde was inactive here (189).

In testing chlorinated benzaldehydes and benzoic acids for their phytotoxicity (inhibition of rape and wheat seed germination), Jones, Metcalfe & Sexton (66) concluded that 2,3,6-trichlorobenzaldehyde was more active than the acid. Assuming that the aldehyde acts after conversion to the acid by oxidation or dismutation, they refer to Michlin & Borodina (97), who established the presence of an aldehyde mutase in germinating peas which is capable of using aromatic aldehydes as substrates.

Thus, there are many indications, indeed, that aldehydes often may not be active per se, but only after conversion to the corresponding acid and that their inherent activity possibly is more of an inhibitory type. All questions pertaining to this problem are not yet satisfactorily answered, however. 2,3,6-Trichlorobenzaldehyde, in comparison with other aromatic aldehydes, is difficult to oxidize and does not undergo a Cannizzaro conversion to alcohol and acid (66). Here, especially, is it desirable to see if the corresponding acid can be formed by plant enzyme preparations.

On the other hand, differences in activities between neutral aldehydes and the corresponding acids may be due to differences in rate of penetration and distribution. Jones *et al.* (66) point to this possibility with 2,3,6-trichlorobenzaldehyde and Veldstra & Booij (161) attribute the more rapid action of

naphthalene acetaldehyde than NAA in the pea test to a higher penetration rate for the neutral compound.

Nitriles.—Until recently the nitriles corresponding to active acids have attracted but little attention. Indoleacetonitrile and naphthaleneacetonitrile have been reported to be inactive in the pea test (153). Thimann (138) ascribed the activity of a few nitriles to the acid formed by hydrolysis. Zimmerman & Wilcoxon (190) had previously explained the activity of naphthaleneacetonitrile in this way after establishing that it acted only after a latent period in the epinastic response of tomato leaves. Steiner (127) also discussed this possibility when *n*-acetyl-indole-3-acetonitrile proved practically equal to IAA[2] in straight growth, whereas, phenyl-acetonitrile even slightly surpassed phenylacetic acid. The possibility was considered that perhaps the nitrile is transported in the tissue more easily than the acid, causing, after the conversion of the nitrile to the acid, a higher final concentration of the acid at the primary active sites. Convincing evidence is not at hand for any of these suppositions.

Nitriles, however, certainly will play a more important role in growth substance investigations since Jones, Henbest, Smith & Bentley (64) identified the neutral compound from cabbage which is highly active in the *Avena* straight growth test as indoleacetonitrile and studied its activity in different tests along with that of IAA. It is quite clear that neutral growth promoting substances present in plant extracts no longer can be considered to be only indoleacetaldehyde, as was the recent trend, without further evidence.

Jones *et al.* (64) established that the nitrile is more active than the acid in the *Avena* straight growth test, whereas, in the *Avena* curvature test at low concentrations their activities are comparable, and at higher concentrations the nitrile is less active than the acid. In the pea test a slight activity of the nitrile was found only at high concentrations.

Here, again, the question arises whether the neutral compound is active as such, or has to be converted to the acid. Experiments of Jones *et al.*, concerning acid formation from nitrile in the presence of *Avena* coleoptiles, show that only a very small part of the effect of the nitrile in the straight growth test can be ascribed to production of acid in the medium. It might be different inside the cell, however. The rates of polar basipetal transport of nitrile and acid in the *Avena* coleoptile are practically equal, and in some cases the transport rate of the nitrile seemed to be even greater than that of the acid. The conversion of nitrile to acid inside the cell might result in higher concentrations of acid at primary active sites than that attained by administration of the acid. It is interesting that indole- and naphthalene-aceto-nitriles have a slightly stronger action in the beet test (161) than the corresponding acids (166). This may be caused by a more rapid penetration into the tissue. Jones *et al.* consider it possible that the lower activity of the nitrile in the curvature test is due to greater lateral spread of the compound across the coleoptile as compared to the acid.

In several tests on other types of responses of plants to growth sub-

stances, including inhibitory actions, the nitrile was less active than IAA or inactive except for cambial initiation in *Salix* and induction of parthenocarpic fruits in tomatoes in which the activity of both compounds was of the same order. In the latter responses, however, the acid acts directly while with the nitrile there is a delay of about three weeks, indicating the possibility that the nitrile has to be converted to the really active compound, possibly IAA. A plausible intermediate in such a conversion, indoleacetamide, proved to be inactive in the straight growth test. The reactions which the nitrile undergoes in different plant tissues will have to be studied before any definite conclusion can be reached as to the importance of a conversion of nitrile to acid.

Apart from that, the fact that the nitrile can be more active than the acid is puzzling in many respects. Hessayon (59) found that the mycelial growth of *Fusarium oxysporum* is inhibited more strongly by the nitrile than by IAA, whereas, rather generally in a series of fungicides, substitution of the nitrile for the carboxyl group results in decreasing activity.

In a contribution to the solution of this problem Osborne (108) suggested that the rôle of the nitrile may be, in part, that of a synergist for the acid formed from it. Her data prove that in the pea test such an effect can be demonstrated. These results were corroborated (166), but the synergistic effect of both indole- and naphthaleneaceto-nitriles was found to be weak compared to that of 2,3,5-triiodobenzoic acid or di-*n*-amylacetic acid, for example. Thus, this question is answered incompletely. One may expect, therefore, that this third member in the family of active natural indole derivatives will receive much attention and contribute thereby to our knowledge of growth substance action.

Alcohols.—Because the conversion of indoleacetaldehyde to IAA[2] in the plant cells possibly implies formation of IAA and indole-3-ethanol (tryptophol) by a dismutation reaction, a possible activity of the alcohol would be of physiological importance.

Larsen (80) and Veldstra & Booij (161) established that tryptophol is weakly active in the *Avena* test and in the pea test, respectively. The corresponding naphthalene-1-ethanol recently was shown to be active in the pea test to a comparable degree at 5 and 2.5×10^{-6} *M*, whereas the effect at 1 and 0.4×10^{-6} *M* is weaker than that of tryptophol (166). Naphthalene-1-carbinol is known to be inactive in the pea test [Veldstra (153)].

This may be an indication that an oxidation of the alcohols to the acids *in vivo* is responsible for the activity observed. Formation of the highly active NAA[2] from naphthalene ethanol will cause a perceptible activity much sooner than a conversion of naphthalene carbinol to the active but much weaker naphthoic acid. Direct evidence of this is lacking, however.

In the inhibition of rape and wheat seed germination, 2,3,6-trichlorobenzylalcohol showed the same order of activity as the corresponding acid (66), and the same is true for 2,4-dichlorophenoxyethanol with respect to 2,4-D[2] in cucumber root inhibition (32).

Amines.—As early as 1937, indole-3-ethylamine (tryptamine) was re-

ported to show a delayed action in both the *Avena* and pea test [Went & Thimann (177); Skoog (123)]. This effect was ascribed to conversion to IAA. Enzyme systems from pineapple leaf cells are capable, indeed, of effecting this transformation [Gordon & Nieva (48)], but spinach shows no such activity (180). It would be interesting to investigate the behavior of extracts from pea stem and *Avena* coleoptile tissue.

1-Aminomethyl-naphthalene was found to be inactive, whereas, the tryptamine analogue, β-naphthalene-1-ethylamine, was distinctly active in the pea test (153, 166). Again, as with the corresponding alcohols, transformation to the acids as a prerequisite for activity, would offer a plausible explanation for this difference.

Jones, Metcalfe & Sexton (65) studied a large number of phenoxyethylamine derivatives as inhibitors for oat and rape seed germination. For the primary amines, selective activity is influenced by substituents in the benzene nucleus in the same way as in the phenoxyacetic acid series. The activities, on the average, were lower than those of the corresponding acids. These facts are considered by the authors to constitute evidence for the view that the action of the amines is effected by a conversion to the phenoxyacetic acids. Although here too, direct biochemical evidence has not yet been obtained, the activity of 2,4-dichlorophenoxyethylamine in the pea test (65) and a physiologically similar effect of both the amine and the acid in certain field tests are in support of the hypothesis. The fact may be noted also that the quaternary compounds of hexamethylenetetramine and 2,4-dichlorophenoxyethyl bromide, which may give rise to an aldehyde or to an amine, possess a weak activity in the pea test (65).

Antagonistic and Synergistic Activity of Growth Substance Analogues

Antagonists—Quite recently Bonner & Bandurski (15) surveyed the data pertaining to auxin antagonists and the antiauxin concept. This important field is receiving more and more of the interest it deserves. One may expect that a better understanding of the types of interference in the action between natural and synthetic growth substances and their structurally related analogues will contribute considerably to our knowledge of growth substance action, in the same way as the study of the antibacterial action of sulphonamides informed us about the function of *p*-aminobenzoic acid.

It would appear, however, that the tests used to determine the growth promoting effects are not equally satisfactory for studying antagonistic actions, because secondary inhibitory activities, which are not really antiauxin actions, may interfere quite easily. It is possible that such secondary effects are responsible for the fact that in certain cases [2,4-dichloroanisole (15, p. 67), 2,3,5-triiodobenzoic acid (33, 142), and *trans*-cinnamic acid at higher concentrations (150)] the inhibition caused by the antagonist cannot be completely reversed by the addition of a growth substance. Although the action of phenylbutyric acid in the *Avena* curvature test seems to meet

the requirements for a competitive inhibition (15, p. 66), this is certainly not so in the straight growth test (161, p. 299).

Van Overbeek *et al.* (150) obtained instructive results in their study of the action of *trans*-cinnamic acid versus different growth substances. That the picture may be still more complicated than that of an inhibition of growth promoting activity, may be seen clearly from the results obtained with the combination of *cis*- and *trans*-cinnamic acid. When a greater concentration of *cis*-cinnamic acid is used in combination with the *trans* form than is necessary to completely reverse the inhibition of the *trans* form, the graph (150, Fig. 2B, p. 592) indicates that the *cis* form is more active in growth promotion in combination with the *trans* form than alone!

It is probable that the response of roots to growth substances and their analogues, if used in the study of antagonistic activities, as done for the first time by Burström (28), will provide more clearcut results. Åberg (1) stressed the fact that growth substance concentrations causing a clearly discernible inhibition are so low, that potential antagonists can be applied at much higher doses, without being generally toxic for the root tissue. Roots whose growth has been inhibited by added growth substance thus could be suitable test objects in the search for competitive antagonists. Åberg's supposition that the positive growth effects on roots caused by antagonists probably will be much more specific than the growth inhibition of organs with optimal or sub-optimal auxin content obtained with antagonists, is, in our opinion a very plausible one.

In view of what has become known about the structural relations between metabolites and antimetabolites [cf., Woolley (183)] it is intriguing to reflect upon the modifications of the structure of a growth substance which would produce a competitive antagonist. Really competitive inhibition has to take place on the primary active sites in the cell. A highly active growth substance will be characterized by: (*a*) a specific affinity for these sites, which is chiefly determined by the nature of the ring system and the acidic polar group; and (*b*) a capacity to cause a specific physiological response when fixed to the receptor (in a dynamic equilibrium with free molecules!). This specific response seems to be dependent largely upon the relation between the ring system and the polar group in a spatial sense, in which the type of the side chain and substitution in the nucleus may be of importance as discussed earlier.

A potential antagonist will have to meet requirement *a* to a large extent because otherwise it cannot compete with the growth substance for the sites involved in the growth response. Consequently, one would expect that the strongest antagonists will closely resemble the growth substances with respect to the ring system. Furthermore, potent competitive antagonists very probably will not have to be sought among compounds lacking the carboxyl group or carrying a polar group of a quite different character. Indeed, Åberg, who analyzed the requirements for an auxin antagonist in a comparable way (4, 5), found α-naphthol and 2,4-dichloroanisole to be

weak antiauxins only (102). The same was true for naphthaleneacetamide.

For amines corresponding to the growth substances, in so far as conversion to the acids does not occur, not only the contribution of the carboxyl group to the fixation of the molecule at the receptor (interaction with cationic sites?) is lost, but very probably it is replaced by a repulsive force. In this sense, it is not wholly unexpected that 2,4-dichlorophenoxyethylamine is not antagonistic towards 2,4-D[2] in germination inhibition (65). It is interesting in this connection to draw attention to the findings of Neurath *et al.* (35) concerning specific inhibitors for carboxypeptidase. While β-phenylpropionic acid is a potent inhibitor, β-phenylethylamine has no effect on the enzymatic activity.

Few data are available on the effect of replacing the carboxyl group with —SO_3H. Veldstra & Havinga (162) did not find any antagonistic activity of naphthalene-1-methanesulphonic acid toward NAA[2] in the pea test. Wieland, Fischer & Moewus (179) on the other hand, concluded that indole-3-methanesulphonic acid is antagonistic to IAA because its inhibition of root growth in the cress test is counteracted by a concentration of IAA which, by itself, is growth promoting. These results in the cress test could not be corroborated by Veldstra *et al.* (166), who found the sulphonic acid to be without influence on the action of IAA. In some respects (a slight swelling of the hypocotyls) the action of the sulphonic acid alone reminded one of that of a growth substance. Because of their effects in the beet test (161) one has to conclude that the penetration into the beet cells of both sulphonic acids is of a very low order (too high a dissociation?). It will be interesting to learn how much the stem and root tissues differ in this property. Additional information also might be obtained from experiments with the root test as done by Burström and by Åberg.

It is our conclusion that strong competitive antagonists depend on interference primarily with factor *b*. This is exactly what has been done in most of the antagonists studied by Burström and Åberg. In all cases where highly active antiauxins result, the ring system is either unchanged or substituted in such a way that its adsorption affinity is not lowered. The carboxyl group is still present and the modification implies other changes in the side-chain.

Åberg (2) investigated α-naphthalene-1-methylmercapto-acetic, propionic, and iso-butyric acid (XLIII, LXIV, LXV) together with their -naphthalene-2 isomers (XLVIII–L). The study furthermore included naphthalene-1-methylselenyl-acetic acid (XLVI), its naphthalene-2-analogue, and δ-naphthalene-1-methylselenide-*n*-valeric acid (XLVII). All of them favor root growth in low concentrations, up to 120 per cent of the controls, presumably by antagonizing the natural auxin in the roots. The stimulation at low concentrations is strongest for the naphthalene-2-derivatives. In combination with externally added 2,4-D[2], the root growth inhibitory activity of the latter is counteracted, and normal root growth is restored to a large extent. Here also the naphthalene-2-derivatives are the most effective ones, while the activity decreases in the order acetic, propionic, and iso-

butyric acid. Evidence is presented that the antagonistic action implies a competitive displacement of 2,4-D from primary active sites.

When growth substances which inhibit root growth to a less extent such as phenylacetic and phenylbutyric acid, are used in these mixtures, instead of 2,4-D, the antagonist is capable of overcoming the effect of higher concentrations of these compounds than of 2,4-D. These proportions are comprehensible when the weaker growth substance activity is associated with a lowered affinity for the receptor, as compared to that of 2,4-D. That phenoxyacetic and phenoxybutyric acid are weaker antiauxins than the abovementioned naphthalene-derivatives may find an explanation along similar lines.

There is only one report of a conversion of a growth substance, naphthoxy-2-acetic acid into an antagonist, naphthoxy-1-acetic acid, brought about by changing the position of the side-chain only (2).

The papers by Burström (28, 29, 30, 181) relative to antiauxins have already been discussed by Bonner (15). In the meantime, Burström has studied racemic α-phenoxypropionic acid and its 4-chloro- and 2,4-dichloro-derivatives. He concluded that all of them showed root growth inhibition; that is, they possess regular auxin activity (31).

From Åberg's work (4) it appeared that the (+) form of α-phenoxypropionic acid behaves as a weak growth substance in root inhibition, whereas the (−) form is 100 times less effective and does not show antiauxin activity. Nor did the (−)2,4-dichloro- and (−)3,4-dichloro-derivatives possess such properties in the root test, their antipodes being highly active growth substances [Åberg (5)]. These results, paralleling the effects in the pea test (discussed in a previous section) are partly at variance with those of Smith, Wain & Wightman (126, 126a), who reported that cell elongation promoting activity (straight growth) of (+)α-2,4-dichlorophenoxy-, α-2,4,5-trichlorophenoxy-, and α-naphthoxy-2-propionic acid can be partially or even completely antagonized by addition of an excess of the (−) forms.

As to α-naphthoxy-2-propionic acid, Åberg (3) obtained comparable results in root inhibition, the (+) acid being highly inhibitory (growth substance effect) and the (−) form acting as a strong antagonist. Åberg (5) has established antiauxin properties for a number of other optically active naphthoxy acids and related compounds, their antipodes behaving as growth substances (Table II). Thus in these cases the change of a growth substance into its antagonist merely involves a change in the configuration.

Burström (30), finding α-phenoxy-isobutyric acid (LII), the whole series of its chlorinated derivatives, and α-indole-3-isobutyric acid (LIII) to be highly active auxin antagonists, is inclined to consider their activity to depend specifically upon the isobutyric acid structure of the side-chain. As indicated earlier and especially because of the results of Wain *et al.* and Åberg with α-substituted propionic acids, we prefer to consider the (−) forms of the antagonistic chlorophenoxypropionic acids, in relation to steric hindrance, to be equivalent to the corresponding isobutyric acids. In the

(+) propionic acid derivatives, evidently the methyl group does not interfere with the fitting required for growth promoting activity, whereas in the (−) forms it does. One has to expect, then, the same hindrance from the isobutyric acids. We must admit, however, that in this picture the case of indole-3-isobutyric acid as an antiauxin and (+) and (−)α-indole-3-propionic acid as equally active auxins in straight growth, does not fit. A re-examination of the indole-propionic acids in different tests, including root inhibition, is therefore desirable.

Surveying the results with these antagonist types, one has to conclude that the strongest ones result from modifications of growth substances which affect the spatial form of the molecules rather than their chemical structure. Minarik *et al.* (98) found 3-nitro-4-halogen benzoic acids to be active in stimulating root elongation (cucumber) and antagonistic to the inhibitory action of 2,4-D.[2] This should be further investigated because growth substance activity with substituted benzoic acids requires the 4-position to be free. It is suggestive of the phenoxyacetic acid derivatives where 2,6-di-substitution is compatible with anti-auxin activity (30), but not with growth substance activity.

Synergists.—One may plausibly assume that if molecules of an active compound are functioning in a certain biological system, not all of them will be "on the spot" of the primary action. Part of them may be interacting at sites of secondary importance for the total action while others may be absorbed somewhere in a harmless way or on an enzyme system which decomposes them or excretes them from the system. With respect to the primary action, this implies a waste or loss of active compound where its concentration is sub-optimal.

Analogues of the primary active compound which, for one reason or another, have a higher affinity for these "loss sites," will be less active or inactive because they reach the primary active sites to a less extent or do not arrive at all, apart from the fact that some of them may be inactive essentially because of their modified structure. If such an analogue is applied together with the active agent it will compete with the latter for the loss sites. A higher fraction of the primary active compound will become available for its essential function. If the action was not yet maximal, it will be enhanced or otherwise the same activity may be attained with a smaller amount of active molecules, provided that the part omitted be replaced by an analogue of the type indicated. This means that such a competition for loss sites between active compound and analogue results in an over-all synergistic effect of the combination [Veldstra (155, 159)].

When applying this analysis to growth substances, it was found that such analogues were obtained, if, by hydrogenation of the ring system or by lengthening of the side chain, the growth substance molecule was changed into one of a more lipophilic (or hydrophobic) character (161). Apparently, this strongly influences the distribution of the compound within the tissue (cells).

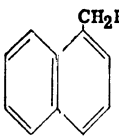
Much earlier, Went considered this problem from a different point of view when he introduced the concept of hemiauxins. These exert an auxin-sparing action when used to pretreat the material (pea test). Most of the hemiauxins (e.g., LIV to LVIII) characterized by Went (174, 176) which meet all structural requirements for primary activity but one, possess a more lipophilic character than the related growth substance and thus seem to fall into the same category as the synergists indicated above. It would seem to us that the synergist concept is somewhat broader than that of the hemiauxins because compounds can become more lipophilic while still meeting all requirements for primary activity as expressed by Went, by substitution of the ring system or by enlarging it.

Thus, 2,4,6-trichlorophenoxyacetic acid (LIX) and 2,4-dichloronaphthoxy-1-acetic acid (LX) are synergists, e.g., for NAA,[2] in the pea test (166), while Thimann (139) found 9-anthroic acid (LXI) and its 9,10-dihydro-derivative (LXII) to be active in the same sense. The latter acids may be considered as the (primary active) 1-naphthoic acid and its hydrogenated derivative to which an extra ring has been added.

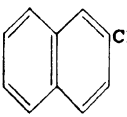
Since hemiauxins and synergists are probably functionally equivalent, we prefer the term synergist because it indicates more directly the function of the compounds. When synergists are used in the pre-treatment technique as used for hemiauxins they enhance the growth substance effect to a lesser extent than when applied simultaneously with the active compound (166). In our opinion, therefore, and also for theoretical reasons the latter technique is preferable for future research.

Thimann & Bonner (142) observed that 2,3,5-triiodobenzoic acid (TIBA) (LXIII) in combination with a low concentration of IAA enables the latter to bring about a disproportionate amount of growth. This effect is more pronounced in the pea test than in the *Avena* straight growth and curvature tests. In the pea test, 2,3,5-tribromobenzoic acid is a weaker synergist (for NAA) than TIBA,[2] whereas the trichloro analogue is only very slightly active (166). Similarly, the lipophilic character of the acids decrease as is shown by their effects in the beet test and their interactions with the oleate coacervate (22, 166). 2,3,5-Trichlorobenozic acid does not enhance the effect of 2,3,6-trichlorobenzoic acid in the pea test, nor are the 2,3,4-, 2,4,5-, 2,4,6- and 3,4,5-trichloro isomers active (166). It would be important to know the synergistic capacity of the complete series of tribromo- and triiodobenzoic acids.

The analysis leading to the synergist concept was originally applied by Veldstra & Booij (161) when investigating the cause of weak activity or inactivity of certain growth substance analogues. Synergistic activity in the pea test was found, then, with acids weakly active as a growth substance such as γ-naphthalene-1-butyric acid (LXIV) or quite inactive such as decalyl-1-acetic acid (LXV). Normal fatty acids, corresponding in molecular size to NAA, are synergists too, although weaker ones. In this normal series, the synergistic activity increases if the carboxyl group is displaced towards



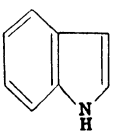
XLIII $-SCH_2COOH$
XLIV $-SCH(CH_3)COOH$
XLV $-SC(CH_3)_2COOH$
XLVI $-SeCH_2COOH$
XLVII $-Se(CH_2)_4COOH$



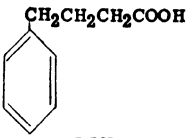
XLVIII $-SCH_2COOH$
XLIX $-SCH(CH_3)COOH$
L $-SC(CH_3)_2COOH$
LI $-SeCH_2COOH$



LII



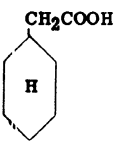
LIII



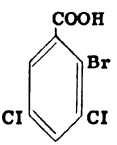
LIV



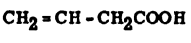
LV



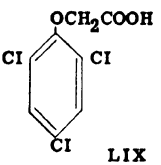
LVI



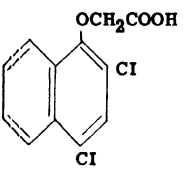
LVII



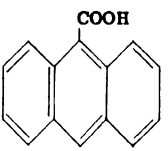
LVIII



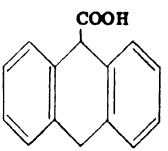
LIX



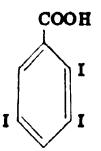
LX



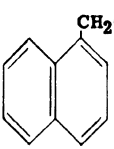
LXI



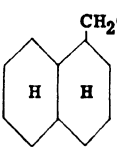
LXII



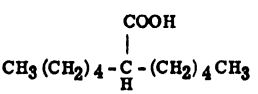
LXIII



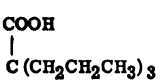
LXIV



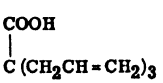
LXV



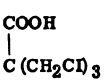
LXVI



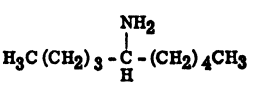
LXVII



LXVIII



LXIX



LXX

the center of the carbon chain. Maximal activity is attained with di-*n*-amylacetic acid (DNAA)[2] (LXVI) which, in a certain sense, is an open analogue of decalylacetic acid. The branched chain isomers are equally active, whereas either lengthening or shortening of the C_5 part causes a decrease of activity (155, 166). A comparable effect is shown by tri-*n*-propylacetic acid (LXVII). Tri-allylacetic acid (LXVIII) is weaker and tris-chloromethylacetic acid still weaker [LXIX, (166)].

Several highly active wetting agents and the amino-analogue of DNAA, 6-amino-undecane (LXX), are inactive as synergists. As, apparently, the molecular size of the compound and the position of the carboxyl group are of importance, it seems, therefore, that this synergistic action implies more than merely a rather unspecific wetting agent or penetrant action. Very probably, the molecules of the synergist "take over" that rôle of the growth substance which is not the primary one or where the active compound is really lost or wasted.

A growth substance may be changed into a molecule with a stronger lipophilic character by omission of the hydrophilic carboxyl group or by modifying this group to a more neutral one. Van Raalte (151) was surprised to find indole to be a synergist for IAA in the induction of root formation. It may be possible, however, that indole can replace IAA at certain sites in the cell (where possibly the carboxyl group is less important for fixation) in the same sense as discussed above. This offers a simpler explanation for its effect than van Raalte's hypothesis, which involves a form of competition at primary active sites and, in our opinion, can result in antagonistic effects only. For this reason both indole and naphthalene were tried in the pea test as synergists. It was found, indeed, that with 10^{-4} *M* they enhance the effect of sub-optimal concentrations of NAA, although the action is a weak one (166).

Osborne (108) applied the synergist concept to indoleacetonitrile, reported to be more active than IAA in the straight growth test (64). In the pea test, where the nitrile is practically inactive, synergism with IAA could be demonstrated and Osborne therefore suggested that this effect be considered as a factor in assessing the activity of the nitrile. Compared with TIBA and DNAA, the nitrile is a weak synergist, its activity being equalled by naphthalene-1-acetonitrile (166). One is inclined to apply the same reasoning to 2,3,6-trichlorobenzaldehyde which is more active than the corresponding acid and than IAA in straight growth (12). In the pea test, where the aldehyde is inactive, no synergistic effect could be found, however (139, 166).

A synergist of the type discussed may show its real potencies if used with a compound which is weakly active or practically inactive as a growth substance because of factors which prevent it attaining the critical concentration at the sites of action. If, however, the lowered activity or inactivity is caused by less and less physiological response of the compound on the site of action, a synergist will be without effect, of course. It is important, therefore, that

DNAA strongly enhances the effect of low concentrations of naphthalene-1-butyric acid and of its naphthalene-2-isomer, whereas no effect is observed with the naphthalenepropionic acids. The low activity of naphthalene-2-acetic acid and naphthoxy-1-acetic acid cannot clearly be enhanced synergistically either (166). Because of these facts one is inclined to deduce that these compounds fail rather fundamentally in exerting a physiological action. For naphthoxy-1-acetic acid, this is now also clear from its antiauxin properties (2).

In many other respects it will be possible to get informations from effects of such types of mixtures, not to be had when assaying growth substances per se.

Growth Substance Activity and pH

From the very beginning of growth substance research the influence of the pH of the medium on autonomous growth and on the effect of applied growth substances has attracted attention and the study of it has often resulted in controversial conclusions [cf. Rietsema (116, 117); Audus (9, 10)]. Growth substances are organic acids of medium or weak acid strength and the proportion of undissociated acid to anion can shift considerably even with small variations in the pH. Apart from the fundamental question of whether the acid or the anion is the active agent, the influence of pH has to be considered in the study of structure-activity relationships because one operates with relative activities in a series of compounds. Simon *et al.* (120, 121, 122, 122a), especially [cf. Albert (6)] have stressed this point and illustrated its general importance when studying the biological effects of weak acids and bases. They suggest that activity be measured at a pH where the compound is present totally in the undissociated form or nearly so, i.e., at a pH of two or more pH units below the pK value. For growth substances (pK from 3 to 5) this theoretical requirement cannot be met, however, because the pH range would be toxic to the test objects. Corrections can be made, however, from a curve describing the quantitative relationship between pH and activity (120).

Some investigators [Bonner (13), Went (174), van Overbeek *et al.* (150)] used corrected values obtained by measuring activity in terms of the concentration of undissociated molecules instead of the total concentration. This method is correct, however, only if it is definitely established that primary activity is due exclusively to the undissociated acid.

There are many indications now, that the latter point of view, which has been held for a long time by the majority of growth substance investigators needs revision because of more recent data which are inconsistent with it. The fact that indole-3-methanesulphonic acid and some related sulphonic acid types (all of which are completely dissociated at a physiological pH) have been found active (166), means that here the anions only can be the active form.

The proportion of acid to anion in the medium, depending on the pH,

certainly will influence the rate of penetration of the growth substance into the cell. But in *Avena* coleoptile sections, for example (117), variations in the pH of the medium practically do not affect that of cytoplasm and vacuole. At a physiological pH, the growth substance molecules in the cell will be present, therefore, largely as anions. Moreover, in so far as pH changes inside the cell are concerned, the important work by Brian & Rideal (22) on the interactions of a growth substance [2-methyl-4-chlorophenoxyacetic acid (MCPA)] with monolayers of protein, lipoid, lipoprotein, and material produced directly from plant tissues, make it quite clear that a shift in pH will cause ionization changes in proteins and protoplasmic membranes which will greatly influence their interaction with the growth substance anion. Since the interaction discussed by Brian & Rideal decreases with increasing pH, thus paralleling the effect of pH on growth substance action, the authors feel that attention should be paid to changes involving the proteins and protein complexes of the plant induced by a pH shift, rather than to the influence of pH on the dissociation of the growth substance.

In this connection we should like to refer to the extensive studies of the interactions between proteins and organic anions by Klotz (70, 73) [also see Teresi & Luck (134, 135)], in which it was shown that the interaction with the protein decreased with increasing pH in a pH range not affecting the amount of anion present. Apparently groups on the protein intimately involved in the binding process were modified (70). This modification appeared to be reversible, and, thus, unlikely to be concerned with changes in the spatial structure of the protein. This influence of a pH shift may imply a decrease of attracting forces or an increase of repelling ones.

Although no definite conclusions can be drawn as yet from the experiments of Burström (30) in which several substituted isobutyric acids were used as antiauxins in root growth, the results indicate that the positive growth action is related to the concentration of the acid anions in the medium. These recent results certainly justify a reevaluation of the older concepts of the effect of pH on growth substance action.

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THE PHYSIOLOGY OF FRUIT GROWTH[1,2]

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Introduction

Definition of fruit.—The empirical concept of what constitutes a fruit is so immediate and obvious that Ulrich (329), in a 370 page book devoted to *The Life of Fruits*, does not even define the word. The extraordinary morphological variation of fruits seen in size, shape, and structure, and the equally variable diversity of the origin of fruit tissues such as ovary wall in a cherry, floral parts and axis in the pineapple, and receptacle in a strawberry have led morphologists toward the establishment of exceedingly complex systems of fruit classification. Despite this extreme diversity in structure and origin, something remains to make us call a certain organ a "fruit." This common denominator is of a physiological nature. It involves the relationship which connects tissues of various origins with the ovules of a plant. Thus, in higher plants, a fruit consists of the tissues which support the ovules and whose development is dependent upon the events occurring in these ovules (206). This concept holds true even for seedless fruits such as the banana, since, in those instances also, ovule primordia were initially present.

If one adopts such a physiological definition, then the diversity of fruit structure and origin becomes more understandable. As has been pointed out by Mangenot (162) in reference to the evolutionary origin of fruits,

> Plants can construct identical apparatus out of very different elements. What is important . . . is not the morphological nature of the materials which are used, but the biological efficiency of the structure which has been built.

The definition of growth.—Thimann (296) defines the word "growth" in plants as "an irreversible increase in volume." Such a definition fails to include explicitly the absorption of materials and energy and their transformation by the plant or organ itself to produce this increase in volume. These operations and the mechanisms by which they are performed constitute the scope of the physiology of growth as used in this review.

The scope of this review.—The terms "fruit" and "growth" having thus

[1] The survey of literature pertaining to this review was concluded in November, 1952.

[2] The following abbreviations are used: ATP (adenosinetriphosphate); DPN (diphosphopyridine nucleotide); TPN (triphosphopyridine nucleotide); 2,4-D (2,4-dichlorophenoxyacetic acid); 2,4,5-T (2,4,5-trichlorophenoxyacetic acid).

[3] Grateful acknowledgment is made to Drs. J. A. Bentley, J. C. Crane, D. N. Duvick, A. F. Faull, M. A. Joslyn, A. C. Leopold, L. C. Luckwill, A. E. Murneek, H. M. Sell, M. L. Stehsel, F. S. Steward, J. Strauss, and W. H. Thomas for sending manuscripts in advance of publication.

been defined, the subject of this article can now be made clear. It deals with growth processes and, more precisely, with the growth processes of the tissues which support the ovules of a plant. The story of the growth of fruits begins in the floral primordium, in which the origin of the fruit tissues can be found. Many authors, however, have implicitly considered that actual fruit growth begins at pollination. Such a view is not justified, for pollination may occur at different stages in the development of the ovules and ovaries. In orchids, pollination precedes the differentiation of true ovules. In most higher plants, the development of the ovules and the ovary is much more advanced when pollination occurs. In certain cases, the development of an ovary into a fruit can proceed even without pollination, as in the cucurbits (210). In this review, the physiology of fruit tissues will be considered beginning with the flower primordium except that the aspects of fruit physiology which concern nongrowing processes such as ripening [see Biale, (14)], as well as the details of pollen physiology, will be left aside. After a glance at the principal techniques used in this field, the general patterns of fruit development will be described. Then will follow a discussion of the mechanics of the growth phenomena, including a study of the sources of the energy available for building up the tissues and the synthetic processes at work in elaborating protoplasm, cell walls, and vacuoles. This complex biochemical work is controlled by growth centers through the release of growth regulators, which will be examined last.

Methodology

Growth measurements.—The most direct way to measure the "irreversible increase in volume" which results from the growing process is, of course, to measure volume itself. Direct volume measurements can be made on fruits that are located and shaped in a way that allows the use of the method by water displacement (81).

When fruits have a regular geometrical shape, their volume may be calculated from one or two linear dimensions. With such fruits, one must be certain that the shape of the fruit remains unchanged from the beginning to the end of the experiment. The fruits of some cucurbits fulfill this requirement (262). Thus, one may be justified in measuring one dimension only to get an index of the growth of the whole fruit instead of the more delicate volume determinations. The length and the width are sometimes interchangeable, as in the silicule of *Capsella bursa-pastoris*, in which a linear relationship exists between these two dimensions (321). In other fruits, for instance, the silicule of *Lunaria biennis*, this linear relationship between length and width fails at a certain point so that two "laws of disharmony" are made apparent (320). Be it direct volume determination or measurement of length and diameter, these methods have the important advantage of being easily performed on the same fruit while it remains attached to the plant and continues to grow.

Periodical sampling.—Chemical analyses, on the other hand, require

the destruction of the fruit. Thus, it is not possible to follow the chemical changes in the same fruit, but it is necessary to sample periodically a certain number of comparable fruits. In actual practice, however, it is not as simple as it may seem to obtain comparable fruit. Some plants bloom at one time of the year, and subsequent fruit development spreads over a considerable period of time during which the weather often changes. Climatic conditions are known to exert a profound influence upon the chemical composition of fruits (water, sugars, ascorbic and other organic acids, etc.). To minimize this cause of error, one might resort to plants which bear flowers and fruits throughout a long period of time, such as members of the genus *Citrus*, the family Solaneaceae, etc., which would allow fruits at various stages of development to be gathered at the same moment. Even with these, however, an old fruit may have been subjected to environmental conditions different from those of a young one. The best solution to this problem seems to be the use of climatically controlled rooms, such as those at the California Institute of Technology (352) in which reproducible growth can be achieved.

In vitro culture.—The study of fruit nutrition has remained in a vague and general status because of the inadequacy of the techniques. When studying the factors which are responsible for fruit growth, one is generally compelled to experiment on the whole plant. For example, fertilizers are applied around the root system and the growth of the fruits is observed. How can such a method enable us to determine which fraction of the fertilizer is essential for the development of the stem, the leaves, or the fruits? How can we distinguish between a direct effect on the fruits and an indirect one, i.e., a stimulation of root or leaf growth which, in turn, would promote fruit enlargement? Also, how can we find out what substances are synthesized in the vegetative parts and then translocated to contribute to the development of the fruits?

Recent attempts to obviate these difficulties by using excised flowers and fruits have met with success with a variety of plants including tomatoes, gherkins, beans, strawberries, and tobacco (203, 204). Young ovaries were planted aseptically in test tubes or flasks containing a suitable nutrient medium. Freedom from microorganisms is essential to secure valid results. The most practical way to disinfect tomato flowers has been found to expose them to calcium hypochlorite, which is then washed away with sterile water (204). Traces of the chemical which might possibly remain are rapidly destroyed by CO_2 and acids present in the tissues. A check of mold growth for one week has been obtained by using sulfanilamide (142). The development of excised flowers into mature fruits has been achieved *in vitro* under strictly sterile conditions. The growth curve of such fruits has the sigmoid appearance which is usual with fruits remaining attached to the plant. On the other hand, test tube fruits were generally much smaller than those grown on the plant. They often showed aborted seeds, except in the case of pollinated gherkins from which viable seeds were obtained (203, 204). Although at its beginning, the new technique of culturing excised fruits

in vitro promises to become a valuable tool in many investigations concerned with fruit physiology.

The Life History of a Fruit

Growth curves.—When establishing growth curves of fruits, authors generally combine the fruit tissues and the seeds as when volume or diameter of the ovary is plotted against time. Be it by volume obtained by direct determination (81) or by calculation (262), fresh or dry weight (81), diameter (203, 206), etc., one generally obtains the S-shaped curve which is characteristic of the growth of both animal and plant organs and organisms. A more detailed study of fruit growth requires a division of the life of the fruit into periods: (*a*) the initiation of the fruit tissues; (*b*) the pre-bloom development; (*c*) the post-bloom growth; and (*d*) maturation and senescence.

The initiation of the fruit tissues.—Although many people speak of a fruit only after pollination, it remains true that the tissues which constitute this fruit do not arise *de novo* at the time the fruit is "set." These tissues derive from cells which have been initiated a long time before. A study of the initiation of fruit tissues might include, therefore, first, the initiation of the floral primordia and second, the differentiation of the floral parts inside these primordia. The first aspect of such a study has been excellently reviewed by Lang (138) and will not be considered here. The second phase deserves much more investigation than has been devoted to it so far. On the whole, climatic factors, especially light and temperature, seem to influence the fate of the floral primordium. In hermaphroditic flowers, the tomato for example, such an effect can be detected (103), but it is more apparent in dioecious and monoecious plants in which flowers with and without pistils are separated. Although genetical factors certainly play a role in determining sex in plants (2, 42, 43), environmental conditions can superimpose on the genetic effect in regulating the formation of pistils and, thus, of fruits. In dioecious plants, such instances have been reported for *Melandryum rubrum* (27), *Morus alba* (249, 251, 252), *Cannabis sativa* (24, 306), etc. Among monoecious plants, *Zea mays* (248, 250), *Ambrosia trifida* (119, 164), *Cucumis sativus* (55, 307), *Cucurbita pepo* (203, 210), and *Ficus carica* (331) have been studied, particularly. In general, during winter days in which light and temperature are at a low level, the formation of pistillate flowers is promoted. A detailed study of this effect in *Cucurbita pepo* revealed an endogenous tendency to produce at first pistilless flowers (male flowers) and then pistillate flowers (female flowers). This development could be hastened or delayed by light and temperature treatments. The fact that growth of the pistil was affected became evident when ovaries of increasing sizes developed successively, the whole evolution culminating in the formation of parthenocarpic fruits (203, 210). The biochemical effect of low night temperatures upon the development of the pistil is not yet elucidated. The production of female flowers in cucurbits by the use of α-naphthalene

acetic acid at sites where male flowers are normally produced points toward auxin metabolism (135, 136, 210).

Pre-bloom development.—The ovary primordium, once initiated, usually develops concomitantly with the flower. The factors responsible for this growth are often the same as those which promote flower initiation since maintenance of the inductive photo- and thermoperiod increases the rate of development of the floral primordia. Not all the biochemical mechanisms inside the flower bud remain unchanged, however. For example, a drop in the auxin level had been correlated with floral initiation in certain plants, whereas subsequent development of the flower seems to be dependent on a renewed auxin supply (147). In tulips and hyacinths, conditions different from those governing floral initiation are necessary to ensure the development of the flower bud (Blaauw and co-workers, see 350).

Many investigators have found that, in the flower bud, ovary growth occurs mainly through cell multiplication. This holds true for cucurbits (260), tomatoes (102), cherries (317), apples (158, 267, 318), etc. Cell division gradually ceases at the time of anthesis, while cell enlargement begins and becomes responsible for the later part of fruit enlargement. The S-shaped growth curve of the ovary, however, does not indicate when this change takes place, so that Sinnott (261) considered that, from the point of view of the growth process, distinction between cell division and cell enlargement was not important. The regular increase in ovary size goes on regardless of cellular changes. The important entity seems to be the organ rather than its constituents. It is true, nevertheless, that cell division apparently continues longer in the ovary of large-fruited varieties, the final size of these fruits being a consequence of the increased number of cells as well as of an increase in the average cell size (102, 260).

A critical phase: anthesis.—Once started, the smooth sigmoid curve which reflects the growth of the fruit does not unfold itself automatically. Should pollination be prevented, the flower often drops off, and growth of the ovary is brought to a rapid end. The maintenance of the ovary on the plant is a prerequisite of fruit growth, but not its cause. As a matter of fact, unpollinated ovaries of certain species such as *Cucumis anguria* do not drop if unpollinated; yet they stop growing (203, 206). In addition, flowers that normally abscise, such as those of the tomato, would stop growing if unpollinated, even though they are planted *in vitro* on the same medium as that which supports the growth of pollinated flowers (203, 204). The cessation of the growth of the ovary which occurs in unpollinated flowers at the time of anthesis constitutes a challenging problem, for we are dealing here with cells that are capable of growing, yet fail to do so.

The stimulus which ensures the continuity of fruit enlargement through the anthesis period comes from the pollen. In many plants, it is difficult to separate clearly the effect of pollination alone from that of fertilization (syngamy). In more primitive plants, however, the two effects can be easily

distinguished. In *Ginkgo*, for example, pollination stimulates the formation of an important prothallium enclosed in a differentiated integument. If no pollen is received, these tissues do not develop. In tropical orchids, also, a long period of time separates pollination from fertilization. In this material, the growth stimulating action of pollen has been demonstrated unambiguously (51, 63, 104). Fecundation occurs much later and gives a second growth stimulus. The significance of this stimulation by the male gametes for the development of the female tissues has been summed up by Mangenot (162) for the whole plant kingdom. In higher plants pollen has a definite, yet often limited, effect on several mechanisms which control fruit growth: it inhibits the development of the abscission layer which would bring about flower drop; it also stimulates the growth of the fruit itself (84, 366, 367, 368). What is still more important, the pollen grain contributes two sperm nuclei which unite with the egg cell and the fusion nucleus, respectively, thus giving rise to the fertilized ovule which soon takes up the leading role in controlling fruit growth.

Post-fertilization development.—Among the numerous species in which the stimulating action of the developing ovules on fruit growth has been established, one might mention the strawberry, in which removal of the ovules at almost any time after fertilization stops growth in the fleshy receptacle (200, 202). In fruits in which ovules are few, such as the stone fruits, three growing periods have been distinguished (313, 315). During Period I, the fruit enlarges rapidly and the integuments of the seed develop, but the embryo remains small. During Period II, the embryo develops, but the growth of the fruit stands still. As embryo growth ceases, the fruit enters a new period of active enlargement, which constitutes Period III. These observations point toward a certain competition between developing embryos and fruits. Such a view is strengthened by the correlation found by Tukey between embryo abortion and earliness in stone fruits (314, 316). The fact that fruit enlargement is slowed down when active seed growth takes place can be observed also during the development of dry fruits. In these fruits, only Periods I and II exist, i.e., at first a simultaneous development of the fruit and of the ovule, then a period during which the seeds alone grow. Such a succession of events may be found in the garden pea (16), in *Lunaria biennis* (320), and *Ervum hirsutum* (321). A distinction between three stages in the postfertilization development has recently been made for the apple (151), the fig (44), and the blueberry (369). In the strawberry, if the over-all diameter is plotted against time, a rather smooth sigmoid curve is obtained; if, however, the diameter of the achenes is deducted from the total, then a decrease in the growth rate of the receptacle can be observed 12 to 14 days after pollination, after which the receptacle resumes growth a week later (203, 206).

In summary, the life history of a fruit can be divided roughly into two main growing periods: preanthesis during which growth proceeds mostly by cell division, and postfertilization during which cell enlargement is the promi-

nent feature. The latter period includes the bulk of the enlargement of fruits and is often considered alone. A critical phase separates the two growth periods: anthesis and pollination. The end of fruit growth is marked by the period of maturation and senescence, which has been dealt with in detail by Biale (14) and Ulrich (329) and will not be reviewed here. The remainder of this article will be concerned with the physiological and biochemical processes which underlie the life history of fruits as just described.

Energetics of Growth

The over-all respiration curve.—Respiration, the process by which animal and plant tissues obtain the energy needed for growth, is generally studied with the aid of manometric techniques which measure the O_2 absorbed and the CO_2 evolved. A complete set of data on the respiratory activity of fruit tissues from the initiation of the primordium to the maturation of the fully grown organ would be desirable. Unfortunately, our knowledge in this respect is still fragmentary. While respiratory curves have been established for the period following fertilization, data on the matter become rare when the time of pollination is considered and practically nonexistent for the preanthesis period.

The emission of heat by certain inflorescences and flowers (palms, *Arum*, *Cereus*, etc.) indicates a high rate of respiration. Detailed studies of the respiration of slices of *Arum* spadix have recently been made by James & Beevers (115). In such instances, however, the site of the highest respiratory activity may be the stamens rather than the ovary, so that an over-all picture cannot give much information on respiration of the tissues of the future fruits.

Pollination and fertilization strongly stimulate the respiratory rate of ovaries (355), but the specific effect of each of these phenomena are often difficult to separate in time. This is not so with tropical orchids, in which fertilization occurs several months after pollination. Working with slices of orchid ovaries, Hsiang (105) showed that pollination alone could cause a 3.5 fold increase in respiration some 48 hr. after the application of pollen to the stigma.

If considering the period of fruit life which follows fertilization, then respiratory data become more plentiful. Respiratory studies have been made on such fruits as the apple (125), the pear (322, 329), the grape (71), the tomato (38, 82, 327), *Sorbus* (362), *Bryonia dioica* (323), *Solanum dulcamara* (325), and even the dry fruits of *Syringa vulgaris* (328). The experimental data show, in general, similar trends in the different species. If one considers the respiratory activity per fruit, then the curve obtained rises regularly from the time of fertilization up to maturation. If the respiratory rate is expressed per unit weight, then four periods are made apparent, namely: (*a*) a period of high respiratory rate, characteristic of the young fruit, (*b*) a period of much lower respiratory rate while cells enlarge enormously, (*c*) a sudden rise which has been termed "climacteric" by Kidd (125) and

which marks the onset of the maturation process, and (*d*) a decrease during the senescence and breakdown of the tissues. Among these periods, only the first two correspond to actual growth and hence, are considered in this review. The continuous drop in the respiratory rate per unit weight during (*a*) and (*b*) is mostly due to an enormous increase of the vacuoles in fruit cells. When expressed on a per protein basis, respiration remains practically constant throughout the growing period of the fruit (110).

The data given in the literature generally concern the tissues of the fruit and those of the ovules taken as a whole. One could ask, therefore, what is the respiration of the fruit alone as compared to that of the seeds. Some indication can be obtained from Ulrich's measurements on *Hedera helix* (324). In this plant, seed tissues become predominant over fruit tissues after a certain time, and the shape of the respiratory curve is significantly modified. In stone fruits, an arrest in the growth of the pericarp is noticeable at the time the embryo develops. At the same time, the respiratory rate of the whole fruit levels off. When the embryo finishes growth and the pericarp resumes enlargement, then the respiratory rate increases again (243, 326). These observations bring up the question: what is the specific respiratory rate of each fruit tissue? It was found by Hackney [(329) p. 171] that the production of CO_2 is higher from slices taken near the skin of the apple than from those taken inside the flesh.

Among the external factors which influence the overall respiratory activity of fruits, the following may be mentioned: concentration of O_2 and CO_2; temperature; humidity; and nutrition. The partial pressure of oxygen plays an important role in the respiration and growth of plant organs. Reduction of the oxygen concentration to 1 per cent caused a marked reduction in the fruit set of excised tomato flowers (142). Respiratory activity increases very rapidly with temperature (Q_{10} about 2.5) up to a maximum, after which a sharp drop occurs. A good example of this behavior is furnished by the strawberry, in which the maximum is situated around 35°C. (74). Humidity influences the respiration of fruits in storage, but little study has been done on the effect of humidity on growing fruits. As to the effect of nutrition, nitrogen fertilization has been reported to increase the respiration of picked apples (268).

The fuel: the metabolites.—An indication of respiratory substrates in fruits can be obtained from measurements of the respiratory quotient (R.Q.). A relationship between the R.Q. and stage of development is difficult to establish because the R.Q. varies appreciably with temperature. Using a seedless variety of grape, in which, therefore, seed respiration does not interfere with that of the fruit, Gerber (73) obtained the following data: between 0° and 20°C., the R.Q. of the grape is 0.7; around 20°C., it reaches 1.0; at 30°C., it is 1.2; and at 35°C., it becomes 1.6. These facts have been interpreted by Gerber to indicate that under 20°C. sugars are incompletely respired and malic acid is formed, at 20°C. sugars are completely metabolised, at 30°C. malic acid is chiefly respired, and at 35°C. tartaric acid also is re-

spired. This indicates that sugars and organic acids may constitute the chief metabolites used up in fruit respiration. We will now review briefly these two types of metabolites, leaving aside other compounds that may be used in fruit respiration but which are less known.

(*a*) Sugars: The concentration of sucrose, rather than glucose or fructose (125, 127), most nearly parallels the rate of respiration of a fruit during its development. Young ovaries are generally green, as are also sepals and bracts which often remain attached to the growing fruit until maturity. All these structures may contribute photosynthates to the fruit. It remains true, nevertheless, that leaves exert a profound influence upon fruit growth. Reduction of the leaf area below a critical value reduces fruit growth in pineapples (334) and tomatoes (142). Such a reduction is especially reflected in a lowering of the total sugar content, as in the apple (329, p. 223).

As tested by the *in vitro* technique, sucrose seems to be one of the essential components of the medium on which excised tomato and gherkin fruits can be grown successfully (201, 204). Sucrose can be replaced by glucose, but fructose seems to be more effective than these two sugars in promoting fruit set in tomatoes, when a 3 per cent concentration is used (142). Aside from the better known sugars, mannoketoheptose is found in the avocado (133), and lactose is particularly abundant in the fruits of *Achras sapota* (329). The sugar-alcohol sorbitol may exist in rather large quantities in pears (3 per cent of the fresh weight) and be used up in respiration (177). In the Kelsey plum, young fruits may contain as much as 1 per cent sorbitol when the total sugar concentration is 4 per cent of the fresh weight, but accumulation of the sugar-alcohol up to 2.8 per cent begins only after stone growth is completed and the concentration of reducing sugars has reached a constant level. These observations led Donen (50) to suggest that sorbitol rather than hexoses was stored in the plum. There was no obvious relation between sorbitol and sucrose concentration. Sorbitol in plums, as in pears, disappears rapidly during storage.

Starch, the common form of sugar storage in plants, is found in the actively growing fruit during the time when carbohydrate material enters this organ in quantity. As growth slows down and maturation sets in, starch rapidly disappears. This is especially conspicuous in fruits which are rich in starch, such as the banana (11, 13). The relationships that may link together the metabolism of sucrose, reducing sugars, and starch during fruit set have been studied in several fruits, for example, the banana (13) and the tomato (172). A banana inflorescence may bear flowers and fruits at the same time. Sugar determinations in various parts of the inflorescense gave the following results: in the peduncle, the proportion of reducing sugars diminishes while that of sucrose increases as one moves from the base toward the flowers and fruits; neither starch nor reducing sugars is found in the flowers, but only sucrose; in the fruits, the sucrose level is low, reducing sugars are practically nonexistent, but starch is very abundant. In the tomato, when growth of the ovary is stimulated by either pollination or the application of auxins

the concentration of sucrose decreases, while there is an increase of both starch and reducing sugars as well as starch phosphorylase activity (293). The effect of the developing seeds on fruit growth is reflected in the sugar concentration, a richness in total sugars of the ripe fruit being associated with high seed number (129, 192).

(*b*) Organic acids: Di- and tricarboxylic acids are very abundant in fruit tissues. While they often amount to 1 to 2 per cent of the fresh weight in apples and pears, their fraction may reach as much as 4 per cent in black currants (80) and 7 per cent in peeled lemons (258). A small portion of these acids is present in salt form, but the bulk remains in the acid form. The identity of these acids can be ascertained by many methods. The older methods, using lead salt precipitation (128), diazomethane esterification, etc., are cumbersome and difficult to put on a quantitative basis. Micromethods have been devised (233, 234), and manometric techniques using enzyme preparations have been described (330). The new technique of chromatography (28, 176) offers many advantages, especially identifying acids which have been left aside in the standard techniques [in plums, 17 to 45 per cent of the total acids have not been determined (227)]. Using such a technique, Hulme (111) has isolated recently a new acid from the apple, L-quinic acid.

What, then, are these polycarboxylic acids that are found in fruits? They are principally malic (pome fruits), citric (citrus fruits), tartaric (grapes), and isocitric acid (blackberries). Instead of showing the predominance of one particular acid, as in the fruits just mentioned, many fruits contain a mixture of these principal acids (301).

The general pattern in growing fruits is one of progressively increasing organic acid content. A decrease in acid ocurs during ripening, when growth has been completed. This sequence is apparent in the apple (5, 109), the black currant (80), the orange (259), and the grape (75). In addition to this general trend, the organic acid content varies also with external factors, such as climate and nutrition. It is well known that fruits grown in a cold and rainy climate are sour. Peynaud (223) found a correlation between climate and the tartaric acid content of grapes grown near Bordeaux, France. During a dry, sunny period, the acid content could decrease to half its value, according to the variety; it increased again during rainy periods. An effect of climate, especially of temperature, on the organic acid metabolism has been demonstrated in leaves of *Bryophyllum* (232, 361). Relatively low temperatures at night stimulate the formation of organic acids, whereas temperatures higher than 30°C. cause the acid level to decrease. The compound which is mainly responsible for this acidification is malic acid (338). This explains why the presence of a suitable CO_2 concentration is essential. In fact, experiments with tagged CO_2 actually show that CO_2 is fixed by the leaves to produce malic acid in a reaction similar to the Wood & Werkman reaction (305). Studies concerning the effect of temperature on ripening in stored fruits also indicate that the lower the temperature, the higher the acid content. In Gerber's opinion, organic acids are metabolised in respiration at

high temperatures, whereas, at low temperatures, they are actually formed. The temperature necessary to induce the respiration of malic acid is lower than the one for tartaric acid. Accordingly, fruits rich in tartaric acid (grapes) require higher temperatures for ripening than fruits which are rich in malic acid (apples). Citric acid needs a still higher temperature to be metabolised, so that fruits which contain this acid remain acid when mature (currants, citrus fruits, etc.). Finally, some nutritional effects on the organic acid content have been reported, such as an increase in the citric acid content of grapefruits following Mg or K fertilization (263). On the other hand, the form of nitrogen which is made available to the plants greatly influences the organic acid content, the malic acid content being 20 times higher when nitrates are given instead of ammonium salts (340).

The site of production of organic acids in fruits seems to be analogous to that of sugars, i.e., they are probably translocated from the leaves. This can be inferred from the fact that acidity in grapes and in apples is higher in the center than at the periphery of the fruit (71, 73) and that a reduction in the leaf area decreases the acid content (9). Organic acids are assumed to be localized in the cell vacuole. A theory has been put forward by Kidd *et al.* (126) in which the formation of acids in the apple and their secretion into the vacuole are considered as side reactions of the cell-wall metabolism.

The role of the various organic acids in fruits is a complex one, for it affects not only respiration but also protein synthesis. We know that these compounds constitute a general pool of raw materials on which the cell can exert its chemical activities in many ways, adding a carboxyl group or taking it away, transferring NH_2- groups, etc. The role of such acids is, thus, a central one in cellular metabolism. It is no wonder, therefore, that Leopold & Scott (142) found a distinct influence of these acids on fruit set in excised tomato flowers. At a 10^{-4} *M* concentration, malic acid was the most effective acid, whereas at 10^{-3} *M*, fumaric acid scored higher than malic, α-ketoglutaric, citric, or succinic acids, all of which increased fruit set from 35 to 45 per cent over the water controls. The outstanding effects of both malic and fumaric acids may be related to the stimulation of malic and fumaric dehydrogenases by parthenocarpy-inducing auxins (294). On the other hand, oxalic and acetic acids had practically no effect on fruit set. The inefficiency of acetic acid seems surprising in view of its growth-promoting effect on the oat coleoptile (21, 300) and of its importance in animal respiration (36). It is known that acetate metabolism requires pantothenic acid as a constituent of Coenzyme A, which is present in the tomato (122). In a recent study, Leopold (144) has been able to show that if pantothenic acid is added (10 p.p.m.), acetic acid (up to $10^{-3}M$) does increase fruit set as much as malic acid. Fluoroacetate counteracts this effect.

The bulk of the acids present in fruits are of the same nature as those operating as intermediates in the Krebs cycle. The comparatively large amounts found in fruits, however, point toward other functions. A direct role for malic acid in the respiration of fruits has recently been suggested

(143). It involves the decarboxylation of malic acid by malic decarboxylase in the presence of Mn^{++} ions to yield pyruvic acid and CO_2. At the same time triphosphopyridine nucleotide (TPN) is reduced, which starts a sequence of oxido-reduction reactions involving glutathione and ascorbic acid. The experimental facts are that glutathione and Mn^{++} ions increase fruit set of excised tomato ovaries over the malic acid level; the addition of TPN (in the form of liver concentrate) or of ascorbic acid further increases fruit set. On the other hand, fruit set is inhibited by the addition of the products of the reaction, CO_2 and pyruvic acid. The presence of malic enyzme and of glutathione reductase has been reported in the avocado, the cantaloupe, and the cucumber (4). The two latter fruits are very rich in these enzymes, especially the cucumber whose activity, on a dry weight basis, is three times that of wheat germ.

The machinery: enzymes and coenzymes.—Only scattered bits of information exist about respiratory enzymes in fruit tissues. Alcohol, fumaric, glutamic, citric, lactic, malic, and succinic dehydrogenase activity has been detected in pollen (212, 341) and some information has been reported about diphosphopyridine nucleotide (DPN) in the pollen of *Salix* and *Populus* (342). Glucose-6-phosphate dehydrogenase has been reported present in avocadoes, cantaloupes, and cucumbers, the latter fruit showing an activity three times superior to that of the highest activity known for plant tissues, that of wheat germ (4). On a dry weight basis, the isocitric dehydrogenase activity of the cucumber fruit is twice as great as that of wheat germ. A stimulation of malic and fumaric dehydrogenase activity (up to 300 per cent of the controls in the first case, and 200 per cent in the second) has been reported by Teubner & Murneek (294) as a result of auxin applications to tomato ovaries; the activities of succinic and glutamic dehydrogenases, on the other hand, were slightly depressed. Data on one of the constituents of DPN[2] and TPN,[2] namely niacin, include the amount in flowers and ripe fruits (20) and studies on the B-vitamin changes during growth of cucurbit and tomato fruits (358, 359). In general, high vitamin levels occur during the early growth period of the fruit, when cell multiplication is active; a decrease in the concentration of all B-vitamins is apparent during subsequent growth by cell expansion.

Among the recent studies concerning flavoproteins in inflorescences, the work of James & Beevers (115) on the *Arum* spadix is of special interest. This organ has an unusually high rate of respiration which is insensitive to cyanide poisoning. Addition of riboflavin and adenosinetriphosphate (ATP) increases the oxygen uptake by the expressed sap. A compound which had the properties of a flavoprotein was finally extracted from the *Arum* spadix, as had been done previously with *Sauromatum* (332). The respiration of the avocado fruit also is insensitive to cyanide and is probably mediated by a flavoprotein (175). Determinations of riboflavin in cucurbits and tomatoes have shown that, on a dry weight basis, the concentration of this vitamin decreases from pollination to ripening (358, 359).

Aside from studies on pollen (213, 214, 254), little is known about the cytochrome and cytochrome oxidase systems in flowers and fruits. Hackney (90) failed to isolate cytochrome C in apples, but cytochrome oxidase activity has been found in this fruit as well as in the tomato (241). On the other hand, seeds are known to be rich in cytochromes, since Goddard (77) isolated cytochrome-*c* in pure form from wheat embryos.

Data on other heme-containing enzymes, such as catalase and peroxidase, are more numerous, perhaps because the techniques for detection and measurement of these enzymes have been known for some time. Catalase, which decomposes hydrogen peroxide into water and molecular oxygen, has been detected in pollen (149, 212, 220, 341), in the flower buds of several species (30, 360), in tomato fruits (86), and orchid ovaries (105). The early conclusion of Gustafson and co-workers (86) was that high catalase activity accompanies a high respiratory and growth rate. Wittwer (360) associated high catalase activity in pear flower buds and corn flowers with synapsis and syngamy. A marked drop in catalase activity followed fertilization and lasted while the corn grains were rapidly developing and accumulating dry weight. A somewhat concordant picture has recently been presented for tomato fruits, in which the oxidase activity strongly decreases as fruit growth proceeds while the activity of carbonic anhydrase increases. At the beginning of the maturation period, catalase activity increases again (309). On the other hand, Hsiang (105) observed a marked stimulation in the catalase activity of *Cymbidium lowianum* some 20 hr. after pollination. This first effect was followed by a gradual drop of the catalase activity 50 to 75 hr. after pollination, and by a new rise three days later. As to peroxidase, which catalizes the decomposition of H_2O_2 with simultaneous oxidation of a variety of phenols and aromatic amines, it has been found in the pollen of many plants (212) and in many fruits (58, 215, 216).

The copper-containing enzymes, polyphenoloxidase and ascorbic acid oxidase, seem to be abundant in fruits. The first one has been reported present in many species (58, 215, 216), and its substrate, the phenols, has been measured quantitatively in peaches (226), plums (227), etc. Ascorbic acid oxidase is particularly abundant in cucurbit fruits (290) from which it has been purified (52, 231, 285, 308), but it is also present in tomatoes (113), bananas (284), string beans, and various flowers (187, 272).

The substrate for ascorbic acid oxidase, ascorbic acid, is also abundant in fruits. In certain species, ripe fruits seem to be the organs which contain the highest concentrations of ascorbic acid, as shown by the following data for *Capsicum* (in mg. ascorbic acid per 100 gm. fresh weight): fruits 300 to 340 mg.; leaves, 100 to 170 mg.; peduncles, 32 mg.; stem, 10 mg.; and roots, 0 mg. (270). Thanks to the importance of ascorbic acid in animal metabolism, extensive data on the vitamin C content of fruits are available. For example, concentrations of this substance not only in various species, but even in different varieties within one species are given for the plum (225, 229), peach (224, 229), apple (291), etc. Among fruits that are richest in

ascorbic acid rank certain tropical fruits, such as *Detarium senegalense* (2,000 mg. per 100 gm. edible fruit), *Actinidia chinensis* [500 mg. (329)], *Psidium guajava* (95 to 300 mg.), and *Mangifera indica* [15 to 115 mg. (179)]. Three fruits of the temperate zone, the rose hip (400 to 1,500 mg.), the black currant (100 to 400 mg.), and the strawberry [52 to 107 mg. (94)] come before the citrus fruits (20 to 90 mg.). Sometimes the petals of the flower are richer than the fruit: 420 mg. against 84 (edible portion) in *Citrus trifoliata* (269), 121 mg. against 95 in *Nymphea*. In the latter case, the ovules at anthesis are still richer [253 mg. (272)].

According to Peynaud (224, 229), 90 per cent of the ascorbic acid of the peach is not free in the vacuole, but "fixed," and can be liberated through acid hydrolysis. This observation is of special interest since Newcomb (198) has reported that ascorbic acid oxidase is bound to an insoluble fraction of the cell, namely the cell wall. Many authors have found that ascorbic acid concentration in fruits increases from the center to the periphery, and that the colored portions are richer than the less colored ones (224, 225, 291). The latter is true only for a given variety and does not mean that an apple variety which is red contains, *ipso facto*, more vitamin C than a variety which remains green (291). The correlation between color and high vitamin C content within the same variety is probably a consequence of high insolation, for exposure to full sunlight is known to increase the ascorbic acid content (61, 94, 159, 187, 240). Temperature also has an effect (3).

The origin of the ascorbic acid present in fruits is not quite clear yet. It is true that a large leaf area per fruit results in a high ascorbic acid content of the fruits (187). Shading of the leaves decreases significantly the ascorbic acid content of strawberry fruits. A 5 to 6 day lag period in the response of the ascorbic acid level in the fruits to the shading of the leaves suggested that a precursor rather than ascorbic acid itself was translocated from the leaves to the strawberries (240). In the guava, Le Riche (145) noted the progressive disappearance of sucrose while fruits were developing, and the concomitant accumulation of fructose and ascorbic acid. He concluded that ascorbic acid synthesis proceeded from the glucose fraction. This interpretation is in agreement with Moldtmann's (187) experiments, in which leaves produced more ascorbic acid when infiltrated with glucose. Recently, Smith (265) proposed a scheme for ascorbic acid formation in potato tuber slices, according to which fructose-1,6-diphosphate is the precursor of vitamin C through a series of steps involving dihydroxyacetone-phosphate and L-sorbose-1,6-diphosphate. It might be of interest to look for a possible link between the metabolism of sorbose and that of ascorbic acid in fruits. The rise in the vitamin C content toward maturation does not seem to be a general feature for all kinds of fruits. It has been reported in peaches (224), tomatoes (76, 187, 222, 309), and *Capsicum annuum* (187). On the other hand, a slight decrease has been found in bananas (304) and certain grapes (71); in still other fruits, the ascorbic acid level did not vary appreciably

during development. Zilva, Kidd & West (370) reported that the total concentration of ascorbic plus dehydroascorbic acid remained constant throughout the life of the apple, the reduced fraction increasing toward maturity. Giroud *et al.* (76) sought a correlation between richness in carotenoids and ascorbic acid content, and Moldtmann (187) confirmed that the final rise in the vitamin C concentration in tomatoes and *Capsicum* occurs precisely at the time chlorophyll disappears and the coloration due to the carotenoids becomes noticeable.

As to the mechanism of action of the ascorbic system in plants, much has yet to be investigated, although some interesting leads have developed. Polonovski & Robert (230) distinguish three classes of catalytic actions of the ascorbic acid system, namely, oxido-reductions, the hydrolysis of esters, and decarboxylation. That ascorbic acid oxidase may function as a terminal oxidase in plants has been pointed out by James & Cragg (116) and others. In this case, ascorbic acid is oxidized by molecular oxygen to form dehydroascorbic acid. The latter can be reduced again in the presence of glutathione by ascorbic acid dehydrogenase, or by glutathione alone (22). The yield of this reduction can be doubled by catechin (219). Glutathione has been found in tomatoes (222, 309), and catechin, i.e., 3,5,7,3′,4′-pentahydroxy-phenyl-2-dihydrochromane, is present in many fruits, including grape, apricot, strawberry, plum, cherry, green apples and pears, citrus, rose hips, black currants, blackberries, bilberries, *Capsicum*, and *Prunus spinosa* (8, 141, 247). Finally, it should be remembered that the effect of tomato juice in stimulating the growth of excised tomato ovaries over the controls with sucrose only, may be partly due to its content in glutathione and ascorbic acid (201, 204). Indeed, ascorbic acid and glutathione are very efficient in promoting fruit set (142).

The energy: the organic phosphates.—The two main lines through which respiration is known to furnish energy to plant growth are the oxidative and the phosphorylative coupling. The first system produces molecules of DPN^2 and TPN^2 in the reduced state, which yield large quantities of energy when they are oxidized. The second gives rise to energy-rich phosphates, which leads us to study the phosphorus metabolism in fruits.

MacGillivray (161) reported that one half of the total phosphorus in the tomato was located in the fruits. This indicates a high synthetic activity in the fruiting organs, for phosphorus is especially abundant in meristematic and floral tissues (39). Most of this phosphorus is present in the organic form. Pollen is relatively rich in phosphorus, as shown by the data given for many species of both angiosperms and gymnosperms (273, 276). In the apple, the phosphorus concentration decreases from about 12 mg./100 gm. fresh weight after pollination to 1 mg./100 gm. at ripening. Such a drop parallels the curve of the respiratory activity (132). Both pollination and auxin applications stimulate the accumulation of phosphorus in the orchid ovary, whereas the perianth actually looses its phosphorus (105). Redistribution of this ele-

ment inside the plants is thought to occur readily (180, p. 302). Teubner and co-workers (174) reported an increase in the hexose-phosphate fraction after pollination or auxin treatment of tomato ovaries. Phosphomonoesterase and adenosinetriphosphatase activity dropped soon after pollination to increase again markedly afterward (293).

The Building Up of Tissues

Like any other plant organ, a fruit consists of cells with walls, protoplasm, and vacuoles. Up to the time of anthesis, it is the protoplasm which makes up the bulk of fruit tissues. As cell division ceases and cell enlargement sets in during the growth period that follows fertilization of the ovules, the relative volume of the protoplasmic fraction tends to decrease, while the cell wall and the vacuole gain in importance. In addition, the differentiation of certain tissues becomes apparent. Leaving aside fats, pigments, and other miscellaneous constituents, we will review only the information available on the synthetic processes at work in building protoplasm, cell walls, and vacuoles in fruits.

The protoplasm: protein metabolism.—What we call protoplasm is actually a very complex aggregate, which includes the cytoplasm, the nucleus, the plastids, and mitochondria. Nitrogenous compounds make up 40 to 50 per cent of the total dry weight of the protoplasm (180, p. 26). These nitrogenous compounds are chiefly proteins: 60 to 75 per cent of the total nitrogen is protein nitrogen in pollen, 75 to 85 per cent in young ovules (271). Pollination or auxin treatment of orchid flowers results in a marked increase of the nitrogen content in the ovary, with a simultaneous decrease in the perianth (105). In *Lilium croceum*, very rapid protein synthesis in the perianth shortly before flower opening changes suddenly after anthesis, into proteolysis. This proteolysis is so intense that the ovary does not gain immediately as much nitrogen as is being lost by the petals (40). In general, the young fruit accumulates nitrogen as growth proceeds (106). In addition, a further increase in nitrogen is often detectable at the time ripening sets in, as shown in the grape (75, 223), the peach (15), and the apple (107). Peynaud (223) mentions also an increase in the tryptophane content of ripening grapes. A similar increase was quantitatively determined in the strawberry (205). It is possible that the accumulation of nitrogenous compounds in the pericarp at the end of the growing period results from the elimination of the competitive action of the seeds. It is well known that seeds store up large amounts of nitrogenous materials. Carpels may even loose nitrogen for the benefit of seeds (256, 347). When the latter cease to drain to themselves the nitrogen supply, it becomes available again to the pericarp. Another fact of importance is that the protein synthesis which occurs in the ripening fruit is concomitant with an energy yielding reaction: the climacteric rise in respiration (107).

One of the most challenging tasks confronting modern physiology is the

explanation of the mechanism of protein synthesis. A widespread view considers that this synthesis could occur as a reversal of the proteolytic mechanism. Such an hypothesis supposes that both proteolytic enzymes and amino acids are available in the growing fruit. The first requirement seems to be fullfilled, for fruits contain, in addition to the proteases generally found in plant tissues, special ones, such as papain in *Carica papaya*, bromelin in the pineapple, pinguinain in *Bromelia pinguin*, mexicanain in *Pileus mexicanus*, tabernamontain in *Tabernamonta grandiflora*, solanain in *Solanum elaeagnifolium*, etc. (78). Amino acids also are present in fruits. Data concerning apples, apricots, pears, prunes, and avocados have been given (120). Avocados contained the largest amounts of amino acids, pears the least. γ-Amino-butyric acid was present in all the mentioned fruits except in pears. This amino acid has also been found in the apple (112), and in citrus fruits, as has α-amino-butyric acid in certain fractions from the citrus material (310). Changes in free amino acids of the developing corn kernel have been investigated (53), and data on the total amino acid content (after hydrolysis of proteins) are reported for cucumber, okra, pumpkin, summer squash, tomato (157), and the apple (112). Among these amino acids, arginine is of special interest because of its growth-promoting properties (18, 337), and also because it has been found in high concentrations in the growing points of shoots (282). Hydroxyproline, which has seldom been detected in the free state in plants, has been reported present in pollen (6) together with amino acids, and in prunes (120). Unknown compounds have been detected on paper chromatograms of extracts from beans and summer squashes (281).

Thus, both certain proteolytic enzymes and free amino acids are present in fruit tissues. A reversal of the hydrolytic mechanism to a synthetic process is endergonic, however, and necessitates a simultaneous and important supply of energy. It is true that the often cited value of +3000 cal. per mole for the free energy change in the synthesis of a peptide bond by a condensation reaction does not always apply. The free energy change fluctuates between the wide limits of +400 and +4000 cal. per mole (66). Nevertheless, peptide bond synthesis by proteolytic enzymes does not proceed unless some very special conditions are fulfilled. Among them is the rapid precipitation of the peptide formed, which led Fruton (65) to suggest that nucleic acids function as insolubilizing agents for the synthesized products, which would enable the reaction to continue. In fact, it is generally admitted that nucleic acids take an important part in protein synthesis. Amino acids may combine with the phosphorylated groups of nucleic acids with subsequent formation of peptide bonds, after which the nucleic acids would be regenerated (35). Our knowledge of nucleic acids in fruits is still very meager, despite a few data on pollen (275, 343).

The form in which nitrogen is supplied to the growing fruit by the plant is still open to investigation. Nitsch (203, 204) found that in the presence of sucrose, nitrates could support the growth of excised tomato and gherkin

ovaries. This is in agreement with Eckerson's (54) finding that, concomitantly with asparagine and some arginine and histidine, large amounts of potassium nitrate enter the culm when the wheat plant begins to shoot. Some improvement over the nitrate level was obtained when casein hydrolysate was added to the basic medium (117, 203). Leopold & Scott (142) reported that all amino acids, except glutamic and aspartic acids, could advantageously replace nitrates for fruit set in excised tomato flowers. Since, however, no carbohydrates were present in those media, this conclusion is open to question, for the stimulation obtained with amino acids was not greater than that caused by certain organic acids alone. The former may have been deaminated into the latter, so that it is difficult to decide if amino acids were effective as such or simply as carbon sources. In fact, it is stated that addition of inorganic nitrogen to media containing ascorbic acid did not improve fruit set over the ascorbic acid level, which points toward the pre-eminent role of organic carbon. It is true that ammonium sulfate without added carbohydrates gave relatively high fruit set, twice that produced by nitrates. Here again the picture is not clear, for sodium nitrate was used and the effect encountered might be due to the interference of the sodium ion. Urea, and especially thiourea, were found to stimulate fruit set in excised tomato flowers (142). When used in addition to sucrose and nitrates, however, urea gave no improvement over the sucrose plus nitrate level (207). Should this hold true for other fruits also, then the urea sprays which are used in horticultural practice may act primarily on the leaves rather than directly on the fruits. Ureids, nevertheless, may account for over 50 per cent of the soluble nitrogen which arrives to the young fruits of *Acer pseudo-platanus*, while amides make up about 10 per cent of this supply (26). Allantoic acid has been found in large quantities in soja pods (277) and may constitute a form of nitrogen transport to the fruits. Allantoin, however, when added to media containing nitrates and sucrose, inhibited rather than promoted fruit growth in excised tomato ovaries (207). On the other hand, the presence of roots greatly stimulates fruit growth *in vitro* (207). Such an effect might be due to the synthetic activity of roots which are able to produce amino acids from nitrates (199). Roots grown *in vitro* have been shown to excrete into the medium amino acids such as alanine, serine, aspartic acid, valine, leucine, and, above all, the amide glutamine (121). This is in agreement with the high glutamine content of the roots of certain species, such as the common beet (339).

The current search for a mechanism of protein synthesis consisting in a reversal of proteolysis has been alluded to above. In carbohydrate metabolism, neither sucrose nor starch are synthesized through reversals of hydrolysis. By analogy, therefore, it seems more profitable to seek for mechanisms different from those involving peptidases. The fact that amides occur abundantly in seedlings and rapidly growing shoots (*Asparagus*) should be noted, especially since the concentration of these amides seems, in some way,

to be correlated with protein synthesis, whether in stems (37) or in seeds (54, 347). In the apple, Hulme (107) could correlate the increase in protein nitrogen with a decrease in asparagine. This amide has been found also in apricots, pears, prunes, avocados (120), and citrus fruits (310). Smaller amounts of glutamine were detected in apricots, prunes, avocados (120), and citrus fruits (310). The mere presence of glutamine and asparagine in fruits is not sufficient, however, to indicate their metabolic importance. Do these amides, in fact, promote growth? Yes, indeed. It is most interesting to note that Rijven (238) found glutamine to constitute the best source of nitrogen for the growth of excised embryos of *Capsella Bursa Pastoris*, but that asparagine had no effect. Leopold & Scott (142) reported that both asparagine and glutamine are effective in promoting fruit set in excised tomato flowers, whereas neither aspartic nor glutamic acids showed any growth-promoting activity.

Glutamine synthesis in kidney and brain tissues proceeds only aerobically (131) and requires the participation of ATP[2] (57, 278). This amide can, thus, be considered as a secondary reservoir of energy. It is, therefore, not surprising that the exchange of the amide group of glutamine with other amines or amino acids can be achieved by enzymes without the need for an outside source of energy. These enzymes, called amidotransferases, were discovered in cell-free extracts of *Proteus vulgaris* (79). They were able to catalyse the synthesis of glutamo- or aspartohydroxamic acids from hydroxylamine and glutamine or asparagine, but not from glutamic or aspartic acids. A glutamotransferase has been found in plants, but it requires manganese and phosphate for its activation (288). A fact of considerable importance is that glutamotransferase activity is strongly inhibited by amino acids, especially glycine and aspartic acid which compete with glutamine in the enzymatic reaction. Indeed, it has been the writer's experience that amino acids are often inhibitory in tissue culture, whether of excised ovaries, undifferentiated calli, or apical meristems.

Glutathione seems to play a role similar to that of glutamine and asparagine. In this case, the γ-glutamyl moiety is transferred to amino acids to yield different γ-glutamylpeptides. Many amino acids are weak activators of the enzyme which splits glutathione, whereas glutamine and its analogs (such as methionine) are strong activators, and dipeptides, such as glycylglycine and glycylalanine, are stronger yet (346). As in the case of glutamine and asparagine, the synthesis of γ-glutamylpeptides from glutathione can proceed without the addition of outside energy. The reaction is reversible, for incubation of a γ-glutamylpeptide and cysteinylglycine with the glutathione-splitting enzyme results in the synthesis of glutathione in an exchange reaction without calling for external energy (346), a difference with respect to the well-known enzymatic synthesis of glutathione from its components by liver extract which requires ATP.[2]

The cell wall.—The grand period of fruit growth which follows fertiliza-

tion consists mainly in cell enlargement, a process which necessitates an increase in the surface of cell walls. These walls include, roughly, a middle lamella separating the primary walls of two adjacent cells, on which, in turn, are deposited the secondary cell walls.

Cellulose has been isolated from the cell walls of pear fruits (101). Hemicelluloses, which are composed of pentoses and uronic acids, have been found in the apple to increase from fruit set to maturation on a per fruit basis, but to decrease during the same period of time, when calculated on a fresh weight basis (356). Arabans are abundant in the juice of prunes (228).

Fruits are relatively rich in pectic material constituting 2 per cent of the expressed juice of certain prunes and plums (227). The middle lamella is composed of calcium pectate and the primary wall contains protopectin. Both calcium pectate and protopectin are insoluble in water. Through boiling with dilute acids, protopectin yields the neutral and water-soluble substance pectin, which is also found free in fruit cells. The pectin of the apple is made out of polygalacturonic acid which is almost completely esterified with methyl alcohol (253). When demethylated, pectin yields pectic acid which is precipitated out from aqueous solutions by calcium or magnesium ions. It is interesting to note that pollinated cucumber ovaries show a clear cut pectolytic activity, whereas unpollinated ones do not (12). In the young fruit, cell walls are thin and little pectic material can be found. As cell enlargement proceeds, the individual cells tend to become spherical and to get loosened from each other, while intercellular spaces are formed. These spaces are lined with relatively thick pectic layers (312), whether in currants (242), or in apples (34). When maturation sets in, the protopectin content of the fruit decreases and pectin is liberated. These changes in the metabolism of pectic material have been studied extensively as far as ripening is concerned (see 14, 329). On the other hand, their relations to fruit growth have been little investigated. Cell wall extension has been assumed to take place as a result of the loosening of the points at which cellulose fibrils intercross (64). The recent electron microscope pictures, however, make this assumption seem rather unlikely, for they show that cellulose fibrils are interwoven in such a fabric that stretching seems very difficult (189). On the other hand, the continuous phase of the young primary wall of growing cells is made out of protopectin (124, 364) in which cellulose strands form only "an open-lace pattern" (354). The possible importance of pectin metabolism in fruit growth will be discussed briefly in the last section of this review, together with the mechanism of auxin action.

The vacuole: water metabolism.—As fruit cells enlarge, the volume of the vacuoles increase steadily. This is correlated with a large uptake of water, so that water may account for 80 per cent of the fresh weight of the ripe grape and up to 90 per cent of citrus fruits. If can be easily demonstrated that such an uptake of water does not occur if growth is not stimulated. Thus, a pollinated ovary of *Cucumis anguria* grows and accumulates water, whereas

an unpollinated one shrivels and dries out, even though it remains attached to a vigorously growing plant (203, 206). Cut discs of pollinated or auxin-treated orchid ovaries take up significantly more water than discs of unpollinated ones; both pollination and auxin treatments increased slightly the osmotic pressure, but this "did not appear to be sufficient to account for the difference in the amount of water uptake" (104). Similar work, but with discs of potato tubers, led van Overbeek (333) and Hackett (88) to formulate the same conclusion *a fortiori* because they recorded not an increase, but a decrease in the osmotic pressure of the sap when water uptake was stimulated by auxins. The water uptake in all these cases is not a passive, but a metabolic one, and is linked with aerobic respiration. Oxygen is necessary in the case of orchid as well as of potato discs. The use of inhibitors led Hackett & Thimann (89) to conclude that the mechanism for water uptake involves "-SH enzymes, a heavy-metal terminal oxidase, the oxidation of acetate, and a phosphorylating mechanism."

In addition to water, the vacuoles of fruits contain many other compounds, such as tannins and pigments, which cannot be studied here (see 329). The general abundance of tannins in young fruits and its possible relationship to both respiration and the metabolism of pectic substances should stimulate further investigations.

The Control of Growth

Fruit growth involves: (*a*) the inhibition of the mechanisms opposed to growth; (*b*) the "attraction" of food toward the growing organs; (*c*) the production of the necessary energy; and (*d*) the stimulation of the synthetic mechanisms. In fact, many of these operations are linked together. Sometimes energy and materials for synthesis are apparently provided, but, still, no growth occurs. It seems, to quote Prof. C. M. Williams, that "Nature is not so much worried about growth itself, but rather about the control of growth" (357a). This situation appears everywhere in animal and plant physiology, for both animals and plants always tend to develop in an organised fashion. Random growth is rare and causes the grave disturbances of tumor and cancer. The control of growth is especially obvious in the case of fruits. It is worth while meditating upon the fact that fruit tissues, having the potentialties for growth, nevertheless fail to develop unless certain regulatory mechanisms are set in motion. The critical period of anthesis and pollination is such a good example of this fact that, instead of the habitual question: "Why does the ovary grow?", it might be more rewarding to ask: "Why does not the ovary always grow into a mature fruit?" (206). This critical phase in the life of fruits has allowed investigators to penetrate somewhat the mechanisms that control fruit development and to single out two growth-promoting centers: the pollen and the ovules.

The role of pollen.—Male organs already inside the bud exert an influence upon the development of the flower and, thus, of the fruit tissues themselves.

In many cases, stamen primordia are differentiated before the ovary primordium (221). The surgical removal of the unripe stamens in the flower bud adversely affects the growth of the ovary if the operation is performed at an early stage (165, 274). In both corn and rye, large amounts of auxin can be extracted from unripe anthers (96, 360). In fact, the largest amounts of the auxin present in the anther of rye occur when this organ is still green and unripe; as it turns yellow, the auxin content (alkaline extraction) decreases some twenty times while the auxin level in the mature pollen grain is still lower (96).

The more decisive action of pollen on fruit growth, however, is performed during pollination and fertilization of the ovules. This is a truly hormonal effect, for there is no fusion between pollen and fruit cells. The germination of pollen and the growth of pollen tubes inside the stylar tissue pose interesting problems of compatibility. At least two types of difficulties may be encountered: (*a*) the pollen grains may not germinate, or the pollen tubes may burst; and (*b*) the style or the whole flower may absciss before the pollen tubes reach the ovule. Pollen germination seems to be conditioned by proper treatment of the cell wall which, according to Brink (25) and Tsao (311), is made out of cellulose and pectic substances. A suitable osmotic concentration is necessary, as shown by the effects of various sugar concentrations (25, 146). Inorganic substances are beneficial or even necessary for proper pollen germination: for example, manganese sulfate (148), strontium, or calcium salts. The best one for *Crinum* is $Ca(H_2PO_4)_2$ at low concentrations (around $M/3000$) (62). A new and thorough study of the well-known effect of boron upon pollen germination and tube growth *in vitro* for various species of lily led Thomas (303) to detect two optimal concentrations: 10 and 75 p.p.m. On the other hand, a marked accumulation of boron occurs in the pistil, especially in the stigma and style. Data for *Lilium regale* are, in p.p.m. of boron (dry weight basis): perianth, 31.6; anthers, 16.5; filaments, 24.5; ovary, 37.3; style, 68.1; stigma, 59.0 (303). Auxins also have been reported to stimulate the growth of pollen tubes (1, 148, 266), although high concentrations may be inhibitory (154).

When growth of the pollen tube is slow, abscission of the style or even of the whole flower may prevent fruit set. In inhibiting this abscission, applied auxins, such as α-naphthaleneacetamide, have enabled plant breeders to obtain seeds from otherwise sterile strains of petunias, cabbage, red clover, or African marigolds (60) and to achieve new crosses between incompatible species of *Lilium* (59) and even between apples and pears (48).

An interesting effect of pollen, called "metaxenia," is best shown in dioecious plants, such as the date palm. In this case, the pollen influences not only the size of the seeds, but also the size and date of ripening of the fruits. This influence is characteristic of the pollen variety, a similar effect being obtained when one strain of pollen fertilizes female inflorescences of different date varieties (211, 289).

Even though pollen tubes fail to reach the ovules, they may suffice to stimulate the development of the ovary (366, 367, 368). When Fitting (63) showed that dead pollen and pollen extracts could stimulate fruit set in orchids, the existence of a chemical stimulator in pollen was established. This substance was later identified as auxin, the substance which promotes curvature in the decapitated oat seedling (134, 137, 295). The last and most decisive element of the demonstration was contributed by Gustafson (83) who proved that synthetic auxins can promote complete fruit development without pollination. Such fruits are called "parthenocarpic," a term which needs to be clearly understood, for it is often confused with "seedlessness." A parthenocarpic fruit is generally seedless, although seedlike structures may develop, as in holly (70), oranges (283), grapes (348, 349), cucumber (206, 363), fig (45), and pineapple [Stewart, (282a)]. On the other hand, a seedless fruit is not necessarily parthenocarpic, for it may derive from a pollinated ovary, in which ovules have been fertilized but have failed to develop subsequently. The mere observation that a ripe fruit is apparently seedless, therefore, is not a conclusive proof of its parthenocarpic origin. The definition of the term "parthenocarpy" itself as "the formation of a fruit without fertilization of the ovules" has been discussed (206).

The effect of pollen and pollen tubes upon fruit set has generally been ascribed to the auxin they contain, especially after the work of Gustafson (84) who used pollen extracts to induce parthenocarpy in certain species. The calculations of van Overbeek *et al.* (336) indicated, however, that pollen often does not contain enough auxin to account for its effect on fruit set. In fact, although pollens of *Sequoia* (295), hazelnut (365), or orchids seem to be rich in auxin, those of rye (96), *Nicotiana*, *Anthirrhinum* (191), *Papaver*, *Lilium* (209), and *Pyrus malus* (139) yield little auxin. The possibility that pollen might contribute an enzyme or coenzyme which would stimulate auxin formation in the young fruit was investigated by Muir (191). He observed that 35 hr. after pollination, diffusible auxin appears in tobacco ovaries (190). He found next that pollen and ovary tissues of the same species, when incubated together for 24 hr. at 37°C. in phosphate buffer, yielded much larger amounts of auxin than when the two constituents were incubated separately (191). Experiments of a similar nature carried out with other plants demonstrated that: (*a*) many tissues yield auxin when incubated for 24 hr. under non-sterile conditions; (*b*) tryptophan, when added to the mixture, increases the auxin yield under aerobic conditions (208); and (*c*) a slightly alkaline pH stimulates this process. Tryptophan is present in pollen (6, 206, 246) as well as in the ovary (206). It is thus possible that, during incubation, tryptophan might be liberated and oxidized into indole-3-acetic acid by bacteria or atmospheric oxygen. These suggestions do not invalidate Muir's data. They only point out that the whole problem needs reinvestigation under perfectly sterile conditions and under various oxygen partial pressures.

The role of the ovules.—Young ovules may regulate ovary growth in the flower bud, since their destruction by parasites (166) or by artificial means (167) causes a reduction in the size of the ovary and of the flower stalk. Such an effect can be observed only if ovulotectomy is performed early before anthesis. Emphasis upon this time relationship is apparent also in the early report of Katunskij (123) who showed that young ovules produce an auxin which controls the elongation of the flower stalk in *Papaver* and *Crepis*. The auxin level in these ovules decreases to a very low value when the flower opens, at which time the peduncle ceases to grow.

The effect which ovules exert upon flower and ovary growth before anthesis becomes very marked after their fertilization. Misshapen fruits result from the uneven distribution of seeds (97, 152, 202, 239). A direct correlation can be established between seed number and weight of the flesh (192, 202) or length of the fruit (319). Such an effect is mediated through chemical substances, for extracts of immature seeds can set unpollinated tomatoes (151, 235, 360). These extracts are also active in the *Avena* test, and might, thus, contain substances of the auxin type (7, 85, 96, 202). In fact, synthetic auxins can duplicate the effects of seeds in stimulating fruit growth (49, 200). Positive chemical identification, however, has been achieved in only two cases, both from immature corn kernels. In the first instance, in which the *Avena* test was used as an assay, indole-3-acetic acid was isolated (87). In the second, in which the test was the setting of unpollinated tomato flowers, the ethyl ester of this acid was obtained (235). Recent work with paper chromatography by Luckwill (155) has revealed the existence of at least two auxins and an inhibitor in various fruits and seeds. One of these auxins falls into the class of an indole derivative, while the other is apparently not one.

The fact that only fertilized ovules are active in promoting fruit growth after anthesis (202) may give a lead as to what part of the ovule is the determining one. The two tissues which develop as a direct result of fertilization are the embryo and the peculiar $3n$ endosperm. Direct extractions revealed that the latter is richer in auxin than the embryo (7, 96, 151). Luckwill (153) has associated with endosperm development the release of auxin in the apple peduncle. The first auxin production in the apple seed, as detected by the tomato test (150) occurs at the time the endosperm becomes cellular (151). Soon hereafter, this tissue forms an active peripheral meristem which produces a secondary endosperm. At the same time, the embryo starts growing rapidly and digests the central portion of the endosperm. As embryo growth nears completion, a sharp rise in the auxin production of seeds can be observed, which is followed by a decline as seeds mature. Such a variation in the auxin production of seeds has been observed in rye (96), corn (279), strawberries (202, 206), apples (151, 153), and beans (181).

Thus, if the endosperm, rather than the embryo, is responsible for auxin production in seeds, then seeds in which embryos collapse while endosperms continue to grow should produce even more auxin, for they would contain a

larger mass of endosperm tissue. This seems to be the case in an observation of the apple, Lane's Prince Albert, in which a high auxin peak was correlated with a high percentage (80 per cent) of embryo abortion (153). If such be the case, then the selective destruction of the embryo during the second phase of the post-fertilization growth period would eliminate competition between endosperm and embryo and result in larger fruits. Auxin applications at that time on figs (46), apricots (47), and strawberries [Tukey, (316a)] have resulted in earlier and larger fruits than the controls. Although, in the case of apricots, the authors state that "there was no difference between the seeds of the 2,4,5-T[2] treated and those of the control fruits," it might be worth while to check more closely if these treatments did not reduce embryo growth to the advantage of endosperm development.

As a rule, endosperm contains in addition to auxin a complex of factors which stimulate cell division. Some of these are heat stable and cause the random proliferation of plant tissues. The heat stable complex has been found in coconut (32, 336), the barley (178) and corn kernel in the milky stage (32, 197, 280), the ginkgo, the walnut (280), the Jack pine seed (178), and the liquid endosperm of *Allanblackia parviflora* (163). Chemical identification of the "coconut-milk factor" has been actively pursued by Steward and co-workers who claim to have crystallized several of the active components [Steward, (279a)]. A direct action of the coconut-milk factor on fruit parenchyma, however, has not yet been demonstrated either through the culture of excised tomato fruits (203) or through that of apple or cherry parenchyma (33). On the other hand, unautoclaved coconut milk greatly stimulates the development of very young *Datura* embryos (336). Unautoclaved malt extract seems to duplicate this effect (17).

The success of La Rue and co-workers in culturing isolated endosperms of corn (140, 286) and *Asimina triloba* [Lampton, (137a)] is of great interest. These cultures have been established on media containing tomato juice and are much stimulated by yeast extract (286). Since both tomato juice and yeast extract contain auxin, it would be worth while to investigate if endosperm cultures could be established and grown without added auxin. Such an experiment would help to gain a better understanding of the auxin metabolism in this tissue.

The role of auxin in fruit growth.—In the course of this paper, the word "auxin" has been written often. It is a generic term which designates organic compounds other than nutrients (materials supplying either energy or essential elements) which induce growth by cell elongation in suitable plant material, such as stems and petioles. The auxins produced by the growing seeds have all so far been identified with indole derivatives, i.e., indole-3-acetic acid (87) and its ethyl ester (235). The relative activity of various synthetic indole derivatives in setting unpollinated tomato ovaries has been studied recently (257). In addition to indole compounds, synthetic substances of apparently very different chemical formulae have been

reported to stimulate fruit growth. They include naphthalene and naphthoxy derivates, chlorinated phenoxy compounds, substituted benzoic acids, *N*-aryl-phthalamic acids, etc. Vitamin K has also been reported to be active, at least in apples (98). The features that most of these compounds have in common are: (*a*) an unsaturated ring; and (*b*) an acidic polar group, the average spatial position of which lies outside of the plane of the ring. Esters, amides, amines, and nitriles have been thought to be active per se, without preliminary transformation into the corresponding acids (118, 297). The interesting attempt of Hansh, Muir & Metzenberg (92) to define more closely the rules governing the auxin activity of a given chemical have been discussed (19). The outcome seems to be that positions *ortho* to the polar side chain are of particular importance as possible points of attachment in the growth mechanism. Thimann (299) has recently improved this concept. He agrees that the ring does probably combine with a substrate, "but the point of combination is not limited to the *ortho* position and, in fact, varies with the substitution of the ring." In addition, in derivatives other than substituted benzoic acids, one hydrogen *alpha* to the carboxyl group of the side chain must remain free (130, 218). An interesting fact in auxin physiology, although little investigated in fruits, is the synergisms which can be demonstrated between indole-3-acetic acid and compounds which possess almost all the auxin requirements, but are inactive by themselves (351). Such an effect has recently been invoked by Osborne (217) to explain the inactivity of indole-3-acetonitrile on the pea test. Could, perhaps, pollen bring to the ovary a synergist rather than auxin itself?

The origin of indole-3-acetic acid and its derivates in plant tissues has been sought in compounds structurally related to it, such as tryptophan. This amino acid is present in pollen (6, 206), in the young ovary (206), in the developing seeds (206, 255, 256, 279, 292), and in the maturing fruit (108, 205, 223). In *Lilium regale*, the tryptophan content of the ovary plus ovules increases six days after pollination (206). In the achenes of the strawberry, the variations in free tryptophan parallel those of the free auxin content (206). The relatively large amounts of free tryptophan found in the strawberry receptacle, however, have not yet been linked with auxin production. The mechanism of indole-3-acetic acid production from tryptophan in plants has not been quite elucidated yet, especially since the discovery of indole-3-acetonitrile in plant tissues (118). In addition to the "tryptophan-converting enzyme," peroxidase has been reported to oxidize this amino acid (56). So-called "bound auxin" has been reported by Moewus (186) to be present in black currants and cherries. Considering the amounts of tryptophan that are generally present in fruits on one hand, and the ease with which this tryptophan (either already free or liberated by proteolysis) can be transformed into auxin during long incubation at 27°C. (357) on the other hand, one should use great caution in interpreting Moewus' data. In fact, the claims made are too far reaching to be accepted

as presented (with no details relative to pH nor to the degree of asepsis) until confirmation of these results is obtained.

Aside from tryptophan, a substance which is inactive in the *Avena* test, but readily hydrolyzable into active substances at pH 7 to 9, has been found in corn (7, 279) and in the apple endosperm (156). Its exact nature and biological significance (a storage form of auxin?) are not yet elucidated. The difficulty in defining natural auxins chemically comes in part from their extraordinary activity at very low concentrations. This activity in fruit growth may be studied at a physiological and at a biochemical level.

At the physiological level, one of the mechanisms controlled by auxins is fruit drop, whether as "June drop" of young fruitlets, or as "pre-harvest drop" of maturing fruits. Although the apparent effects of the same auxin sprays on these two occasions seem quite opposite, for auxins promote June drop and delay pre-harvest drop, a closer analysis reveals an identical mode of action. In both cases auxins tend to inhibit drop by strengthening the pedicel. Increased vascularization in the fruit pedicel usually follows fruit set (206), and auxins are known to promote vascular development (31, 114, 287). Auxins also seem to inhibit the breakdown of the middle lamella (335), which might be stimulated by the ethylene produced by the tissues, abscission thus depending on an auxin-ethylene balance (72, 91). Both ethylene (93) and auxin (196), however, seem to stimulate the breakdown of protopectin. The differences observed in the effects of auxin applications in June and in September come from differences in the physiological stage of the pedicel. In June, abscission seems to result from active cell division in the "abscission layer"; in September, it is due to a loosening of the cells (10, 160). Most important of all, in June, auxin sprays usually cause seed abortion in the apple (154, 194) and the pear (Nitsch, unpublished data), whereas in September, seeds are enough developed to be insensitive to these sprays. Both in June and in September, the immediate effect of auxin is to make the fruits remain on the branches. In June, however, the collapse of the seeds reverses completely this effect. An abundant literature has been published on both thinning sprays in apples (69, 193), peaches (100), olives (95), etc., and pre-harvest sprays (69, 344, 345).

If we now turn to the biochemical processes that are regulated by auxins, the difficulty becomes great, for many enzymatic reactions have been reported to be affected by these substances (19). A few points only will be stressed here, namely the effects of auxins upon (*a*) carbohydrate metabolism and respiration, (*b*) metabolism of pectic substances, and (*c*) water uptake.

Contradictory reports have been made on the action of auxins upon starch synthesis and hydrolysis. On the one hand, they have been found to accelerate starch hydrolysis (67, 182, 183, 185). On the other hand, starch accumulation in the sterile parts of flowers seems to parallel the development of anthers and ovules (168). Pollination (105, 172) and de-

veloping seeds (169) stimulate starch synthesis. This effect has a time relationship to the period of auxin production by the seeds (153). Indole-3-acetic acid can duplicate the stimulation of starch formation in flowers deprived of ovules (170), the effect being proportional to auxin concentration (171). The same auxin stimulates starch synthesis in Iris stomata, a process which is inhibited by KCN (173). Such an effect could be linked with an accumulation of reducing sugars under the influence of auxin (264) rather than with a direct action upon amylases (23). Whatever may be the exact mechanism, it remains that in the presence of malate or fumarate and sucrose, indole-3-acetic acid can stimulate the overall respiration of sections from oat coleoptiles up to 22 per cent of the controls (41). A direct stimulation of malic and fumaric dehydrogenase activity by auxins has recently been observed by Teubner & Murneek (294). In addition, the activity of oxidases also increases in auxin-treated tissues. Auxin-rich crown-galls contain more tyrosinase, peroxidase, and catalase than normal tissues (195). In the culture of isolated calli, the effect of auxin is reflected by a marked increase in tyrosinase and peroxidase (188), with a corresponding decrease in catalase activity (68, 188). Ascorbic acid oxidase activity also increases in calli cultured on media containing auxins (198). However, when 2,4-D[2] inhibits the growth of seedling roots, the activity of ascorbic acid oxidase is also depressed (353).

That auxins may affect growth through a direct action on the cell wall was suggested early (see 99, 244, 245), but the explanation given was too mechanical. The localization of ascorbic acid oxidase in the cell wall points again toward such an action, but this time through the general cell metabolism, which is a more appealing explanation. Van Overbeek (335) emphasized recently a possible role for auxin in regulating the metabolism of pectic substances, because it stimulates pectin-methylesterase activity (196) and may inhibit the breakdown of calcium pectate in the middle lamella. A calcium-auxin relationship has been suggested for roots (29). In fact, low concentrations of auxin exert a sparing action on calcium ions (287). On the other hand, blocking of cell elongation can be obtained through high concentrations of calcium or magnesium (302).

Whatever the multiple effects of auxins upon enzymatic systems may be, it remains true at present that one of the clear-cut actions which can be demonstrated is that on metabolic water uptake or retention (184). This process is inhibited by lack of oxygen (104, 236) and many respiratory inhibitors (89), as is growth. It is greatly enhanced by auxins which, according to Thimann's scheme (298), direct the respiratory energy toward the active pumping of water inside the cell. The way this energy is channelled may be the high-energy phosphate metabolism, as proposed by Bonner & Bandurski (19), and supported by possible structural interactions between phosphoric acid, the *ortho* position and the *alpha* carbon of the auxin molecule (237).

Conclusion

As a conclusion for this review, the writer had hoped to present an integrated picture of fruit growth. After the available literature had been compiled, it turned out that too many mechanisms were still unexplored. Although the physiology of ripening has been studied extensively, under the pressure of practical problems, many aspects of growth and development in fruits have been neglected. Exceedingly little is known about the physiology of ovary development before anthesis. The biochemistry of pollination is still incomplete. The study of auxin relationships, to which more attention has been devoted, is by no means exhausted, especially in the case of stone fruits. Under these circumstances, a general scheme for fruit growth would remain necessarily vague, and, therefore, uninteresting. It has, thus, been omitted. But rather than discouraging, this state of affairs should stimulate new efforts toward a deeper knowledge of fruit physiology.

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PHYSIOLOGY OF ROOT GROWTH

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The following review deals with the growth of roots in a fairly broad sense. It has been possible in a few instances only to separate from each other experimentally the different kinds of growth, i.e., cell multiplication and cell elongation. Usually growth as measured by length, weight or, less frequently, by volume of roots denotes the sum of an increase in cell number and cell elongation. It has thus been necessary to employ "growth" in this unspecific sense, whereas "elongation" has been used when obviously the process of cell elongation proper has been studied. Many aspects of growth and elongation are common to roots and shoots, but this review has been restricted to problems which are or seem to be specific to roots. The special problems of tissue differentiation, including root hair formation, have not been treated.. The scope has been extended, on the other hand, to include root initiation because this can not be set off sharply from growth in a restricted sense. In addition, problems in root initiation can shed some light on the growth process proper. The review covers mainly the literature from 1950 to May, 1952 inclusive; older papers have been quoted only in order to complete the discussions.

The Normal Growth of Intact Roots

Nearly every scientist working on root growth problems has developed his own experimental technique and method of measuring growth. Only some methods of general interest are worthy of special mention.

Methods.—Microscopic measurements of cell lengths along the root axis in favorable materials enables the construction of fairly detailed graphs showing the grand period of elongation of the individual cell, as well as computations of the actual rates of cell elongation and the duration of the cell stretching process. Grass roots are especially suited for such a purpose because of a convenient pattern of cell divisions in the meristem. Such a method was worked out ten years ago, and this and similar ones have been applied in some recent investigations [Burström (1), Heitz (2), Street & McGregor (3)]. This method also renders possible a distinction between growth by cell multiplication and by cell elongation [Burström (4), Wilske & Burström (5)]. The growth has usually been followed for experimental periods of some hours or, most commonly, days, but Lundegårdh (6) has developed a technique for recording growth over intervals of time as short as four minutes. It is convenient or even necessary under some circumstances to maintain the nutrient environment of the roots constant by growing them in flowing solutions. Recently such methods have been described by Audus (7) and Wilske & Burström (5). A number of new, simple routine methods

have been developed for testing the action of growth substances on roots. They may be satisfactory for a superficial comparison between different substances, but these methods may also suffer from severe errors and do not permit a deeper penetration into the mode of action of the substances. There should be mentioned the *Lepidium* test of Moewus (8), which has been used by other investigators but has recently been criticized by Clauss (9); and two different tests with seedlings of flax worked out by Åberg (10). It appears from the latter's papers, however, that fairly different results can be obtained with the two methods even on the same plant material, which shows that some caution is advisable in interpreting the results of such rapid tests.

Growth and metabolism.—The connection between cell elongation and dry matter production of roots has been dealt with in some recent studies. Brown & Broadbent (11) have verified with pea roots earlier statements that during the normal process of growth about half the production of dry matter takes place during cell elongation; thus meristematic growth and cell elongation are not sharply set off from each other. Wanner (12) has further shown with *Allium* roots that the nitrogen content of the cells increases rapidly during elongation. This does not necessarily imply, however, that dry matter syntheses form an indispensable prerequisite for, or are causally connected with the elongation mechanism. There are, on the contrary, new evidences against such an assumption. Supraoptimal concentrations of indoleacetic acid (IAA) inhibit root growth by reducing cell elongation with only small changes in cell multiplication [Burström (4)]. Derivatives of isobutyric acid, on the other hand, increase cell elongation, also with few significant changes in the number of cells produced. In both cases, the contents of dry matter and organic nitrogen, and, probably, of both cytoplasmic and wall materials, are independent of cell elongation but proportional to the number of cells. The length of the individual cells can increase by about 4:1 without a change in the dry matter content. This auxin-regulated elongation should be identical with the normal one, which, accordingly, is largely possible only with the absorption of water. Kandler (13) has likewise observed that excised corn roots can undergo a considerable elongation without a corresponding dry matter production. Growth inhibition by IAA or vitamin B_1, on the other hand, down to one fifth of the normal root length, are unaccompanied by corresponding reductions in dry matter production. In neither case is the change in dry matter production the primary cause of the growth inhibition.

There must, nevertheless, be an intimate relation between the bulk process of growth and metabolism. This is illustrated, for instance, by the oxygen requirement of roots recently studied by Hopkins, Specht & Hendricks (14). The root growth of tomato, tobacco, and soya bean ceases at an oxygen content of the root environment atmosphere of 0.5 per cent and increases in proportion to the oxygen content, up to that of normal air. This does not necessarily imply a higher oxygen requirement of roots than of aerial parts, because the oxygen solubility must be duly considered.

In this connection, Cooil (15) found that fumarate and malonate promote the growth of the root but not that of the shoot of oats. The action on the roots is not inhibited by iodoacetate, and it is not clear whether the acids affect the growth by means of the respiratory mechanism or not. A sudden increase in temperature from 20°C to 35°C causes, in *Pinus taeda*, a tenfold increase in the rate of root growth, which, however, after one hour drops to a low level again [Barney (16)]. This phenomenon has been explained by assuming a rapid increase in the utilization of nutrients. It appears, however, that the increase is so much greater than that corresponding to a normal temperature coefficient of metabolism that a further analysis is desirable.

Mineral nutrient requirements.—Two mineral nutrient elements are of special interest in connection with root growth, namely, calcium and boron. Haynes & Robbins (17) have verified for Ca and B, and, independently, Presley & Leonard (18) for Ca, that these elements must be continuously supplied in the nutrient medium to insure a normal root growth, and that an accumulation in the roots is not sufficient. It ought to be emphasized, out of respect for forerunners in this field, that this was shown for calcium by Hansteen Cranner in 1910, and in 1937 by Rehm (19) for boron. A further study of the calcium action has revealed [Burström (20)] that the maximal rate of cell multiplication of wheat roots at an optimal pH of 5 to 6 is obtained with a calcium concentration in the nutrient solution as low as $10^{-6}M$ or 0.04 p.p.m. Considering the close correlation between cell multiplication and dry matter production this implies that calcium exerts its maximal nutritional effect at this low concentration, which is on a level with that of a micronutrient element. A rise in the calcium concentration above that level up to $10^{-4}M$ and at a pH above 5 causes, however, an increase in cell elongation. An excess of IAA scarcely affects the roots at all at a calcium concentration of $10^{-8}M$, but prevents the positive effect of higher calcium concentrations. The conclusion has been drawn that calcium, in addition to its nutritional effect at low concentrations, acts as a genuine cell elongation factor in some kind of interaction with auxin. This will be discussed more in connection with the importance of pH. The interaction between boron and auxin, recently questioned by MacVicar & Tottingham (21), has been taken up for investigation by Hemberg (22). He has verified the statement that boron, just as IAA, can promote the initiation of adventitious roots in *Phaseolus* petioles. This is accompanied by a strong increase in the content of bound auxin and Hemberg has assumed that auxin or auxin precursors are involved in the boron effect. A relation between boron and calcium is often indicated by studies of nutrition. Many facts also indicate a growth-regulating system involving a mutual interaction of calcium, boron, and auxin, although direct evidences of the participation of boron in the growth mechanism are wanting.

Root growth and pH.—The interpretation of the effect of these factors on growth is somewhat complicated by the attention that must be paid to the hydrogen-ion concentration. It is necessary to point out that even if some unanimity exists as to the general shape of the growth-pH curve of roots,

a complete disagreement prevails as to the interpretation of this relation. Data in the earlier literature have been subjected to an apparently justified criticism by Lundegårdh (23), who found, with wheat roots, a wide optimum range of pH, perhaps with a peak at pH 6 and with root elongation steeply decreasing towards pH 3. This is in fairly good conformity with Audus' (7) result with *Lepidium*, showing an optimum at pH 5.5 and decreasing growth towards pH 4 and 7. In long-time experiments with wheat roots in complete nutrient solutions, the author (24) obtained a continuously accelerated increase in the growth from the acid side to pH 7. Lundegårdh (6) has found in new experiments with the same material, in tests of short duration, a rapid increase in the root elongation caused by 10^{-3} *M* IAA or 10^{-3} *M* HCl, i.e., at a pH of 2.7 to 3.4. Prolonged treatment with solutions of this pH invariably kills the roots. This growth reaction may, at present, be disregarded as a premortal phenomenon not comparable with the other hydrogen-ion effects on the growth. For the rest, the controversial results have been explained as due to the specific action of Ca on root growth, which is well known from nutritional studies but has been markedly neglected in the growth literature after the introduction of the auxin concept [Burström (20)]. At calcium concentrations up to 10^{-6} *M* an optimum curve for the bulk growth of wheat roots is obtained with a maximum at pH 5 to 6 and little or no growth at pH 4; with 10^{-5} *M* Ca there is still little growth at pH 4.5 but the decrease from pH 6 to 7 has disappeared, and with 10^{-4} *M* Ca even the depression at pH 4.5 is smoothed out. This means that the growth depressions on both sides of the optimum of pH 5 to 6 are counteracted by Ca ions, with the depression near neutrality more easily reversed than that on the acid side. This seems to be of importance in the interpretation of the pH curves.

It has become customary in the growth literature to look for an explanation in the dissociation of native auxin and its importance for growth. The interpretations vary, however. Audus has assumed, in agreement with earlier authors, that the decrease in growth on the acid side of pH 5.5 depends upon an inhibition of growth by a supraoptimal concentration of undissociated auxin acid molecules, and, on the other hand, the decrease towards the alkaline side on a decrease in the concentration of undissociated acid below the optimal one. Lundegårdh (23) on the contrary, is of the opinion that the increase in growth from pH 3 depends upon a positive action of an increasing concentration of auxin anions. The crucial point is, whether auxin is active as a growth factor in the anionic form or as the undissociated acid. Both authors assume axiomatically that every change in the root growth curve depends upon a shift in the concentration of active auxin. This assumption can, in itself, be earnestly doubted, in spite of the fact that it is supported by results from the action of auxins on shoot elongation. It has been shown repeatedly, even in recent studies [Moewus (8), Pilet (25), Slankis (26)], that an external addition of IAA of as little as 10^{-11} *M* may increase root growth but it can not be inferred from the papers that this occurs only on the alkaline side of an optimum around pH

6, which must be postulated from Audus' explanation. It would also follow that wheat roots in a complete nutrient solution with a high concentration of Ca could not show positive growth reactions to added IAA at a pH below 7, which certainly does not hold true. It is, as a matter of fact, not permissible to draw any conclusions as to the action of auxin from the shape of the growth-pH curve.

This is especially obvious if the pH-growth curve is analyzed in detail and also related to the action of Ca [Burström (20)]. Increasing pH at a calcium concentration below 10^{-5} *M* decreases cell elongation, irrespective of the shape of the bulk growth curve. Cell elongation is maximal at pH 4.5 and the lowest Ca concentration permitting any growth. Auxins principally regulate cell elongation. Interpreted in the usual terms of auxin dissociation, the mentioned pH-curve would imply that the roots over the entire range of pH suffer from a deficit of undissociated auxin. Furthermore, increasing the calcium concentration levels the differences between the various acid solutions. This means that the presumed auxin can be replaced by Ca ions, which hardly seems likely. It is also difficult to understand how a hydrogen-ion activity above pH 6 with a dissociation of auxin of more than 95 per cent can be interpreted in terms of a shift in the dissociation of auxin at all. It has finally not been shown that the same pH prevails at the points of action of auxin as in the external solution. At pH 4, on the contrary, the low bulk growth is not due to a low rate of cell elongation, but depends upon an inhibition of cell multiplication, which can be partly restored by the addition of Ca ions. This is probably the well-known action of Ca antagonizing a toxic excess of hydrogen-ions. It has thus been pointed out that probably no part of the pH-growth curve can be explained as simply a change in the dissociation of auxin.

Auxin actions.—This leads to the question of the mode of action of auxin on roots. The author (27) has summarized how four different actions of externally added auxins and related compounds can be distinguished: (*a*) a positive action on the first part of the cell elongation process; (*b*) an inhibiting action on the second part of the same process, which comprises the main part of elongation; (*c*) an antiauxin action exerted by certain compounds; and (*d*) an unspecific toxic action of both auxins and antiauxins, involving a reduction of the rates of both cell multiplication and cell elongation. The first two actions are genuine cell elongation effects not affecting cell multiplication; they are both antagonized by antiauxins.

It is not easy to find out which of these activities are exerted by the native auxins of the roots. Some conclusions can be drawn from the results with the antiauxin-active indole- and phenoxy-isobutyric acid derivatives [Burström (1, 24)]. They can cause an increase of more than 100 per cent in the root length by increasing cell elongation without changing cell multiplication. They thereby accelerate the second phase of elongation and retard the first or initial phase. The first-mentioned action dominates. Externally added auxin is also counteracted. It has thus been concluded that the native auxin has an effect opposite to that of the antiauxin, i.e., the actions men-

tioned under (*a*) and (*b*). These two actions are locally separated [Burström (28)] and are exerted simultaneously and independently of each other. The sensitivity of the roots to action (*b*) is easily changed under the influence of prolonged exposure to auxin and antiauxin, which means that the roots adapt themselves to an external supply of the compounds. With an adaptation to auxin, the resulting positive action (*a*) causes increased cell elongation. This picture of the auxin actions, especially worked out on root material [Burström (27, 28)], will explain the optimum curve of the auxin action on roots as due to two separate actions exerted at different stages of development of the cells.

The normal auxin conditions and their relation to growth have been studied on roots of *Lens* by Pilet (25, 29, 30). It has been shown in his extensive series of experiments that in seedlings the growth rate increases until a length of about 20 mm. is attained and then decreases again to a very low and constant value. What Pilet has studied is probably the grand period of the heterotrophic phase of development of the seedlings, but not the time course of normal elongation, which ought to be noticed. The auxin content at different developmental stages has been determined by a carefully calibrated *Avena* test apparently giving a satisfactory degree of accuracy. Pilet (29) has thus demonstrated by means of diffusion experiments the occurrence of two longitudinal auxin currents, one very strong in the acropetal direction, and a weak basipetal current. This picture does not exactly agree with the general ideal of auxin migration, and Pilet's interpretations are perhaps open to discussion. He has shown, e.g., that from the root of an entire seedling an auxin content of ca. 10^{-5} mols could be obtained, but if either the leaves or the root tip were removed the content decreased to ca. 10^{-9} mols, which seems to indicate that both parts are necessary for a complete circulation. There seems to be no doubt about the existence of the acropetal stream of active auxin, although the strength can be discussed. Pilet further found a lateral, centripetal current, strongest in the meristem region. Unilateral illumination by ultraviolet light causes the auxin to move away from the light; this means that the centripetal current is accelerated on the exposed side. Subsequently the auxin content is decreased by destruction of the hormone. Pilet has thus obviously demonstrated the existence of both a light-induced auxin movement and a light inactivation of auxin in these roots.

The sensitivity of the roots to light has also been thoroughly studied by Pilet (25). The auxin content of the roots increases with increasing length, amounting to ca. 3×10^{-7} *M* in 2 mm. roots and ca. 5×10^{-4} *M* in 20 mm. roots. The younger roots react with a decreased growth rate to ultraviolet light but not to red or blue light, but old roots respond to ultraviolet light with an increase. That the young roots really have a suboptimal content of auxin is revealed by the fact that spraying of one-day old seedlings with 10^{-7} *M* IAA results in a 70 per cent increase in growth. The sensitivity to IAA increases with age resulting in a diminished response, e.g., 12-day old

plants increase in growth by 30 per cent at best with 10^{-11} *M* IAA as the effective concentration. In summary, Pilet's results give a precise and logical picture of roots containing a suboptimal concentration of auxin at this stage of development, which, however, steadily increases in amount. At every phase of development the roots react according to expectations, and consequently, auxin is a positive growth factor for these roots at an early stage. The tropistic responses, which must be highly interesting in such roots, have not been mentioned; thus, the response to unilateral light is probably slight.

Controversial statements are found in the literature concerning the auxin requirements of excised roots. The roots of one out of three investigated pea strains reacted favorably to 10^{-6} *M* IAA in experiments by Naylor & Rappaport (31). Almestrand (32) could not find any positive actions on roots of barley and oats.

A special problem of great interest is the importance of auxins for the mycorrhiza formation. According to Slankis (26) and Levisohn (33), isolated roots of *Pinus* react to low concentrations of IAA and napthaleneacetic acid (NAA) with a dichotomous branching resembling a mycorrhiza, whereas concentrations up to 200 μg. give growth disturbances of the ordinary type. Compared to other roots, those of *Pinus* are very slightly sensitive to auxin [Slankis (34)]. Dichotomous branching caused by IAA was also found on roots of intact plants in sterile cultures; the roots also lose their root hairs, and Slankis has assumed that auxins are given off from the fungus to the host plant in the course of the normal mycorrhiza formation. It is also noteworthy that the *Pinus* roots adapt themselves to IAA thus decreasing in sensitivity. This seems to be a common property of roots [Burström (1, 28)] and will be discussed more later on.

A similar problem has been studied by Kandler (35). He has arrived at the conclusion that bacteria living in the rhizosphere normally produce growth regulators—probably IAA—in amounts, which, according to the nutrient conditions of the plant species in question, may favor or inhibit root growth.

Neither Pohl (36) nor Ashby (37) has reported action other than growth inhibition by the likewise native aldehyde of IAA, although, according to Ashby, the aldehydes of IAA and NAA are only one tenth as toxic as the corresponding acids. Ashby also studied the transformation of the aldehydes to the acids in *Artemisia* roots. Considerable amounts of aldehyde were unaccounted for by the analyses indicating that they are also converted into other compounds than IAA.

Naturally occurring growth inhibitors have been extracted with alcohol from roots of rye, wheat, and barley by Vogt (38); the solubility of these inhibitors is recorded to be less in ether and chloroform than in alcohol. The extracts are supposed to contain auxin as one active principle because in high dilutions they accelerate growth. Also, other unidentified growth regulating compounds are supposed to be present.

The Action of Artificial Growth Regulators

A number of papers in recent years report the action of synthetic compounds, mainly indole and phenoxy derivatives, on root growth; that they specifically concern root growth problems is seldom clear. When roots have been used as the test material in investigations on the relation between chemical structure and growth activity root growth *per se* has not been the objective. Such studies aim at general rules. On the other hand, with our imperfect knowledge of the mechanism of growth it is not possible to decide whether the results obtained can be generally applied, or if they only reflect specific properties of the plant materials studied under experimental conditions often chosen at random. An attempt to summarize and compare the results obtained thus seems to be justified, especially if some general conclusions can be drawn concerning the properties of roots.

Leaper & Bishop (39) studied the growth inhibiting action of differently chlorinated phenoxyacetic acids on *Lupinus* roots. The strongest inhibition was obtained with the 4-, 2,4-, 2,5-, 3,4-, and 2,4,5-chlorine substituted acids. Derivatives with more than three chlorines were not tested. It was concluded that the strongest inhibition requires two unsubstituted carbons in a *para*-position. A more complete picture has been obtained by a comparison of differently chlorinated phenoxy-isobutyric acids [Burström (24)]. The unspecific toxic action of these acids exerted at higher concentrations and corresponding to (*d*) above follows the rule found by Leaper & Bishop, with one important exception, i.e., that the strongest inhibition by far was obtained with the penta (2,3,4,5,6,-)chlorinated acid, which was not studied by these authors. This does not agree with the *para*-position hypothesis. It appears that the toxicity increases if two chlorines are introduced in an *ortho*-position to each other. The positive growth action, on the contrary, exerted by the isobutyric acid series and corresponding to an antiauxin action, is only increased by a substitution of chlorine in the 4-position and is independent of any other mono- to penta-substitutions tested. It is thus impossible at present to formulate a general rule describing the action of chlorine substitution. Neither do these results seem to tally with the *ortho*-position hypothesis launched by Muir & Hansch (40) for the action on shoots [cf., Veldstra & van de Westeringh (41), Thimann (42)].

An obstacle which usually impedes the comparison between separate investigations is the fact that the kinds of inhibitions which substancess exert upon roots is seldom clear. It is certainly wrong to denote every substance inhibiting root elongation as a "growth inhibitor" in the sense that its action equals that of auxin listed under (*b*). Rapid tests, especially, can be misleading, because every toxic substance instantaneously killing the roots must be classified as such an inhibitor. In long-term experiments such cases are more easily eliminated, at least if the growth behavior of the roots is checked by microscopic examination. Every change in growth behavior from that of control roots is said to depend upon the "growth activity" of the compound tested. The action of 2,4-D, to take one example, is assumed to be identical with that of auxin, although very much stronger

and with an added disturbance of the metabolism [van Overbeek, Blondeau & Horne (43)]. However, phenoxyacetic acid does not inhibit the growth of wheat roots [Burström (27)], and α-phenoxy-propionic acid—methylated in the side chain—exerts a regular auxin action of type (*b*), which may be additive to that of NAA or antagonized by an antiauxin. It is at least at variance with general ideas of the importance of ring and side chain to assume that identical activity can be called forth by chlorination of the ring and methylation of the side chain. Wilske & Burström (5) have shown that 2,4-D decreases both cell elongation and cell multiplication simultaneously, which resembles a toxicity of category (*d*). The same action is exerted by 2,4-D and 2,4-dichlorothiophenoxyacetic acid, i.e., if the oxygen of the chain is substituted by sulphur. Every activity disappears, however, if this is oxidized to —SO— or —SO_2—. The same holds true of the corresponding derivatives of 2-methyl,4-chlorophenoxyacetic acid [Jönsson *et al.* (44)]. The significance of this substitution of the side chain is not clear.

Hansen (45) has systematically investigated the growth activity of homologues of the last-mentioned acid on roots and found that the chlorinated acids have histological effects similar to those associated with auxins. The pure toxic effects increase with the chlorination, whereas the unchlorinated compounds with or without a carboxyl in the side chain antagonize the chlorinated acids. Auxin antagonistic actions on roots have been described for a number of substances. The already mentioned isobutyric acid derivatives (1, 24) were hitherto believed to be unique insofar as they antagonize even native auxin, at least on wheat roots. Hence they acted as positive root growth factors increasing cell elongation more than 100 per cent. Hansen (private communication), however, has found the same effect of phenoxyacetic acid at the high concentration of 10^{-4} *M*. Weaker effects have been obtained on flax roots [Åberg (46)]. Other compounds with similar effects have been detected by Åberg (10, 47) among α-(1-naphthylmethyl) derivatives, partly as sulphide or selenide carboxylic acids; also α-(1-naphthol) functions as an antagonist. The antagonism against native auxin is weak or lacking with these substances on flax roots. The diversity of structure of both ring systems and side chains of these antagonists renders it difficult to formulate generalized explanations of the actions (cf. Åberg). They may depend partially upon a reduced absorption of the compounds and partially upon a real blocking of the points of action.

Coumarin acts, according to unanimous reports of several authors [Audus (7), Moewus (8), Goodwin & Taves (48)], only as a growth inhibitor of roots. The last-mentioned authors have emphasized that all unsaturated lactones reacting with SH-groups are root growth inhibitors. Of special interest is scopoletin, 6-methyl,7-hydroxycoumarin, a normal constituent of oat roots, occurring in low concentrations in the tips but in larger amounts in old parts.

A phenomenon which is wholly neglected in most investigations on root growth inhibitors but one which probably explains discrepancies in the literature, is the adaptation of roots to such compounds and the different

sensitivity of cells at varying developmental stages. A striking example is offered by the undecanoic and dodecanoic acids; 10^{-4} and 10^{-5} M solutions kill cells in the process of elongation, thus causing growth inhibitions, probably unrelated to auxin actions [Burström (49)]. This resembles an "adaptation" of the root as a whole to the acids. Meristems are resistant and go on growing; the new derivatives are insensitive to the compounds, just as adventitious roots develop freely in the same solutions. Such cases of specific sensitivity of cells must be taken into account in evaluating results of short-time routine tests. The roots are shorter than usual, appear to be living even under the microscope, and the phenomenon is easily interpreted as a case of inhibition of the elongation mechanism. That a real adaptation to auxin homologues also occurs, has already been mentioned.

The Growth of Isolated Roots

In 1949 the question of the unlimited growth of excised roots seemed to be no longer a problem, and there were even records in the literature of successful growth of roots of monocotyledonous plants. There are reasons today to look upon this question more sceptically. Endeavoring to cultivate roots of barley and oats, Almestrand (32, 50) found that they were unable to grow because cell divisions ceased, although elongation of already present meristematic cells and a certain amount of tissue differentiation proceeded. No known nutrient or growth factor was found capable of maintaining meristematic growth. Wheat roots grew better (51) but with a decreasing rate of cell division. The tips became tapered and finally ceased growing; lateral roots were formed, also growing normally at first. Neither the length of the main root nor the number of laterals formed can be taken as evidences of a favorable development. A survey of the literature revealed results similar to those obtained with wheat to be fairly common in experiments purporting to show unlimited growth. Subcultures are apparently often or generally started from lateral tips. It is emphasized that a criterion of infinite growth must be the unlimited development of one meristem, and that it is not the same thing, if each meristem has a limited term of life, successively followed by lateral ones predestined to the same fate. Roots of corn behave similarly [Kandler (13, 52)] and there are no convincing evidences of unlimited life of root meristems of monocotyledons.

Dormer & Street (53) had, as a matter of fact, shown in 1948 that the classical material—tomato roots grown in White's own medium—certainly form a cambium and sometimes a normal xylem, but seldom a phloem, and that the growth is often irregular. Street *et al.* (54) later admitted, on the basis of continued cultures of tomato roots in White's medium, that it is probably not possible with normally branching roots to keep the main tip alive for any considerable time, and, finally (55) that this problem of the unlimited growth, even with roots of dicotyledons, may not yet be solved. As stressed by Almestrand, the histological behavior of the roots must be considered in evaluating the results of root cultures. Kandler (13) has pointed out that the main tip of corn roots can be kept alive only if lateral root forma-

tion is prevented by repeated excision and subculturing of the main tip, whereas wheat roots, according to Almestrand, do not tolerate subculturing, but instantaneously cease growing.

A scrutiny of the recent literature on the nutrient requirements of isolated roots is thus justified. The micronutrient and vitamin requirements have been in much dispute. The literature has recently been summarized by White (56). The optimal pH for tomato roots is 4.8 to 4.9 [Street & Lowe (57)] and no growth is obtainable at a pH above 5.2 [Street, McGonagle & Lowe (54)]. Normally, the pH of the culture medium increases causing a cessation of growth. This is due, in part, to a precipitation of iron [Street *et al.* (55)], but this "staling" of the medium cannot be wholly prevented by supplying iron in a complex form.

Tomato roots prefer, as is already known, sucrose as a carbon source [confirmed by Street *et al.* (57, 58)]. The latter authors have shown that the sugar absorption ceases when there is a deficiency of phosphorus. They assume that sucrose is taken up as a phosphorylated sugar. Street & McGregor (3) studied the histological effects of sucrose on tomato roots. Concentrations above one per cent retard cell elongation and cause a transverse swelling, but dry matter production is maintained at a maximal level by concentrations of 1.5 per cent and higher. After growing all parts of tomato seedlings isolated from each other, Lee (59, 60) arrived at the surprising conclusion that, although isolated roots grow best on sucrose, the roots of intact plants prefer fructose. Isolated pea roots also utilize only sucrose [Naylor & Rappaport (31)]. Wheat roots, on the contrary, use only glucose [Almestrand (61)] and the absorption of the sugar as well as that of the mineral nutrients parallels growth. Roots of barley and oats neither grow nor consume any carbohydrates tested, but give off nitrogen compounds to the medium, possibly because of autolysis. Kandler (62) has found that corn roots, on the other hand, grow in a modified White's medium to a length of about 70 mm. and give off amino acids to the solution. These are formed from nitrate and can be absorbed again. Hence, no disturbance of nitrogen assimilation occurs in isolated corn roots, a disturbance Almestrand has tentatively assumed for the wheat roots.

The crucial point in root cultures is supposed to be the vitamin requirement. It is just as certain as it is regrettable that the failures to obtain root growth with vitamin additions are seldom published. Almestrand (32), in a study of barley and oats, found no reaction to any of the usual vitamins, to amino acids, or to extracts of yeast and different parts of higher plants. Peptone contains a factor promoting cell divisions, but only in barley; a water soluble cell elongation factor was obtained from endosperm. Wheat roots do not require thiamin or niacin but only pyridoxine (51). Out of nine tested strains of wheat some made fairly good growth even without pyridoxine and did not react to an addition, either. Kandler (52) has noticed a positive action of 2,4-dinitrophenol with corn which has not been verified with wheat (Almestrand, private communication). A strange statement has been made by Rabideau & Whaley (63, 64) to the effect that tomato roots

require thiamin and either niacin or pyridoxine. It is difficult to see how these could replace each other and the question whether perfect growth has been obtained under all conditions should be investigated.

Naylor & Rappaport (31) have demonstrated striking differences between three strains of pea. Roots of two of them grew well in Bonner's medium with casein hydrolysate, L-cystein, and L-tryptophan, whereas the third failed to do so. The labile state of isolated roots and perhaps the lack of harmony with the external conditions result in a periodicity of root elongation. Kandler (65) has tentatively given an explanation which does not involve endogenous forces.

The controversial opinions as to the action of IAA on isolated roots have already been mentioned. Bein & Schopfer (66) claim that 10^{-11} *M* 2,4-D increases the growth of corn roots by 70 per cent. More remarkable, perhaps, is the finding of Levine (67, 68), who reports that roots of sunflower, tobacco, and carrot are favored by carcinogenous compounds. No effect of IAA was obtained on root formation, but instead a callus formation was demonstrated (69). This leads to the problem of root initiation.

The Initiation of Roots

Levine's works, especially those with calluses, exemplify the differential action of IAA on root and shoot initiation. A callus grown in a wholly synthetic medium with 5 p.p.m. IAA forms an amorphous mass of cells, with an initiation of root initials; transferred to an IAA-free medium it develops whole plants with normally growing roots. The action of auxin induces a dedifferentiation, a retrograde change to an embryonal stage, including a formation of root initials. Shoot initials, on the contrary, are only formed at a lower concentration of IAA, and further growth and development of the roots is possible only in the absence of external IAA. Thus, the conditions for root initiation and root growth are, as is already well known, very different. The consequences of this fact will be discussed in relation to the recent literature.

The initiation of adventitious roots by auxin is treated in several papers [e.g., Bouillenne & Bouillenne-Walrand (70)]. Pilet & Pfister (71) made careful observations of the anatomy of their material (*Iris*) and van Raalte (72) pointed out that root initiation is further increased by the presence of 10^{-3} *M* indole. A special reference is justified, for reasons given later, to the "comb-like" initiation of adventitious roots from the pericambium of *Pinus* [Slankis (34)] and pea roots [Hansen (45)], implying that the root initials are laid down so close together that they merge in a longitudinal ribbon with a spiny edge. These initials do not develop further in the auxin solutions.

The participation of auxin in determining the polarity and the polar root initiation is not clear. Kulescha & Camus (73) have determined the auxin content of roots of *Lactuca* and arrived at the conclusion that the correlative inhibition of shoot formation from the distal end is independent of the content of auxin. Warmke & Warmke (74), on the contrary, found with the same material as well as *Taraxacum* roots that distally applied auxin in-

creases root formation at that end, whereas a proximal application induces roots in both ends. That the concentration of auxin determines the initiation of not only roots but also shoots, follows from the fact that the auxin antagonist, ethylene chlorhydrin, in some instances caused shoot formation at both ends. The formation of adventitious roots from stem nodi of sugar cane is weak if the stem is in a normal position, stronger in an inverse position, and attains a maximum if placed horizontally (Hes (75)]. This is interpreted as due to the distribution of auxin but direct evidence is lacking.

In spite of all these results and the earlier ones, there still remains the question whether auxin alone is sufficient for root initiation, or if other compounds—the hypothetical rhizocaline—are required. It is then necessary to disregard the obvious fact that the general nutrient conditions play a prominent part in the process. Kramer (76) has resorted to this possibility to explain the formation of adventitious roots above the water-line in flooded plants. It ought also to be behind the action of organic nutrients in the experiments of Gregory & Samantarai (77).

White (78) has dismissed the rhizocaline concept with the sweeping assumption that it has turned out to be identical to auxin, an opinion shared by many investigators but far from unanimously accepted. Pilet (79) adheres to the rhizocaline theory and has found additional support in the formation of adventitious roots from *Ramondia* leaves. The initiation is increased by IAA. Pilet carefully studied the distribution of the root initials over the leaf surface. He concluded that the auxin concentration, although important enough, is not the only decisive factor. Torrey (80), studying pea roots, explicitly assumed the existence of two different substances, IAA and an unknown one, which, together, were able to explain the well-known fact that laterals can be induced either by the addition of IAA or by decapitation. He assumed that the unknown substance is transported acropetally, is normally consumed, but is accumulated by the root-forming operations. It is obvious that the new evidence in support of the rhizocaline theory is rather weak and the whole question still uncertain; even if an unknown factor is required for root initiation it seems to be premature to discuss it in terms of a specific hormone.

Nevertheless, some general considerations by Bouillenne (81) in the theory of rhizogenese are fruitful. He started with the opinion, useful, at least, as a working hypothesis, that three factors are required for root formation: (*a*) a hormone formed in the leaves (rhizocaline); (*b*) auxin; and (*c*) a "cell factor" restricting root initiation to certain cells and consisting of the respiratory system and a certain nutrient condition of the cells. New evidence for (*a*) is not presented, but it is interesting to follow up the consequences of this line of thought.

This hypothesis requires, as also assumed by Bouillenne, that excised roots, usually cultivated in weak light, must gain the capacity of synthesizing rhizocaline. Bouillenne considers rhizocaline not only a factor for the initiation of roots but for the continuous functioning of the meristem. An excised root must consequently either start producing a new compound or be supplied with it externally. This is undoubtedly worth attention in con-

nection with the difficulties already mentioned in growing isolated roots unlimitedly, and the well-known peculiar light requirement of such roots. Bouillenne also stressed the necessity of a histological identification of the growth factors by separating from each other those causing cell division, cell elongation, and tissue differentiation. That is what has been introduced by Almestrand in root culture research. Bouillenne emphasized that the development of a root meristem involves three phases: (*i*) nuclear divisions; (*ii*) cell divisions in certain given directions; and (*iii*) differentiation, which apparently includes the growth by elongation. He set forth the intricate question, "Does a nucleus entering a prophase know that its descendants are going to form a root meristem?"

Considering the presumed genetical uniformity of the mitoses and the obvious omnipotency of living plant cells, i.e., the lack of cytological determination in embryonal tissues, this question ought to be answered by "no." Auxin, especially in high concentrations, undoubtedly promotes mitoses under exceedingly varying conditions. Regular cambial divisions, irregular divisions of proliferating tissues, and root initial formation in pericambia and similar locations can be mentioned. This is certainly no activity leading specifically to root formation. Thus, the possibility under (*i*) cannot explain the differentiation of embryonic tissue into a root meristem. The next one is (*ii*) that the cell divisions are laid in a specific pattern, necessarily leading to a root meristem. The failure of the histogen theory and the complete lack of co-ordination between cell divisions and differentiation in an apical meristem seem to exclude even this possibility; therefore, differentiation remains as the process giving the character to the root tip. This means that a root initial does not possess the properties of a root, distinguishing it from other meristematic groups of cells until differentiation has started. It is convenient here to consider again the comb-like formation of root initials and raise the question whether or not they have any properties of roots. They do not develop into morphologically distinguishable roots under the external circumstances of high auxin concentration leading to their initiation, but do so only when the auxin supply is drastically lowered. The same generalization seems to hold true for artificially enhanced root formation, which has, for instance, also been stressed by Kandler (13). The pertinent question with regard to the rhizocaline theory is whether the differentiation of the root is positively caused by the low auxin content or specifically by some other factor, and the development only prevented by a high auxin content.

An interesting point is the localization of the longitudinal growth of roots. It has been shown [Burström (82), Sandström (83)] that di-*n*-amylacetic acid inhibits cell elongation in wheat roots, specifically in the rhizodermis. This leads to a condition of a normal formation of rhizodermal cells, which, however, do not elongate but are continuously torn apart and shed off, while the elongation of central parts goes on fairly unimpeded. Hansen (45) also noticed that in treating pea roots with phenoxy compounds the whole cortex can loosen and be ruptured, the stele continuing to elongate. The cortical parts are thus dispensable for root elongation, which is carried on

by the stele or the adjacent layer. It is well known that in shoots, on the contrary, elongation is regulated by the surface layers, and the medullary cavity of a stem forms a counterpart to the ruptured cortex and rhizodermis of roots. Reverse conditions seem to prevail in roots and shoots, and these manifest themselves early in the ontogeny. It may be assumed that one characteristic of roots, as distinguished from shoots, is a certain pattern of growth by elongation, but whether this is determined by some hormonal factor, auxin or the presumed rhizocaline, or by the cell factor of Bouillenne (81) remains an open question.

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HERBICIDES[1,2]

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Introduction

Two reviews on the topic of herbicides have already appeared in the *Annual Review of Plant Physiology* [Norman *et al.* (174); Blackman *et al.* (22)]. In addition, the agricultural uses of growth regulators were reviewed by Nickell (173), van Overbeek (226), Rademacher (185), and Thorup (224). This paper is restricted, insofar as it is feasible, to papers which have appeared since these reviews.

It is now over 12 years since Templeman discovered the selective herbicidal action of naphthaleneacetic acid [Blackman, Holly & Roberts (19)], 10 years since Slade, Templeman & Sexton (208) and Zimmerman & Hitchcock (247) recognized the exceedingly strong hormonal and herbicidal effects of 2,4-dichlorophenoxyacetic acid (2,4-D), and 8 years since the announcement of 2,4-D[2] and 2,4,5-T[2] (2,4,5-trichlorophenoxyacetic acid) as weed killers by Hamner & Tukey (100). The phenomenal rise to popularity of these materials is a familiar story. It is of interest to note the extreme versatility of the hormone-like herbicides and to consider the variety of compounds used in weed control. Though noted in a previous review [Crafts & Harvey, (47)] it still seems pertinent to point out that 2,4-D and its analogues may be used in four rather distinct ways: (*a*) as contact or knockdown sprays for killing annual weeds, temporarily holding back growth or blossoming of perennials, and for preharvest desiccation of crop plants; (*b*) as selective sprays for controlling weeds, either annual or perennial, in a number of crops such as cereals, pastures, flax, etc.; (*c*) as translocated sprays

[1] The survey of literature pertaining to this review was completed in December, 1952.

[2] The following abbreviations will be used: BAL, 1,2-dithiol derivative of glycerine; CMU, 3-(*p*-chlorophenyl)-1,1-dimethyl urea; Cl-IPC, metachloral derivative, isopropyl-*N*-phenylcarbamate; 2,4-D, dichlorophenoxyacetic acid; 2,4-DI,* 2,4-dichloro, 5-iodophenoxyacetic acid with radioactive iodine; E.H. 2, dichloral urea; IAA, indoleacetic acid; IPC, isopropyl-*N*-phenylcarbamate; KOCN, potassium cyanate; MH, maleic hydrazide; MCPA, 2-methyl, 4-chloro-phenoxyacetic acid; N-1, N-1-naphthylphthalamic acid; PCP, pentachlorophenol; PMAS, phenylmercuric acetatate; 2,4,5-T, 2,4,5-trichlorophenoxyacetic acid; TCA, trichloroacetic acid, sodium salt.

[3] The writer is deeply indebted to Mrs. Barbara Kean for assistance in searching the literature; to R. L. Weintraub, V. H. Freed, R. J. Foster, D. H. McRae, J. Bonner, H. B. Currier, S. A. Peoples, and J. van Overbeek for manuscripts in the process of publication; and to his associates at Davis for reading portions of this manuscript.

for low-volume application to annuals and either low-or high-volume spraying of perennials; and (*d*) as temporary soil sterilants [Akamine (3)] for perplanting, pre-emergence [Arceneaux & Herbert (11)], or postemergence treatments of crops, and general application on noncropped areas. For soil sterilization, the hormone-like weed killers are often formulated in combination with TCA[2] (trichloroacetic acid, sodium salt) or carbamates because the latter kill grasses and so complete the sterilizing action.

The first herbicial growth regulator to be used in quantity in the United States was 2,4-D. It appeared as the acid formulated with Carbowax 1500 but soon was marketed as the acid mixed with sodium carbonate, and then, in quick succession, as the sodium and ammonium salts and as the alkyl esters; ethyl, butyl, and isopropyl were the most popular. In Britain, 2 methyl, 4 chlorophenoxyacetic acid (MCPA) was sold as the sodium salt (Methoxone) and as various salts and esters. Dust formulations of the salts and acids were tested and are still used in certain localities. Their application by airplane was soon prohibited in the United States and they were replaced by liquid formulations.

The salt formulation of 2,4-D gave trouble in hard water and liquid formulations of amine salts soon appeared. These are the most popular for general use, particularly for selective spraying in cereal crops. The light alkyl esters proved hazardous in areas of mixed farming because they volatilized and harmed sensitive crops such as cotton, tomatoes, grapes, beans, etc., [Daines (53)]. To meet this situation, esters of higher molecular weight were tested and they are gradually replacing the light esters. Examples of these are the butoxyethanol ester, the propyleneglycolbutylether ester and the tetrahydrofurfuryl ester. Others are being tested, and many will undoubtedly be marketed [King & Kramer (132); Anderson *et al.* (7)].

With the great interest in the hormone-type weed killers, many compounds were tested and it was soon found that 2,4,5-T and its salts and esters had different selectivities than 2,4-D. These compounds proved particularly toxic to woody species and the development of their use in brush control has paralleled that of 2,4-D for controlling herbaceous weeds. They are now available in formulations of the emulsifiable acid and as the amine salts and light and heavy esters.

Though it is not the function of this review to list all of the papers reporting screening tests the following are given for the convenience of those who are interested in this phase: Cuthbertson (52); Everist (73); Everson & Dunham (75); von de Goor (96); Green (97); Kopitke & Langford (136); Osborne & Wain (179); Osborne *et al.* (180); and Shaw (198).

The practical use of the hormone-like herbicides is considered in agricultural journals, weed conference reports, experiment station publications, and many other places. Only a few are cited here which seem to the writer to cover specific uses particularly well: Holly *et al.* (117); Hurwitz & Lachover (122); Loustalot (149); Marcelli (151); Moore & Myers (164); Offord (175); Overlord & Rasmussen (181); Stahler (212); Stewart *et al.* (214) Strykers (216, 217); and Wurgler, (244).

When 2,4-D proved useful as a temporary selective soil sterilant in pre-emergence weed control, it seemed desirable to find a form that would have little or no contact action on susceptible crops. As a result of synthesis and testing of many compounds, Herbicide No. 1 of the Carbide and Carbon Chemicals Division, Union Carbide and Carbon Corporation, was discovered. This is sodium 2,4-dichlorophenoxyethylsulfate [Finn (77); King (131); King *et al.* (133)]. A number of analogues of this compound have similar properties and several have been tested. The unique property of these compounds is that they have little or no hormone effect when sprayed directly on 2,4-D susceptible plants but after incorporation into the soil they are broken down by soil organisms releasing 2,4-D for action on the roots of young germinating seedlings of weeds [Carroll (34)]. The above named compound, marketed under the name of Crag 1, is being effectively used for weed control in a number of crops. It can be used safely where 2,4-D would be extremely hazardous.

To summarize, 2,4-D, 2,4,5-T, and MCPA[2] are being widely sold as formulations involving the acids, the sodium, ammonium, and various amine salts, and the light and heavy esters. In addition, a modified form having special properties is being used through the soil. Actually, many millions of pounds of chemicals are being sold under hundreds of brand names and within less than a decade an important and fascinating agricultural practice has become established. Most of these facts are familiar and they are being repeated here for the sole purpose of emphasizing the point that all of this research, testing, and business involves a small group of organic substances, the chlorophenoxy compounds. If one examines the whole array of herbicides he finds not a long list but a relatively short one consisting of the arsenites, borates, chlorates, thiocyanates, and phthalamates and the sulfamates, carbamates, and chlorophenoxyacetates, hydrazides, and chlorophenates.

With the exception of certain oils and chlorobenzenes, nearly all of the important herbicides are acids and salts or esters of acids. While it is unwise to overgeneralize concerning the toxicity of compounds having such diverse modes of action, it seems that a review of our knowledge of one important group might serve a useful purpose as an approach to more general studies on toxicity. It is proposed here to examine mainly the work on the hormone-like herbicides, with such briefer consideration of other materials as space will allow.

Mechanism of Herbicidal Action

Absorption.—To exert herbicidal action on a plant, a chemical applied to the foliage must first penetrate the cuticle of the leaf or stem. Fogg (82) concluded that chemicals in aqueous solution do not enter the leaf via the stomata but diffuse through the cuticle. Crafts & Reiber (48) postulated that activation of dinitro-*o*-cresylate solution by ammonium sulfate, buffered it on the acid side thus presenting a constant supply of undissociated dinitro-*o*-cresol molecules for diffusion across the cuticle and absorption by

living cells of the epidermis and mesophyll. Crafts (40) suggested further, that, for ready entry through the cuticle, 2,4-D and other herbicides should be in a nonpolar form.

Simon & Blackman (205) have shown, for a number of organisms, that the toxicity of a weak acid or base varies with the hydrogen-ion concentration of the medium. Simon (202) points out, however, that in applying dinitro-*o*-cresol to mustard seedlings as a spray, the volume of the seedlings is large in comparison with that of the spray solution and the toxicity will not necessarily be determined by the pH of the spray solution.

Simon & Beevers (203, 204) extend this reasoning to the pharmological tests on laboratory animals and the *Avena* curvature test. Finally, Simon, Roberts & Blackman (206) point out that when *Lemna minor* is grown in nutrient solution containing the toxicant, or when leaf disks of *Brassica alba* are vacuum infiltrated, the pH relation holds. They conclude that the pH effect is masked when leaves are sprayed with a relatively small volume of the dinitro solution and that "it is not to be expected that pH changes will have much influence on the toxic effects of other weak acids used as herbicides." While it is true that the influence of pH on toxicity is not as great in the case of herbicidal sprays as in the case of organisms growing within or on a buffered medium containing the toxicant, this does not mean that use of acid salts to activate dinitro-*o*-cresylate sprays is not a practical procedure. Sodium dinitro-*o*-cresylate is still used in large quantities in this country and ammonium sulfate is always included because experience has proved that it about doubles the toxicity and so halves the dosage required. When one realizes that this herbicide sells for $3.65 to $3.85 per gallon whereas ammonium sulfate costs around three cents per pound it is apparent that the use of this activator as a buffering medium is very economical. From one and one-half to six pounds of ammonium sulfate per gallon of cresylate concentrate are used depending upon the degree of selectivity required.

Reasoning from the fact that undissociated dinitro-*o*-cresol enters the plant through the cuticle more readily than does the dissociated cresylate, Crafts proposed (40) that nonpolar compounds in general enter leaf tissues more readily than polar ions. This led to the testing of other substituted phenols dissolved in oils as toxicants, with the result that dinitro-sec-butyl- and amyl-phenols and pentachlorophenol were introduced as general contact weed killers. The same generalization apparently applies to the chlorophenoxyacetic acid compounds [Mitchell (162)]. Toxicity apparently increases through the following series when used on leaves: sodium salt < ammonium salt < amine salts < acid < esters [McNew & Hoffman (157)]. Polarity of the compounds decreases in this same order and pH affects absorption in the same way as it does that of the dinitro compounds, penetration showing a steady increase through the pH range of 10 to 2 [Crafts (44)].

A very practical aspect of this relation is the formulation of emulsifiable 2,4-D acid herbicides. The American Chemical Paint Company has distributed for experimental trial a formulation (No. 638) which is proving very effective against a number of difficult species such as Russian knapweed,

hoary cress, white horse nettle, and poison oak. In dry habitats these weeds tend to develop very waxy leaf coatings. Furthermore all of these species are deep-rooted perennials and extensive translocation is required to control their deep and ramifying roots. The acid form of 2,4-D seems best suited for penetration and translocation under these critical conditions.

A special aspect of 2,4-D absorption has been given attention by Hansen & Buchholtz (106). They studied absorption by corn and pea seeds and found greater absorption at pH 4 than at pH 7. Top growth and survival of seedlings were both correlated with pH, increasing through a culture series from pH 4 to pH 7. Everson & Dunham (75) found aqueous solutions of 2,4-D to stimulate radicle growth at low (0.1 to 1.0 p.p.m.) concentrations and to inhibit it at concentrations from 100 to 1000 p.p.m. Water impermeable seeds were less affected than those readily permeable. Okumoto (176), studying 19 varieties of vegetable and field seeds, found some very sensitive to temperature differences, others relatively insensitive. Brown & Weintraub (29) found no temperature effect on leaf repression in bean between 22° and 34°C.

One further aspect of absorption needs attention. That is the role of adjuvants such as surfactants and cosolvents. Mitchell & Linder (163) proved that surfactants increased absorption and translocation of radioactive 2,4-D in bean seedlings. Ennis (74) showed that Carbowax 1500 would increase absorption of 2,4-D from aqueous solutions. In greenhouse experiments he found that oil as a carrier increased absorption of the isopropyl esters of 2,4-D and 2,4,5-T[2] over water as a carrier. A 5 per cent oil emulsion was somewhat less effective than straight oil in increasing absorption. In field plots oil emulsions including stabilizers were almost as effective as straight oils as carriers and were more effective than water plus surfactant.

Translocation.—Having penetrated the cuticle, the herbicide molecules must next part from this lipoidal medium and enter living mesophyll cells. In simple words, they must pass from a lipoidal to an aqueous medium. This requires that they be somewhat polar. Experiments have shown that 2,4-D ions of the sodium salt enter leaves slowly; aliphatic esters enter rapidly but do not part from the cuticle and translocate well; and 2,4-D acid and heavy ester molecules pass through cuticle, mesophyll and phloem with relative ease [Crafts (44)].

Day (57, 58) made detailed studies on the absorption and translocation of 2,4-D acid. He found this compound to traverse the cuticle, epidermis, and mesophyll at a linear rate of around 30μ per hr. Once inside the phloem, it moved at rates of from 10 to 100 cm. per hr. Because dosage had no effect on linear rate of movement within the phloem and plugging of the phloem near the ground level by steam ringing slowed movement out of the leaf 20 cm. away, Day concluded that the mechanism responsible for rapid longitudinal movement in the phloem operated by pressure flow of the assimilate stream. For a detailed review of 2,4-D translocation see Crafts (43), pages 257 to 263; also Blackman *et al.* (22), page 211; Woodford (242); and Mitchell (162).

As indicated above, translocation of 2,4-D and similar systemic toxicants

evidently occurs in the phloem with assimilates. Rohrbaugh & Rice (195), Weintraub & Brown (237), and Davis & Smith (56) all showed that translocation of applied 2,4-D depends upon the presence of carbohydrates and that any one of a number of sugars will induce translocation from starved leaves.

Recent work using radioactive isotopes as indicators has shown that 2,4-D is rapidly absorbed and moved in bean plants [Fang *et al.* (76)]. Young plants absorbed and moved the indicator more rapidly than old ones; absorption and translocation were not correlated with dosage applied; and small amounts of the growth regulator were broken down and metabolized in the bean plants with a consequent loss of radioactive CO_2. Studying absorption and translocation of 2,4-DI*[2] (2,4-dichloro, 5-iodophenoxyacetic acid with radioactive iodine) by oats, wheat, corn, beans, sunflowers, dandelion, and plantain, Gallup & Gustafson (90) found the monocots to absorb the indicator more slowly than the dicots. Translocation was also less rapid in monocots and the authors postulated a block in the intercalary meristem of the monocot leaves. Anatomically such a block has not been demonstrated. Blair (23, 24) studied absorption and translocation of 2,4-DI*[2] and 2,4,5-T by mesquite, and Riess *et al.* (192) and Earle *et al.* (69) used radioactive 2,4-D in studies on alligator weed. Time series experiments, using carboxyl-labelled radioactive 2,4-D in bean plants, have confirmed Day's results [Crafts (44)]. This indicator was absorbed and translocated into the roots of young bean plants within 30 min. after application. If 15 min. are assigned to absorption (Tween 20 was used in the indicator solution) and 15 min. to translocation, the linear rate of movement must have been over 100 cm. per hr. When one leaf was treated there was little movement into the opposite leaf and accumulation in the apical bud required 12 hr. or more. Plants that gave no bending response because of a water deficit moved the indicator as far and as fast as well-watered plants. Much more indicator was moved to the stem and roots when the droplet was placed over the midrib than when it was placed on the edge of the leaf.

Analysis of all of the data presented on absorption and translocation of 2,4-D and similar compounds indicates rapid penetration of the cuticle, ready migration across the mesophyll, and very rapid transport in the phloem along with assimilates produced in the leaves. The acids and heavy esters seem best adapted to absorption and translocation by perennial weeds and woody species, presumably because they have a balanced solubility in lipoid and aqueous phases. It seems agreed [Crafts (40); Blackman (17)] that injury to the mesophyll and phloem is not desirable; such injury, by stopping photosynthesis and plugging the sieve tubes, should retard or stop downward translocation to the roots. Much experimental and observational evidence indicates that this is true.

Absorption by roots.—A third phase of the mechanism of herbicidal action involves root absorption and subsequent translocation. Skoog (207) showed in 1938 that indoleacetic acid is readily absorbed by roots and trans-

ported upward in plants in the transpiration stream. Slade, Templeman & Sexton (208) found in 1940 that naphthaleneacetic acid applied to the soil would kill seedlings of *Sinapis arvensis* with little or no injury to oats. They later tried a number of chlorophenoxy compounds and proved the high toxicity of 2,4-D and MCPA.[2] Evidently these compounds are rapidly absorbed from the soil and the early appearance of epinasty and growth irregularities indicates their ready transport to the foliar organs. Crafts, (38, 41) reported the high toxicity and slow leaching from soils of 2,4-D as has Muzik *et al.* (169). Norman *et al.* (174) reviewed the literature on pre-emergence use of 2,4-D through the soil and Stamper & Chilton (213) described the use of this method in sugar cane culture. Harvey (108) stressed that there is no difference in toxicity between two water soluble forms of 2,4-D, namely the sodium and triethanolamine salts, and the oil soluble butyl ester. Either the ester is rapidly hydrolysed in the soil or the roots, unlike the leaves, do not distinguish between polar and nonpolar forms of 2,4-D. Crafts (40) has found that under humid conditions in the tropics, 2,4-D acid was more persistent in soils than were the salts. Persistence of 2,4-D in soils has been reviewed by Norman *et al.* (174). Workers at Camp Detrick have been active in this field, showing that microroganisms are definitely involved. Audus (14), Flieg & Pfaff (81), Hernandez & Warren (110), Jensen (125), and Stopp & Freter, (215) have made additional observations.

Even more interesting than the persistence of 2,4-D in soils is its apparent persistence in plants [McIlrath *et al.* (155)]. In cotton experimentally treated it is carried over into seeds produced by the treated plants. In cotton plants displaying obvious 2,4-D symptoms resulting from volatilization of an ester formulation used on a neighboring field, seeds produced subsequent to exposure showed no effects [Randall (186)].

In summary, it seems that 2,4-D, 2,4,5-T, MCPA, and similar compounds, as well as a number of dinitro and chloro substituted phenols enter plant leaves through the cuticle and that the oil-soluble, nonpolar forms penetrate most rapidly and in greatest quantity. Passing through the cuticle these compounds enter living cells. The dinitro and chloro substituted phenols kill the mesophyll if applied in sufficient concentration; in contrast, the hormone-like compounds pass through the meosophyll, are released into the phloem, and there tranlocated with synthesized food materials in the assimilate stream to regions of utilization. Dinitro and chloro substituted phenols kill roots if absorbed from the soil. The hormone-like compounds are absorbed by roots and translocated by the transpiration stream to the leaves where they accumulate and bring about injury or death.

One of the most unique features of the hormone-like materials is their ready absorption and translocation through vascular tissues. Evidently they are sufficiently like normal metabolites of plants to be handled by protoplasmic structures with little immediate injury. In this connection it is interesting to note that the tissues involved in this absorption and translocation are mostly mature. In contrast, the cells that accumulate these

compounds are mostly meristematic and are the cells that respond by increased water uptake, high turgor, abnormal division and growth, high respiration, and eventually death.

Another point in passing is the fact that it is the ready translocation of 2,4-D in plants that makes possible the low-volume application method. This method is used on millions of acres of grain and is now coming into use on sand sage, mesquite [Fisher (78); Fisher *et al.* (79); Fisher *et al.* (80)], and other woody species. For experimental testing of the method see Holly (116) and Blackman *et al.* (20).

Mechanism of auxin action.—While it is necessary that a herbicide of the hormone type be readily absorbed and rapidly translocated, in the final analysis it is its ability to kill cells and destroy plants that is important. What do we know about the chemical mechanisms involved in the toxic action of the chlorophenoxyacetic acid compounds?

In approaching this problem one soon finds that it is impossible to make a clear distinction between auxins and the hormone-like herbicides. With both of these types of compounds it is impossible to separate toxic effects from regulative effects. In starting it might be well to note the responses and the primary cell process, respectively, involved: (*a*) cell elongation—water uptake and growth; (*b*) root initiation—cell division and growth; (*c*) cambium activation—cell division; (*d*) callus stimulation—cell division and growth; (*e*) correlative inhibition of lateral buds—apical dominance, dormancy; (*f*) development of fruit—parthenocarpy; and (*g*) abscission inhibition—cell wall biochemistry.

A basic cell process that may be involved in any or all of these responses is protoplasmic streaming; this may be stimulated by both auxins and 2,4-D. Low concentrations of both auxins and the hormone-like herbicides may stimulate or accelerate plant responses; high concentrations usually inhibit them. It is with the inhibitory responses that we are vitally concerned in the consideration of herbicides. Weintraub points out that studies on herbicidal effects should stress effects not common to auxins in general [Weintraub (236)]. While death of tissues is amenable to accurate study [Greenham & Cole (98)] it is not a convenient phase of herbicidal activity to work with, particularly where dormancy and regeneration are involved, as with woody species. Even with laboratory tests various aspects of growth and its inhibition are easier to observe and measure, hence the emphasis on coleoptile sections, seedlings, and young greenhouse test plants.

In his analysis of studies on herbicide mechanism, Weintraub (236) points to two approaches to the problem, the backward and the forward, as he terms them. In the backward approach one takes the end effects of herbicide action, that is aberrant tissues, malformed leaves, or even dead plants and attempts by chemical analysis or histological studies to trace the course of action and arrive at the processes involved. Innumerable derangements are caused by 2,4-D and death itself may be secondary

resulting from decreased photosynthesis, increased respiration, impaired translocation, and even from fungus or bacterial invasion.

Many have found relationships between 2,4-D application and chemical composition of plants (Klingman & Ahlgren, 134). The well known effects on protein and carbohydrate composition have been reviewed by Blackman *et al.* (22) and Bonner & Bandurski (27). Work by Asana *et al.* (12), Freiberg & Clark (86), Rhodes *et al.* (191), and Wort (243) indicates a lowering of N and K absorption, abnormal distribution of N and K, breakdown of leaf proteins, and a temporary decrease in the per cent dry weight followed by an increase as the plants dried. The latter changes resemble those occurring to plants in the autumn and they recall the fact that 2,4-D application brings about early appearance of autumn coloring in many plants. Almost innumerable cases of change in composition resulting from treatment with growth regulators have been described. In a recent paper Rhodes (189) describes work on tomato plants treated with MCPA[2]. Using residual dry weight (total dry weight less available carbohydrate) to measure growth, Rhodes found strong inhibition shortly after treatment. Net assimilation rate was greatly reduced and the uptake of total N and P was somewhat reduced. In the plant as a whole there was no evidence for a depletion of available carbohydrate although the increase for treated plants was less than that for the controls. The tops resembled the whole plants in carbohydrate content but the roots differed. Whereas the roots of controls and of plants receiving no mineral nutrients showed a steady increase in available carbohydrates, the roots of plants receiving nutrients showed a slight increase followed by a decrease to a low level throughout the experiment. Rhodes suggested this may explain the strong herbicidal action of MCPA on vigorously growing plants and it indicates plants should be treated at the low point of root reserves.

An increase in the content of certain unsaturated lactones related to coumarin has been noted [Fults (89); Hamner *et al.* (102)]. Scopoletin and β-methylumbelliferone are examples. These compounds are noted for their effects on the sulfhydril enzymes that are thought to be important in growth. However, neither the two compounds named above nor a variety of other coumarin derivatives are able to bring about typical 2,4-D responses, nor do they seem to be directly involved in 2,4-D herbicidal action [Weintraub (236)]. Since much of the analytical work on 2,4-D treated tissues has involved long exposure to the herbicide, grossly abnormal or even dead tissues have received attention. Obviously the results contribute little to our knowledge of the initial responses to this chemical.

Studies on enzymes have not been especially fruitful. Plants treated with 2,4-D show increased activity of phosphatase [Olsen (177)] and pectin methoxylase [Neeley *et al.* (172)]. Catalase, peroxidase, and amyloplastic enzymes may be either increased or decreased according to the tissues and methods used. Work on purified enzymes *in vitro* has led mainly to negative

results [Wagenknecht *et al.* (234)]. Lipase activity was found to be reduced by 2,4-D and this reduction was less in wheat, a resistant plant, than in castor bean, a susceptible one. Goldacre (94) indicated that indoleacetic acid oxidase was activated by 2,4-D but later testing proved that activation was brought about by dichlorophenol [Weintraub (236)].

Turning now to the forward approach it seems reasonable that the first step should be a separation of the many aspects making up the total response of a plant to a herbicide into a number of more simple processes. Assuming that the effects of a 2,4-D treatment result from an interaction between the applied material and some cellular constituent that plays a major role in metabolism we might concentrate on this interaction and study it in detail. Since formative responses are the most convenient to observe and measure they are very useful. Moreover, as Weintraub points out, there is a high degree of correlation between formative activity and herbicidal activity whereas the majority of compounds which are active as auxins possess little or no formative activity. 2,4-D has strong formative activity but is ineffective in the *Avena* curvature test.

In attempting to identify the cellular constituent with which a particular compound interacts it is suggested that a knowledge of the structural requirements of the reagent for maximum response might be useful. For auxin activity, Koepfli, Thimann, & Went (135) set forth the following in 1938: (*a*) a ring system as the nucleus; (*b*) a double bond in this ring; (*c*) a side chain; (*d*) a carboxyl group, or a group readily converted into a carboxyl, on the side chain at least one carbon atom removed from the ring; and (*e*) a particular space relation between the ring and the carboxyl group [Thimann, (222); Bonner & Bandurski, (27); van Overbeek, (226); Nickell (173)].

It is not the function of the present review to cover in detail the relations of structure to function of growth regulators. In this volume this topic is covered by Dr. H. Veldstra, a scientist who has contributed much to the subject. For additional reference material the following may prove useful: (8, 26, 27, 45, 67, 68, 83, 86, 95, 102, 103, 104, 113, 127, 128, 130, 135, 138, 143, 165, 166, 172, 182, 183, 188, 190, 194, 200, 209, 210, 222, 223, 225, 226, 229, 230, 231, 232, 233, 235, 236, 241, 248).

Theories range from those involving a physical mechanism in which the regulator, having surface activity, changes interfacial relations at the plasma surface, to strictly chemical mechanisms involving salt or amide linkages with a protein moeity. One theory proposes that phenoxyacetic acids are metabolized in the plant generating energy-rich phosphate bonds by a process involving cyclization of the acid molecule. In one of the more critical analyses Thimann (223) suggests a rather loose combination between the ring of the activator and some substrate within the cell, the balance of the substitutions in the ring being more important than firmness of any one.

Summarizing briefly it seems that definite molecular configurations are responsible for auxin activity and very probably these same features are

responsible for herbicidal properties. Whereas a decade ago the requirements for auxin activity seemed well defined, today the testing of hundreds of compounds has revealed many exceptions to the early rules. Probably eventual understanding will involve a rather flexible mechanism of chemical and physical binding in which a balance of forces consummates the union of the regulator molecule with its substrate. It seems that such a concept might throw some light on selectivity of the hormone-like herbicides.

If speciation in plants is related to specificity of plant proteins it might well be that slight variations in spacing or even in critical electron density at certain positions on the aromatic nucleus of enzyme proteins in plants might result in rather large variations in the tendency of growth regulator molecules to react.

Fate of 2,4-D in plants.—Work with radioactive 2,4-D indicates that this compound enters plants readily and is rapidly distributed throughout the vascular tissues [Crafts (44)]. Possibly its failure always to cause death results from an inability to attach effectively to enzymes involved in the critical processes of metabolism. Mature cells are characterized by a lack of response to 2,4-D. Again, changes in enzymes accompanying the maturation of cells may bring about this altered reactivity. Finally, as shown by Weintraub *et al.* (238), 2,4-D may be fairly rapidly metabolized by plants with an attendant release of radioactive CO_2. The very uniform distribution of radioactivity in young cotton leaves of plants treated by applying a droplet of radioactive 2,4-D to a single cotyledon suggests the possibility that such metabolically released CO_2 might be synthesized rapidly into cell constituents and laid down in expanding leaves. Weintraub (236) points out that within a few days after application of 2,4-D to buds, both side-chain carbon atoms can be found distributed among acids, sugars, dextrins, starches, pectins, proteins, and cell wall constituents. Several other tagged compounds have been isolated [Holley, *et al.* (114, 115); Jaworski & Butts (124)]; one of these is a water-soluble, dialyzable compound hydrolyzable to an organic acid. It may be a hydroxylated derivative of 2,4-D. Another, termed "unknown 1" by Jaworski & Butts (124), may be a glycoside.

Inactivation of 2,4-D by riboflavin in light has been demonstrated by Hansen & Buchholtz (107). By a series of critical experiments these authors show that both *in vivo* and *in vitro*, 2,4-D is rendered nontoxic to corn seedlings by illumation in the presence of riboflavin. One end product of the reaction was identified tentatively as 2,4-dichlorophenol.

Brian & Rideal (28) attacked the problem of growth regulator activity by studies on adsorption and surface activity using the Langmuir trough. With MCPA[2] as their regulator and employing monolayers of a series of long-chain, surface-active compounds they determined their interactions through ranges of pH values. With octadecyl trimethyl ammonium chloride they found MCPA to interact in the ionic form above pH 4.0. It also interacted in low concentration with long chain amines and ketones. Adsorption of MCPA to monolayers of protein, lypoid, lipoprotein, and juices from the

living tips of wheat, cress, and tomato plants suggests that species susceptibility may result from adsorption of the regulator to sites not concerned in physiological response to the regulator. To the reviewer it seems possible that the same technic might be used in studies on the role of multipoint attachment as indicated by interaction correlated directly with regulator activity. With gliadin between pH 4.0 and 6.0 Brian & Rideal found such a correlation.

One further comment seems pertinent at this point. The British workers, including Brian & Rideal, Simon, Blackman, and others viewing the role of pH in herbicidal action, consider mainly entry of the chemical into cells through plasma membranes and they stress the buffering action of the living cells as limiting the degree of association of such herbicides as the DN (dinitro) compounds, 2,4-D, 2,4,5-T, MCPA, etc. In pointing out the common use of ammonium sulfate as an activator of DN compounds, and the great effectiveness of the ester and acid formulations of the phenoxyacetic acid compounds the reviewer is emphasizing the fact that on weeds the first movement of the chemical is through the cuticle. Here plant buffers such as those of the plasma and the vacuolar sap are not present. The spray solutions as they dry down on leaves concentrate and probably increase in buffering action. And the association into relatively nonpolar molecules conditions the herbicides for movement across the fatty surface layers into the epidermal and mesophyll cells. Here the herbicide molecules undoubtedly dissociate and react in their ionic form. Particularly in the phloem where rapid transport occurs, ionization must be complete because phloem sap is usually around pH 7.0 and highly buffered. Finally, interaction by multipoint attachment at the site of maximum physiological response (meristems) must take place from the ionic form for the protein-regulator complexes postulated involve linkages or bonding through ionic structures. Only during passage through the cuticle is ionization inhibitory to ready movement, and here the principal plant buffer systems are not concerned.

Whatever the nature of the processes involved in the breakdown of 2,4-D in the plant, they are apparently fairly rapid and in some cases at least, as indicated by recovery rates, fairly complete. Undoubtedly in plants in which they account for a fair to large proportion of the applied chemical they constitute an effective detoxification mechanism. This might explain the ability of some species to tolerate rather large doses of 2,4-D despite the fact that the herbicide is rapidly absorbed and translocated. Such an effect necessarily entails involvement of enzyme systems and again the intimate relations of the 2,4-D to the enzyme molecules may determine the specificity of the attendant apparent response or lack of response. Herein may lie clues to the selectivity and specificity of action of the various hormone-like herbicides.

Relation between 2,4-D and endogenous auxin: antiauxins.—Most growth regulators stimulate growth at low concentrations and the more active ones may inhibit growth at higher concentration, herbicidal action being an

expression of this latter effect. In treating plants with various compounds certain ones seem to act as auxin antagonists or antiauxins [Bonner & Bandurski (27)]. Van Overbeek *et al.* (228) have reported on the antiauxin activity of *trans*-cinnamic acid. Burström (31, 32, 33) has described a number of compounds having stimulatory and inhibitory action on root growth. Stimulatory response to isobutyric acid derivatives is taken to represent antiauxin activity. Åberg (1, 2) has discussed additional ones. Although antiauxins may find more direct agricultural use as defoliants [van Overbeek *et al.* (228)] they may eventually find use in the herbicide field. Meanwhile their use in fundamental studies on the mechanism of auxin action is providing information of an essential nature [Hitchcock & Zimmerman (112)].

Most work with auxin antagonists has involved compounds having molecular configurations similar to auxin but lacking one or more essential features or being sterically incompatible. Action of such compounds is usually explained on the basis of competitive inhibition [Bonner (26); Bonner & Bandurski (27); van Overbeek *et al.* (229)]. Leopold & Klein (141, 142) have shown that maleic hydrazide, a heterocyclic compound having practically no resemblance to normal auxins, acts as an inhibitor of indoleacetic acid and naphthaleneacetic acid. It is also known to counteract the effects of 2,4-D [Currier *et al.*, (50)]. In a separate paper, Leopold and associates (143) show that chelidonic acid, a compound having some resemblance to maleic hydrazide, inhibits growth, but that this inhibition is overcome at concentrations up to $1\times10^{-4}M$ by indoleacetic acid. In this way chelidonic acid and coumarin are similar. This similarity and the similarity in structure of chelidonic acid and γ-lactones suggested that chelidonic acid may inhibit growth in the same way as unsaturated lactones. Since the reagent BAL[2] (1,2-dithiol derivative of glycerin) is known to counteract the inhibitory action of lactones, Leopold and co-workers used this material and showed that it relieved the action of chelidonic acid at concentrations from 0.5 to $1000\times10^{-4}M$. BAL is a sulfhydryl containing reagent.

Weintraub (236) points out that 2,4-D treatment reduces the auxin content of buds, the reduction being proportionate to the 2,4-D dosage. Conversely, the formative action of 2,4-D is nullified by added auxin and quantitative studies indicate that 80 molecules of IAA[2] are required to antagonize one molecule of 2,4-D. In a sense, then, 2,4-D may be spoken of as an antiauxin.

Seeking again the mechanism involved in these responses it seems that one characteristic effect of 2,4-D is a disturbance of the normal processes of differentiation. Antagonism of this by indoleacetic acid suggests that the function of the latter may be the ordering of normal cell divisions, perhaps by inhibiting nonpolarized division. On the basis of this assumption, Weintraub (236) suggests that if normal development depends upon polarized divisions in meristematic cells it may be controlled by endogenous auxin acting as a coenzyme and fixed to cellular substrates by multipoint attachment. Structurally related compounds such as 2,4-D might compete

for these substrates and bring about a deficiency of the auxin complex which is essential to normal growth. Mild deficiency may cause malformations, severe deficiency proliferation, galling, and death. Relative affinities may be the basis for observed structural specificity of herbicides. A natural consequence of multipoint attachment is that excess of either auxin or applied regulator leads to a decrease in the auxin complex and to growth inhibition because some auxin or regulator molecules will attach to one site, some to the other, mutually hindering each other from attaining the requisite multipoint attachment. The resultant unpolarized divisions might bring about abnormal growth, derangement of metabolic and physiological processes, and ultimately death [Weintraub (236)].

Variety of plant response.—The foregoing consideration of the mechanism of action of growth regulators is given to indicate the trend of research and thought with regard to the newer herbicides. While such work is fascinating and productive of fundamental knowledge it should always be kept in mind that the ultimate objective of the use of herbicides is plant control. Reasoning from elongation of *Avena* sections to inhibition and cell death is hazardous. In fact, any testing that does not include application to whole living plants that results in their ultimate death may be very misleading. In such testing a variety of crop plants and weeds should be used if selectivity is to be discovered.

A great variety of plant responses has been used in herbicide testing [Wain (235); Hitchcock & Zimmerman (112)]. For instance, the straight growth test of coleoptile or root measures principally growth activity, promotion, or inhibition. The bending test of beans is used to measure the rate of absorption and translocation, and the amount of material absorbed and translocated [Day 57, 58)]. Rates are measured by periodically removing treated leaves and detecting the minimum time required for a given response. Formative effects are used as a measure of activity and to distinguish between different types of growth regulation. They are also used as a measure of the minimum amount of material required to injure plants in studies on drift and vaporization of the chlorophenoxyacetic acids. Stimulation of cell division, root initiation, seed germination, and formation of parthenocarpic fruits are additional responses that have been used to study growth regulators. For examples of these tests see Blackman (18); Hitchcock & Zimmerman (112); Norman *et al.* (174); Wain (235); and Zimmerman (246). Measurement of injury to, and death of, experimental plants following spraying, dipping, dusting, and other types of treatment is a standard technique for studying herbicides and examples would be too numerous to mention. The point of emphasis here is that this method should be included in any comprehensive plan of screening and testing of herbicides. For a critical consideration of experimental design of toxicity tests see Blackman (18); Fromm (87); Muzik, *et al.* (170); and Sampford (197).

MCPA and 2,4,5,-T.—Testing of the chlorophenoxyacetic acid compounds soon proved that MCPA and 2,4,5-T had somewhat different selec-

tivities than 2,4-D. Comparison of MCPA and 2,4-D [Blackman (18); Blackman, Holly & Cox (20); Mullison (167); Templeman & Halliday (219); Templeman & Wright (221); Woodford (242)], has shown the former to be somewhat more selective than 2,4-D, particularly when clovers, flax, and other crops having leaves that are hard to wet are concerned. MCPA, as sold, may contain up to 40 per cent by weight of impurities. Hansen (105) has shown that some of these have toxic properties. The use of 2,4,5-T against brushy species is becoming common practice [(10); Barrons (15); Blair (23, 24); Cornelius & Graham (36); Coulter (37); Day (59); Fisher (78); Fisher *et al.*, (79, 80) Halls & Burton (99); Hewetson (111); Hull *et al.* (120); Hull & Vaughn (121); Leonard (139); McNamara (156); Minshall (161); Mullison *et al.* (168)].

Crop injury by 2,4-D.—Despite the great relative selectivity of 2,4-D there have occurred many cases of injury both to sprayed crops and to crops and miscellaneous plants accidentally exposed to 2,4-D. Effects of 2,4-D on corn have been studied by Rossman & Staniforth (196), Hoshaw & Guard (119), and Elle (70); on barley by Dersheid (64), and Dersheid and associates (65); on flax by Dunham (66); on cotton by Gifford (93); on rice by Kaufman (129); on wheat and barley by Olsen *et al.* (178), Gautheret (92), Longchamp, Roy & Gautheret (146, 147); and on barley and oats by Anderson (6). Recently Unrau & Larter (225) have shown that treatments with 2,4-D cause mitotic irregularities at all stages of growth in one barley and two wheat varieties. In view of the nature of these effects it is difficult to understand why so little trouble has been experienced with seed from treated cereals. Meletti (158) has shown that 2,4-D acid stimulated mitosis in *Pisum* and *Vicia* roots and hypocotyls with many polyploid cells being formed. Lateral roots were frequent. These studies on 2,4-D injury prove that cotton, grapes, tomatoes, and a good many ornamentals and garden plants are highly sensitive to 2,4-D, and that many of the so-called tolerant plants may be seriously injured if exposed at particular periods of their development.

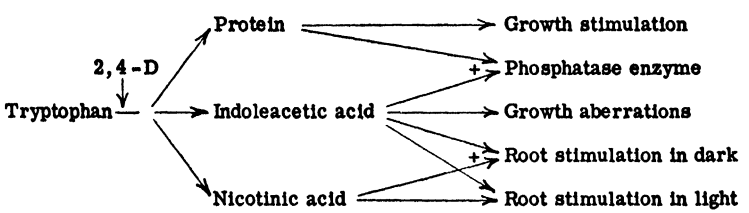
Kratochvil (137) has studied the effects of various herbicides on soil microorganisms. He found that TCA[2], PCP (pentachlorophenol), E.H. No. 2 (dichloral urea), and IPC (isopropyl-N-phenylcarbamate) reduced soil microbiological activity; 2,4-D, 2,4,5-T and endothal (disodium-3,6-endoxohexahydrophthalate) had no effect at the rates used; E.H. No. 1 (sodium 2,4-dichlorophenoxyethylsulphate) now known as Crag 1, had a stimulatory effect.

From the foregoing consideration of the mechanism of action of auxins and herbicides it is apparent that the problems are complex. Actually thousands of compounds have been tested, a great variety of plants have been used, and many responses have been measured and recorded. From the nature of the chemicals used and the responses observed it seems probable that molecular attachment of the applied chemical to substrates in plant cells is involved and that enzymatic reactions are brought about.

Some would favor a mechanism wherein the applied chemical molecules attach directly to proteins or amino acids to form a complex immediately concerned in metabolism; others suggest competition between applied molecules and endogenous molecules for space on receptor entities, aberrant metabolism resulting from the disturbance of normal balance; still others would go farther and place the responsibility for injury and death upon discrete substances produced as a result of abnormal metabolism. It seems obvious that any satisfactory theory is going to have to consider the multiplicity of responses and the complexity of the causal reactions.

Taking into account growth stimulation and growth inhibition by the same reagent, depending upon concentration, and recognizing the great array of morphological responses elicited by hormone-like herbicides, the writer proposed in 1949 (42) a scheme that might merit consideration. Wildman, Ferri & Bonner (240) have suggested that tryptophan may undergo two fundamental transformations, one to a protein, the other through indolepyruvic acid to indoleacetic acid. If 2,4-D could be shown to affect the equilibrium betweeen tryptophan and these two products a broad interpretation is presented. If protein is favored, stimulation of growth might result, whereas increase in auxin might bring about growth aberrations.

Galston has recently pointed out (91) a third product that might come from tryptophan, namely nicotinic acid. He shows that nicotinic acid and indoleacetic acid in the dark favor root initiation while in the light they favor shoot growth. Might not the two schemes be combined into a more complete picture as follows:



If 2,4-D should regulate the above transformations, increase in protein might explain the growth stimulation of cotton shoots, grape tendrils, and seedlings observed in light, and root growth in the dark. Increase in indoleacetic acid might inhibit growth and bring about the morphological aberrations commonly observed on 2,4-D treated plants. And in proper combination indoleacetic acid plus nicotinic acid might stimulate root initiation and growth as observed on treated plants. On the other hand a favorable combination of protein and indoleacetic acid might result in increased phosphatase which in turn would account for depletion of stored food. Such a combination of effects might explain the stimulation found from low application of 2,4-D, the translocation into and accumulation in meristems of 2,4-D resulting in injury and death from intermediate dosage, and the

immediate contact killing and restricted translocation resulting from heavy application of the readily absorbed esters [Crafts (42)].

Mode of Action of Some Other Herbicides

Carbamates.—Literature on the carbamates has been reviewed by Freed (84) and Ivens & Blackman (123). Two of these compounds have proved to be effective herbicides, isopropyl-*N*-phenyl carbamate (IPC[2]) [David, (55)] and the metachloro derivative of IPC (Cl-IPC[2]). Templeman & Sexton (220) first suggested that carbamates had possibilities as selective herbicides. IPC proved to be the most effective of the alkyl esters and it showed selective toxicity against grasses. Several years passed before practical application methods were worked out. The compound was relatively insoluble in common solvents, it acted through the soil as a mitotic poison on roots, and it was rather rapidly lost from the soil by microbial decomposition or volatilization [Anderson *et al.* (7)]. Currently it is being used in car-lot quantities in the United States, particularly on the West Coast where it has proved effective in the control of winter annual grasses, chickweed, and certain other weeds in alfalfa, Ladino clover, bird's foot trefoil, flax, perennial grass nurseries, and certain winter grown vegetables, notably spinach, peas, and asparagus. IPC is also finding use in other countries, as indicated by the reports of Maskova (152).

The deep green color resulting from treatment with IPC is from increased chlorophyll content. Treated plants contain from 18 to 28 per cent more chlorophyll per unit leaf area or per gram dry weight than comparable untreated plants. Nitrogen content is also high and reserve carbohydrates are low. Respiration shows an initial inhibition, then stimulation, and finally decline. Dehydrogenases of the four carbon cycle are nearly completely inhibited by $1\times10^{-4}M$ IPC. Growth and respiration inhibition are overcome by inositol, a behavior characteristic of narcotic treated plants. Dehydrogenase inhibition probably acts at the metaphase of cell division [Freed (85)].

Cl-IPC is more resistant to attack by soil microorganisms but more volatile than IPC (Anderson *et al.*, 7). It is finding increasing use as a preemergence and postemergence herbicide [De Rose (62)] against annual and perennial grasses [Ciferri & Bertossi (35) King (131)] in summer row crops, notably cotton.

The action of the carbamates has been shown to be similar to that of colchicine. Ivens & Blackman (123) using ethylphenylcarbamate, found in treated barley and pea roots a disruption of mitosis with accumulation of arrested metaphase states. In barley roots there was increase in size of cells, many polyploid nuclei, varying degrees of chromosome contraction, many multinucleate cells, and a great increase of nuclei in the metaphase condition compared with anaphase and telophase. Concentrations that inhibited root extension interfered with cell division and gave rise to nuclear abnormalities. It has been postulated that these compounds inside the cells associ-

ate with the lipophilic side chains of the proteins in the spindle leading to an intramolecular precipitation, folding of the protein chains, and disintegration of the spindle [Ivens & Blackman (123)]. Though these conclusions are still tentative they give a clue to the mechanism of toxic action and indicate the direction to be taken in future research. Further, they give some insight into the nature of the herbicidal action and explain why the carbamates fail as sprays on foliage but kill plants through the roots at relatively low dosages.

Freed (84) points out that to control quackgrass IPC should be applied to well-grown plants, that oil as a carrier is essential to success, that disking after application increases effectiveness, and that late summer application is better than earlier. Addition of maleic hydrazide further increased effectiveness of the treatment. In tests on annual grasses Freed found that application of IPC at the time of planting was superior to later treatment, that adequate soil moisture was necessary to bring the IPC into intimate contact with the roots, and that treatment was effective during cool winter months but decomposition in summer was so rapid that treatments were ineffective.

The carbamates are available as wettable powders and as emulsifiable liquids and are being produced by a number of chemical concerns. They seem to offer great promise for the control of grasses and certain other weeds and their use will undoubtedly expand greatly as growers recognize their role in chemical weed control and learn to handle them intelligently.

Trichloroacetic acid (TCA).—The sodium salt of this acid is being widely used as a grass killer. It is highly soluble and may be applied in aqueous solution as a spray or it may be applied dry or in solution to the soil. Its principal action is through the soil on the roots and its principal use is on perennial grasses. As used in weed control it is required at rates of 80 to 160 lb. per acre to kill Johnson and Bermuda grasses [Moore & Myers (164); Stamper & Chilton (213)]; against water grass, crabgrass, and similar summer annuals it is applied at dosages as low as 5 lb. per acre. Acetates and acetic acid are known to penetrate living cells readily and they are common ingredients in killing fluids for preserving plant and animal tissues [Robbins *et al.* (193)].

Barrons & Hummer (16) describe chemical and biological testing methods for TCA. This chemical has a contact action that is distinct from its systemic effects on susceptible grasses. It produces a prolonged dormancy of buds on rhizomes exposed to it in the soil. The buds may recover if removed from the treated soil. Seeds will germinate in soils containing sufficient TCA to kill seedlings. Death usually occurs in the cotyledon stage. Roots show less injury than foliage. Barrons & Hummer suggest that possibly in susceptible plants, TCA inhibits synthesis of an essential substance which is present in the seed in sufficient quantity to provide for growth of the seedling, particularly the roots. Finding that TCA is absorbed by both tolerant and susceptible species but that it disappears quite rapidly

from susceptible plants whereas it could be found in quantity in the sap of tolerant species, they postulate that it may be metabolized in some manner with resultant growth reduction and growth aberration. They suggest that the TCA applied to foliage may react with the protoplasm and so be used up without expressing its toxic effects on the roots. There is very little evidence for translocation of TCA in plants. For lists of tolerant, intermediate, and susceptible plants the reader is referred to the original article. TCA is being used to control grasses in sugar cane fields [Loustalot (148)].

Phenylmercuric acetate (*PMAS*[2]).—This compound, in addition to having fungicidal properties, is useful for controlling crabgrass in lawns, being selective against the seedling stages. The chemical is a white crystalline solid, relatively insoluble in water. In commercial preparations it is formulated by means of a cosolvent [De France *et al.* (60); De France & Simmons (61); Robbins *et al.* (193); Simmons & De France (201)]. Toxicity of phenylmercuric acetate in soils has been studied by Levi & Crafts (145). In three soils, sterility against the test plant (oats) was attained at 200, 300, and 450 p.p.m., respectively. In Yolo clay loam growth was about 16 per cent of normal at 680 p.p.m. By the second run, crops were normal in all four soils.

In percolation texts toxicity was confined to the top 10 cm. of soil when 85 cm. soil columns were completely moistened from above. In leaching tests, water up to and including 20 surface cm. did not displace the toxicant from the top soil fraction.

Phenylmercuric acetate is now formulated as a granulated material for dry application. The soluble chemical is spray dried on expanded vermiculite which is dyed green to match the color of turf.

Potassium cyanate (*KOCN*).—This chemical is proving to be a useful selective contact herbicide against broad-leaved weeds in onions, asparagus, and peas. It also gives promise in the control of crabgrass in lawns and in gladiolus. It is a white crystalline solid readily soluble in water and is applied in aqueous solution in concentrations of from 1 to 2 per cent.

Disodium-3,6-endoxohexahydrophthalate (*Endothal, Niagrathal*).—An active defoliant and potent contact herbicide, this new chemical is finding use in many situations [Poland (184)]. As a preharvest desiccant it has been found effective on legume seed crops, dry beans, soy beans, potatoes, rice, corn, small grains, flax, and milo. Special formulations are used for cotton and nursery stock defoliation.

Endothal has shown promise as a selective pre-emergence herbicide in sugar beets, table beets, onions, soy beans, potatoes, gladiolus, certain crucifer crops, and peanuts. It is particularly effective against seedling grasses including wild oats. As a post-emergence spray it is used in sugar and table beets, onions, cranberries, white potatoes, and gladiolus. It is being tested and shows some promise against grasses in flax, joint grass in winter wheat, and summer annual grasses between cuttings in alfalfa. It is also useful as a nonselective soil treatment against quackgrass, giant fox-

tail, barberry, and other shallow-rooted weeds and weedy shrubs and trees at rates up to 10 gallons per acre. Endothal is more effectively absorbed by roots than by foliage. Incorporation of ammonium sulfate in the formulation greatly enhances its toxicity. This chemical apparently has no effect on the soil microflora [Kratochvil (137).

3-(p-chlorophenyl)-1,1-dimethyl urea (*CMU*[2]).—This is a nonvolatile, noncorrosive, nonflammable organic compound having extremely high toxicity to plants when absorbed from the soil by roots. It is a solid, sparingly soluble in organic solvents and very slightly soluble in hydrocarbon solvents and water. Its water solubility is around 230 p.p.m. at 25°C. Several aromatic substituted ureas are active but only the asymmetrical ones are effective. *Para* substitution of the aromatic nucleus is more effective than *ortho* or *meta* [Freed (85)].

Used in acetone solution CMU proved highly toxic when applied as a spray to a variety of plants [Bucha & Todd (30)]. It is now being formulated as 80 per cent wettable powder for application either dry or in suspension in water. As with all soil-borne herbicides, CMU requires soil moisture to be effective. Applied to dry soil it remains ineffective until washed in by rainfall or irrigation. Once incorporated in the moist soil it is picked up by plant roots and very soon symptoms of chlorosis of leaves and die-back of shoots appear. As a pre-emergence material against seedling weeds it is being tested at dosages of from $\frac{1}{4}$ to 2 lb. per acre. Against established annual and shallow rooted perennials it is used at 20 to 40 lb. per acre. On deep-rooted perennials 80 lb. per acre are recommended. At the latter rates it kills all vegetation and maintains sterility for a year or more [McCall (153, 154)]. CMU has given satisfactory soil sterilization on Canadian railroad ballast. CMU and 2,4-D proved compatible and the results additive [Snyder (211.)]

CMU has a different herbicidal action than any chemical previously used in soil tests. Plants grow for several days in soil that has had the chemical thoroughly mixed in it. After 10 to 14 days tips of cereal plants turn yellow and start to burn back. Soon the whole plants are involved and they usually die within a week or ten days. Freed (85) has suggested that the nitrogen metabolism is disturbed and this may well be, for the yellowing plants resemble those suffering from lack of nitrogen. Chemical studies show CMU treated plants to be low in ammonia and nitrate nitrogen but high in protein. Treatment lowers nitrate absorption and carbohydrate reserves become depleted. Tests at Davis, California, prove that CMU and arsenic are compatible and the idea of using CMU with white arsenic for lasting soil sterilization is being studied. CMU should provide sterilization during the year of application and the arsenic should maintain sterility for many years. CMU is undergoing tests as a pre-emergence herbicide at low rates of application. While it is highly toxic at rates as low as $\frac{1}{2}$ lb. per acre it remains to be proved if it has sufficient selectivity to be useful by this method. Cabbage, cucumber, onion, lettuce, red clover, and cotton are quite susceptible. Corn, peas, beets, oats, flax, potatoes, and carrots are

somewhat tolerant. Much more testing under different conditions of temperature, precipitation, and soil type are needed before recommendations for pre-emergence use can be given.

Maleic hydrazide (*MH*).—This is a white crystalline solid having acidic properties which has been formulated as the diethanolamine salt and as the sodium salt. It is a growth inhibitor, a grass killer, and induces prolonged dormancy in treated plants. As a growth inhibitor it seems promising for holding back growth of hedges, lawns, bulbs, and stored roots, and for inhibiting second growth on cotton, beans, alfalfa, and similar crops where late vegetation interferes with harvest. As a grass killer it has proved effective against many species. It seems to offer most promise for controlling Johnson grass, Bermuda grass, and quack grass [White (239)]. Literature on this chemical has recently been summarized by Zukel (249). Its behavior in California soils is described by Levi & Crafts (144). Tatum & Curme (218) describe responses of young corn plants to maleic hydrazide.

Little is known concerning the mode of action of maleic hydrazide. It has been known for some time that this compound is antagonistic to 2,4-D [Currier, *et al.* (50)] and auxin [Andreae (8); Leopold & Klein (141, 142)]. The latter reported that maleic hydrazide and the auxins IAA and naphthaleneacetic acid, show mutually antagonistic effects. From the standpoint of molecular configuration it is difficult to reconcile this with the concept of competitive inhibition, either through a general similarity of molecular form or through the more exact concept of multipoint attachment. Crafts, Currier & Day (45) concluded that MH has hormone properties, not on the basis of molecular structure, but on the basis of its formative effects on leaves, its inhibitory effect on growth, its nullification of apical dominance, and its apparent ability to be translocated in plants and produce regulatory responses at a distance from the point of its application. MH differs from many auxins in that it shows no stimulatory effects [Leopold & Klein (142) Figure 1). Weintraub (236) disagrees with Leaper & Bishop's (138) concept of a direct relation between 2,4-D toxicity and MH toxicity.

N-1-naphthylphthalamic acid (*N-1*).—This new chemical is a monobasic acid, crystalline in form, relatively insoluble in water, only slightly soluble in common organic solvents and highly toxic to a number of plants. It seems promising as a pre-emergence material. A summary of published work and a description of current testing is given in N.P. information sheet No. 1 of the Naugatuck Chemical Division, United States Rubber Company (9).

Other compounds.—Many additional compounds having phytotoxic properties have been described by Alamercery *et al.* (4), Allen & Skoog (5), De Rose (63), Emmert & Klinker (71), Fromm and O'Donnell (88), Hamner & Tukey (101), Miller (159), Minarik *et al.* (160), Ready *et al.* (187), Shaw & Bissett (199), Simmons & De France (201), and Zimmerman & Hitchcock (248). Aslander (13) has described new methods for use of an old herbicide, sodium chlorate and Loustalot & Ferrer (150) and Young & Carrol (245) have studied the breakdown of pentachlorophenol (PCP) in soils.

Oils.—In general, four types of oils are used as herbicides: (*a*) light distillates of the Stoddard Solvent type used as selective killers of annual weeds in crops of the carrot family [Blackman and Ivens, (21); Crafts, (39); Emond, (72)], forest tree nurseries [Holmes & Ivens, (118)], cranberries, etc., and for pre-emergence treatment where no toxic residue is permissible; (*b*) light aromatic fractions of the Edleanu extract type used as general contact sprays for general weed control and for preharvest desiccation of many crops; (*c*) heavier aromatic fractions, i.e., catalytically cracked bottoms, used as general contact sprays, particularly when grasses are present in the weed population and for winter spraying and between crop spraying of alfalfa and other legumes in the production of certified seed; and (*d*) various fuel and by-product oils, including smudge pot oil, diesel oil, slop and wash oils, and various bottoms from refining processes. These oils are used as general contact sprays and they are particularly effective in controlling grasses.

For pre-emergence treatment, *b*, *c*, and *d* all leave toxic residues, i.e., the heavier oils are the more persistent. This persistence of the heavy oils is desirable where they are used for complete control of weeds in non-agricultural areas but it may be fatal to crops in pre-emergence application.

Since the last review [Blackman *et al.* (22)], Currier (49) has published results of studies wherein plants were treated by vapors as well as by spray application of the liquid fractions. Currier concluded that toxicity increased in the order: benzene, toluene, xylene, and trimethyl benzene in both vapor and spray treatments. Resistance of plants to both vapor and spray increased in the order tomato, barley, carrot. Activity of the vapors diluted with air, on a molar concentration basis, is roughly 30 times that with water as the diluent, and 3000 times that in paraffin oil; hence, the nature of the diluent is important.

Currier concluded that the mechanism of acute toxicity of the four hydrocarbons studied probably involves disorganization of the outer plasma membrane of the cells as a result of the solvent action on the lipoid components. Partition phenomena probably determine the concentration of hydrocarbons at the point of action. Carrots showed relative resistance in all treatments and Currier suggested that the basis of selectivity may lie in the character of the cell wall or of the outer plasma membrane. Dallyn & Sweet (54) attacking the problem with a somewhat different approach have concluded that oil toxicity is tied to response by the plasma membranes of cells.

Crafts *et al.* (46) proposed a new classification of oil toxicity. Including data from their addendum this might be outlined as follows:

Contact toxicity: (*a*) Acute burning of foliage by light aliphatic and olefinic oils; (*b*) Acute toxicity from aromatic hydrocarbons; (*c*) Acute toxicity produced by refined parathenic oils that have become oxidized.

Systemic toxicity: (*a*) Chronic injury caused by high boiling non-aromatic oils; (*b*) Chronic injury caused by polycyclic aromatic oils. Such oils must

be diluted to produce typical chronic injury; undiluted, their acute toxicity is so high that systemic effects are masked or possibly actually inhibited.

Myers (171) studied the toxicity of oils to *Paspalum dilitatum* and found the boiling range to be more closely correlated with toxicity than was aromaticity. The most toxic oils were those having a mid-boiling-point of 300°C. or more. Since he was measuring regrowth of a perennial grass Myers' results emphasize the importance of chronic toxicity where killing of such weeds is concerned.

Leonard & Harris (140) conducted detailed studies on the toxicity of aliphatic oils on cotton, soybeans, nutgrass, and Johnson grass. They concluded that olefines were more toxic to the weeds and the crop plants than paraffines and branched chain isomers were slightly less toxic than straight chain compounds. The speed of injury decreased as the length of the carbon chains increased from 6 to 14 carbon atoms. The lighter oils are effective against grass seedlings; against mature nutgrass and Johnson grass the heavier, more persistent oils were more effective. However since toxicity becomes less with increasing chain length dodecane was more effective on nut grass than tetradecane. Soybean hypocotyls were more sensitive to hydrocarbons than cotton hypocotyls, the latter withstanding several applications of decane. Octane was safer than decane on soybeans while dodecane was quite effective in killing nutgrass. Ten and 12 carbon hydrocarbons were most effective against Johnson grass while tetradecane and tetradecene produced chronic toxicity on both crop plants and weeds.

Johnson & Hoskins (126) studied oxidized spray oils. They found visible injury of bean leaves to be correlated with the acidity of oxidized oils and proved the acids to be, in their experiments, 36 times as toxic as peroxides. Oxidized oils materially reduced O_2 uptake by leaf fragments, reduced leaf respiration 30 per cent, and prevented synthesis of sucrose from glucose. They did not inhibit the hydrolysis of infiltrated sucrose nor did they have any *in vitro* effect on the activity of the cytochrome oxidase system of bean leaf brei. The oils used by Johnson and Hoskins are not those commonly used as herbicides but such oils are used as carriers and solvents in many formulations of various herbicidal mixtures and knowledge of their toxic affects is required if they are to be used wisely. Heringa & Swarbrick (109) have described the effects of refining on two spindle oil fractions obtained from crude oils of different geographic origins.

In a recent report, van Overbeek (227) attempts to explain the toxic action of oils on the basis of a solubilization of the plasma membrane. This follows the same general principle as the theory of Currier (49) but goes more into detail. It brings work in theoretical colloid chemistry to bear on oil toxicity and provides a very attractive mechanism which explains many of the observed plant reactions. Whether this theory can reconcile the differences between acute and chronic toxicity on the basis of rate remains to be proved.

Shale oil is a new material for agricultural use and Bohmont (25) de-

scribes physiological investigations, phytotoxicity studies, and field trials with crude shale oil, shale oil vapors, naphtha, and kerosene fractions. In addition to the expected phytotoxicity, shale oil naphtha at 2 to 10 gal. per acre acts as a plant stimulant.

Currier & Peoples (51) studied the toxicity of *n*-hexane, hexene-1, cyclohexane, cyclohexene, and benzene. All produced acute toxicity symptoms and toxicity increased in the order in which they are named. Barley plants were more susceptible than carrot. From the determination of the coefficient of distribution of the hydrocarbons between air and water, air and oil, and water and oil, toxicity was found to correlate best with the air/oil distribution where plants were treated with vapors. When treated with true solutions of the hydrocarbons in water the minimum lethal dose expressed as per cent saturation increased in the order benzene, cyclohexene, cyclohexane, hexene, hexane; on a straight concentration basis this order is reversed, hexane being low in solubility compared with benzene.

Calculation of theoretical hydrocarbon concentrations in the cell lipid by use of distribution coefficients gave rather constant values of about 2.0M; differences in absolute toxicity were not great. Differences in toxicity, therefore, are related to molecular structure only in the sense that structure determines physical factors that affect the distribution of the oils between the cells and their surrounding medium.

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FLUORESCENT SUBSTANCES IN PLANTS[1]

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Introduction

Fluorescent substances have two properties in common: that of absorbing radiant energy and that of reradiating this energy, usually at longer wave lengths. This reradiation takes place with extreme rapidity, often in less than 10^{-7} seconds, according to Pringsheim & Vogel (153), and is to be distinguished from phosphorescence, a slower energy transfer which can be observed as an afterglow following the extinction of the exciting radiation. Fluorescence may be excited by x-rays and by ultraviolet and visible radiation. The spectral distribution of the fluorescence may range all the way from the ultraviolet to the infrared. This review will be limited arbitrarily to compounds of botanical origin which fluoresce in the visible range. Phosphorescence will be dealt with only insofar as this process is not distinguishable experimentally from fluorescence itself.

Fluorescence is a very interesting and useful property of chemical substances. It enables one to detect, to extract, to isolate, to identify, and to measure minute amounts of fluorescent compounds. Identification and quantitative determination can often be carried out on crude preparations. In certain instances, the fluorescence of substances can be excited *in vivo*, thus permitting the localization of the compound within the organism. When these substances are involved in biological processes, observations on their fluorescence may elucidate the nature and rates of these processes.

The reader is referred to the following books dealing with various aspects of fluorescence phenomena: Danckwortt (36), DeMent (38), Dhéré (43), Förster (64), Haitinger (83), Pringsheim & Vogel (153), and Radley & Grant (155). Extensive bibliographies will be found in these references.

Occurrence of Fluorescent Compounds in Plants

Much remains to be learned about the fluorescence of organic compounds. Favorable conditions such as the physical state of the substance, the pH of the solvent, and the wave length of the exciting radiation, must exist if the phenomenon is to be observed. Investigators are frequently uninterested in fluorescence and may omit or make only casual reference to this property.

Since published information on fluorescent compounds of botanical origin is so widely scattered, bibliographical data on this subject have been compiled in Table 1. Although this table is very incomplete, it will demonstrate the ubiquity of fluorescent compounds in plant material and will serve as a guide to the extensive literature already available. Only those com-

[1] The survey of the literature pertaining to this review was concluded in September, 1952.

pounds which are known to occur in plants and which fluoresce in the visible range when dissolved in solvents are included. Most of them are excited by the 366 mμ lines of the mercury arc spectrum. The compounds have been placed in natural groupings which have been arranged as nearly as possible in order of increasing complexity. Individual salts of organic acids and alkaloids have not been separately listed. The following data, when available, are given after each compound: color of fluorescence in solution, nature of the solvent if not water; references to fluorescence spectra, pH-fluorescence curves, quantitative fluorometric methods, ultrachromatographic methods; genera or families in which compound is found, except where compound is generally distributed. Secondary sources are frequently cited for economy of space. General references to investigations on groups of compounds are given opposite the group name.

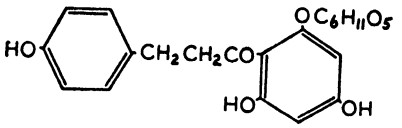
TABLE I

FLUORESCENT COMPOUNDS IN PLANTS*

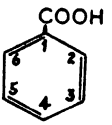
Compound	Data and References on Fluorescence
HYDROCARBONS	
β-Carotene	Sm, in xylol (44)
Lycopene	Sm, in xylol (44)
Phytofluene ($C_{40}H_{64}$)	Greenish; *Lycopersicum*, *Pyracantha* (215), *Acacia* (186)
Phytofluenol	Greenish (214)
PHENOLS	
Laccol [$C_6H_3(OH)_2 \cdot C_{16}H_{29}$]	Blue; S(22a, 45); *Rhus*, *Semeocarpus*
Moreacol [$C_6H_2(OH)_2(CH_3) \cdot C_{16}H_{29}$]	Blue; S(22a); *Rhus*
Phloroglucinol (1,3,5-trihydroxybenzene)	Blue (38); C (9); *Pelargonium* (9)
Phlorizin (=phloridzin) (see Formula I, p. 287)	Light green (106); *Rosaceae* (142)
AMINO ACIDS AND PROTEINS	C(66, 150, 151, 213)
Phenylalanine	Bluish-white phosphorescence (37)
Tryptophan	Greenish in H_3PO_4 (17); bluish-white phosphorescence (37)
Tyrosine	Blue phosphorescence (37)
Proteins	Blue (198); bluish phosphorescence, Sm (37)
BENZOIC ACID DERIVATIVES (see Formula II, p. 287)	
Anthranilic acid (2-amino)	Blue, maximum at pH 3.5; H(103, 193); violet in organic solvents (38); C(193); *Corynebacterium*, *Bacillus*, *Neurospora*, *Zea* (193)
Benzoic acid	Violet (106); in gum benzoin (142)
Damascenine (methyl ester of 2-methylamino-3-methoxy)	Blue (97); *Nigella* (142)
Gentisic acid (2,5-dihydroxy)	Blue; C(56, 193); *Gentiana* (142)
Protocatechuic acid (3,4-dihydroxy)	Blue (106); *Illicium* (97)

Salicylic acid (2-hydroxy)	Violet (36, 106)
Salicylic acid, sodium salt	Violet (36); Q (172)
Salicylic acid, methyl ester	Greenish (36); *Betula, Gaultheria* (142)
QUINONES	
1,4-*Naphthoquinones* (See Formula III, p. 287)	
Phthiocol (2-methyl-3-hydroxy)	Violet in EtOH (43); *Mycobacterium tuberculosis* (3)
Vitamin K_1 (2-methyl-3-phytyl)	Whitish (2)
Anthraquinones (see Formula IV, p. 287)	
Catenarin (1,4,5-trihydroxy-β-hydroxymethyl)	Yellow-green in HAc (156); *Helminthosporium*
Cynodontin (1,4,5,8-tetrahydroxy-2-methyl)	Red in conc. H_2SO_4, greenish yellow in HAc (156); *Helminthosporium*
Helminthosporin (4,5,8-trihydroxy-2-methyl)	Green in HAc; *Helminthosporium* (28)
Munjistin (1,3-dihydroxy-2-carboxy)	Green in HAc and NaOH; *Rubia* (142)
Purpurin (1,2,4-trihydroxy)	Yellow in EtOH (38) in Et_2O (142); *Rubia* (142)
Rhein (=cassic acid) (4,5-dihydroxy-2-carboxy)	Yellow, with maximum between pH 4–6 (102); *Cassia* (163), *Rheum* (195)
Tritisporin (1,3,5,8-tetrahydroxy-6-(or 7-)hydroxymethyl	Yellow-green in HAc (156); *Helminthosporium*
Hypericin (see Formula V, p. 287)	Red; C(149); Sp(44, 46); *Hypericum* (21)
Oxypenicilliopsin	Red; Sp (44, 46); *Penicilliopsis*
CINNAMIC ACID DERIVATIVES (see Formula VI, p. 287)	C, I (192)
o-Coumaric acid (2-hydroxy)	Green in alkali (38); C (192); *Melilotus, Lavandula*
p-Coumaric acid (4-hydroxy)	C, faint blue on filter paper (192); *Prunus, Thea, Trifolium*
Caffeic acid (3,4-dihydroxy)	Whitish blue especially from pH 8–11 (77); C (192); *Coffea, Solanum, Nicotiana*
Ferulic acid (4-hydroxy-3-methoxy)	C, blue on filter paper (192); *Opopanax, Pinus*
Chlorogenic acid (depside of caffeic and quinic acids)	Faint blue (77); C (94, 192); *Solanaceae* (94, 152); *Compositae* (152); *Convolvulaceae* (171); *Coffea, Strychnos*
COUMARINS† (see Formula VII, p. 287)	H, I (80, 81); C (192)
Ayapin (6,7-methylenedioxy)	Blue; H (81); *Eupatorium*
Esculetin (6,7-dihydroxy)	Blue, pH 7–11; H (80); C (192); *Aesculus, Euphorbia*
Esculin (6-glucosido-7-hydroxy)	Blue; Sm (140); H (80); C (192, 193); Q (107); *Aesculus, Gelsemium, Bursaria*
5-Geranoxy-7-methoxy	Blue H; (80); *Citrus*
Herniarin (7-methoxy)	Blue in H_2SO_4; H (80); C (192); *Herniaria, Lavandula, Matricaria*

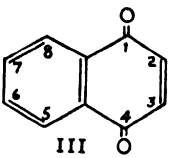
Ostruthin [6-(3,7-dimethyl-2,6-octadienyl)-7-hydroxy]	Blue above pH 7; H (80); C (192); *Imperatoria*
Scopoletin (6-methoxy-7-hydroxy)	Blue, strongest above pH 7; H (79, 80); Q (79); *Solanum* (4), *Nicotiana* (15), *Avena* (79) and numerous other genera.
Umbelliferone (7-hydroxy)	Blue above pH 6; H (80); C (192); *Umbelliferae.*
FUROCOUMARINS†	H, I (81)
Bergaptol	Blue; H (81); Bergamot oil
Oreoselon	Blue in H_2SO_4; H (81); *Peucedanum*
Sphondin	Blue; H (81); *Heracleum*
PYRANOCOUMARINS	
Dihydrobraylin	Blue in EtOH and HAc; a derivative of braylin, isolated from *Flindersia* (6)
Luvangetin	Blue; H (81); *Luvunga*
Seselin	Greenish blue; H (81); *Seseli, Skimmia*
Xanthyletin	Blue; H (81); *Citrus, Luvunga, Xanthoxylum*
ANTHOCYANINS‡ (see Formula VIII)	C (8)
Delphin (3′,4′,5′,7-tetrahydroxy-3,5-di-β-glucosido)	Green in Na_2CO_3 (159); *Delphinium, Salvia, Verbena*
Gesnerin (4′,7-dihydroxy-5-β-glucosido)	Green in H_2SO_4 (164); *Gesnera*
Malvin (3′,5′-dimethoxy-4′,7-dihydroxy-3,5-di-β-glucosido)	Green in Na_2CO_3 (166); *Malva, Primula*
Pelargonin (4′,7-dihydroxy-3,5-di-β-glucosido) (=salvinin, monardin)	Green in NaOH (38); strongly fluorescent (165); *Pelargonium, Centaurea*
FLAVONES§ (see Formula IX)	C (70)
Apigenin (4′,5,7-trihydroxy)	Blue in conc. H_2SO_4 (43); C (70)
Datiscetin (2′,3,5,7-tetrahydroxy)	Green in conc. H_2SO_4; *Datisca* (142)
Fisetin (3,3′,4′,7-tetrahydroxy)	Green in dilute alcoholic NaOH (142), yellow when adsorbed on cellulose (145); *Rhus* (142)
Flavone	Violet blue in conc. H_2SO_4 (43); *Primula*
Kaempferol (3,4′,5,7-tetrahydroxy)	Bluish green in H_2SO_4 (38, 43); C (70)
Lotoflavin (2′,4′,5,7-tetrahydroxy)	Greenish blue in conc. H_2SO_4 (43); *Lotus*
Morin (2′,3,4′,5,7-pentahydroxy)	Green in weak acid plus trace of Al ions (35, 175); used as pH indicator (112); C (70)
Quercetin (3,3′,4′,5,7-pentahydroxy)	Green in conc. H_2SO_4 (38); C (70)
Rhamnetin (3,3′,4′,5-tetrahydroxy-7-methoxy)	Greenish in conc. H_2SO_4 (38); C (70)
PTERIDINES (see Formula X)	Blue; H (103, 157)
2-Amino-4-hydroxy-6-R	Derivatives probably present in plants as precursors of pteroylglutamic acid
Rhizopterin (2-amino-4-hydroxy-6-CH_2-N(CHO)*p*-benzoic acid)	Blue, particularly above pH 7; H(157); *Rhizopus* (161)



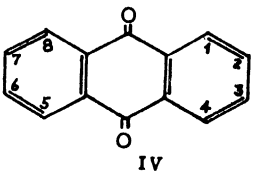
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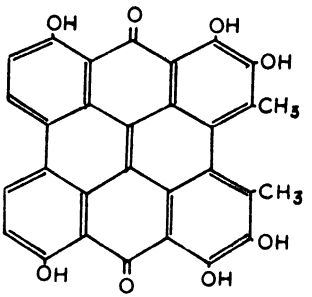
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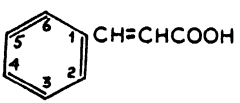
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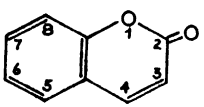
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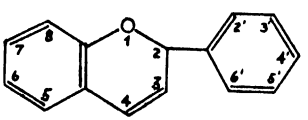
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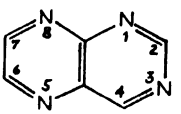
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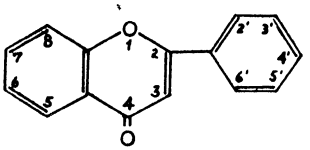
VII



VIII



X



IX

ISOALLOXAZINES
(see Formula XI, p. 290)

Lumichrome (6,7-dimethyl)	Green; Sp, in MeOH (98); H (103)
Lumiflavin (6,7,9-trimethyl)	Green; H (103)
Riboflavin (6,7-dimethyl-9-(D-1′-ribityl))	Green; Sp (98); colored photograph of fluorescence (191); H (55, 103, 118, 180); Q (33, 39, 89, 113, 146, 170, 180, 185, 209)
Flavin-adenine dinucleotide	H, fluorescence maximum pH 2–3 (206)
Flavoproteins	Sometimes fluorescent (206)

PURINES
(see Formula XII, p. 290)

Adenine (6-amino)	Fluorescence in solution at 1 mg./ml. (187)
Guanine (2-amino-6-hydroxy)	Green in NaOH, blue violet in NH_4OH
Xanthine (2,6-dihydroxy)	Green in NaOH, violet in NH_4OH and H_2SO_4
Nucleic acid (yeast)	Bluish green in NaOH, blue in NH_4OH and H_2SO_4
	Blue (187)

TETRAPYRROLES	
Chlorophylls	Red; Sp (43), Sm (44); S, *in vivo* (68)
Chlorophyll-*a*	S, in Et_2O (217); S, *in vivo* (68); Q (76)
Chlorophyll-*b*	S, in Et_2O (217)
Protochlorophyll	Sp, in MeOH and Et_2O (42)
Phycocyanin	Red; Sm (167); Sp (47); S, *in vivo* (68)
Phycoerythrin	Orange; Sm (167); Sp (47); S (68)
Porphyrins	Red; Sp (43), Sm (44); H (60, 61); C (18, 47); I (18, 61)
Coproporphyrins	H (95); Q (162, 179); I (61, pp. 398, 480); yeast (104)
Protoporphyrin	I (62)
Uroporphyrins	S (82); H (201); Q (162); *Vicia* (82); legume root nodules (110, 111)
ALKALOIDS	Structure and isolation (88)
Alstonidine	Fluorescent in acid solution (142)
Alstonine	Blue; *Alstonia* (88)
Atropine	Violet (106); *Atropa*
Berberine	Yellow on filter paper; C (138); QC (73); *Berberidaceae, Hydrastis* (138)
Chelerythrine	Violet (142); oxidized in air to blue-fluorescing compound (88); *Chelidonium, Sanguinaria*
Ergonovine (=ergometrine)	Blue; Sp (196); *Claviceps*
Ergotamine	Bluish; Sp (196); *Claviceps*
Ergotoxine	Bluish white; Sp (196); *Claviceps*
Harmaline	Blue; S, in absolute EtOH (13); *Peganum* (142)
Harmalol	Green; *Peganum* (142)
Harmine	Blue; *Peganum, Banisteriopsis* (142)
Hydrastinine	Blue (10, 52, 142); Q (20); an oxidation product of hydrastine from *Hydrastis*
Lunacridine	Blue; *Lunasia* (88)
Lunacrine	Blue; *Lunasia* (88)
Lunamarine	Blue in EtOH; *Lunasia* (88)
Narceine	Green in neutral, blue in acid solution (106); *Papaver*
Papaverine	Violet in neutral, green in acid solution (106); *Papaver*
Perlolidine	Blue in acid; an oxidation product of perloline (158)
Perloline	Green in EtOH, $CHCl_3$ and alkaline solutions above pH 8 (141); *Lolium* (210)
Quinine‖	Blue in acid; S (13), Sm (86); H (40, 103); Q (22, 41, 109); *Cinchona, Remijia*
Sanguinarine	Bluish violet in organic solvents (88), yellow in acid, blue in alkaline solution; H (77); *Sanguinaria* (142)
Solanine	Blue (106); *Solanum* (142)
Solanidine	Green (106); *Solanum* (142)

Theobromine	Blue-green in EtOH (38); *Theobroma, Cola, Thea* (142)
MISCELLANEOUS	
Aureomycin	Blue in alkali, yellow in acid in presence of Ca and Mg (174); Q (124, 173); *Streptomyces* (142)
Eleutherol (see Formula XIII, p. 290)	Blue, pH 2–10; *Eleutherine* (176)
Floridorubin	Green; *Rytiphlaea* (59)
Rotenone (see Formula XIV, p. 290)	Blue in $CHCl_3$ and other organic solvents; Q (63); *Derris, Lonchocarpus* (97, 142)
α-Terthienyl (see Formula XV, p. 290)	Blue; *Tagetes* (216)
Thiochrome (see Formula XVI, p. 290)	Blue; H (55, 103, 119); Q (32, 33, 39, 87, 109); isolation from yeast (120); an oxidation product of thiamin.
Uracil (see Formula XVII, p. 290)	Green in NaOH, violet in NH_4OH and H_2SO_4 at 1 mg./ml. (187)

* C, ultrachromatography or filter paper chromatography; H, pH-fluorescence curve; I, isolation from a plant source; Q, quantitative fluorometric method of determination; S, fluorescence spectrum; Sm, wave lengths of fluorescence maxima given; Sp, photograph of the fluorescence spectrum; Et_2O, ethyl ether; EtOH, ethanol; HAc, acetic acid; MeOH, methanol; R, an organic substitution.

† The only derivatives listed are those which have marked fluorescence in aqueous solution when irradiated with the 366 mμ mercury arc lines. The following additional compounds have been detected as fluorescent spots on filter paper chromatograms sprayed with 2*N* NaOH (192), whose fluorescence may be due in some cases to impurities or decomposition products: bergapten, coumarin, daphnetin, imperatorin, limettin, osthol, skimmin, xanthotoxin [see also (80, 81)]. Fraxin from *Diervilla* has been reported as fluorescing greenish blue in ethanol and ammonia (137).

‡ The chlorides of the 5-substituted glycosides are fluorescent (165). For natural occurrence see (71, Table 16; 122).

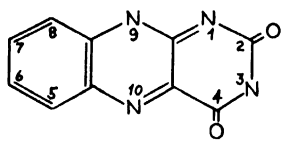
§ Flavones occur either as aglycons or as glycosides in many plants (71, Table 11).

‖ The following alkaloids of the quinine series are also blue fluorescent: cinchonidine, cinchonine, epiquinidine, epiquinine, quinidine (86, 142)

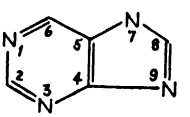
DETECTION AND LOCALIZATION OF FLUORESCENT COMPOUNDS

Fluorescent compounds can often be detected *in situ* by examining living tissues under ultraviolet light. Extensive studies of the spontaneous fluorescence of many plants have been carried out by Klein & Linser (106), who have also described, in *Aesculus*, the morphological distribution of the fluorescent glucoside, esculin, at various stages during germination and development (106, 107, 108). The fluorescence of roots has been reported for a great many species [Gentner (72); Linsbauer (128)]. Goodwin & Kavanagh (78) examined representatives of each of the major groups of vascular plants; the only ones not exhibiting fluorescence were heavily pigmented fern roots, from which fluorescent acetone extracts could be prepared. The fluorescence

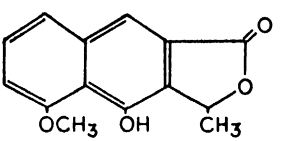
of the following plant parts have been studied: xylem [Vodrážka (199); Wislicenus (212)]; seeds and fruits [Metzner (143); Oka (148)]; seed coats of cycads [Schnarf (177)]; pollen [Berger (12)]; latex of *Euphorbia* and various *Apocynaceae* [Linsbauer (129)]; and fleshy fungi [Mafei (134)]. Plants infected with certain virus diseases exhibit blue fluorescent areas around the lesions. Best (14) observed this phenomenon in tobacco leaves infected with tomato spotted wilt virus. He later identified the fluorescent substance as scopoletin and described its distribution within the plant (15, 16). Andreae



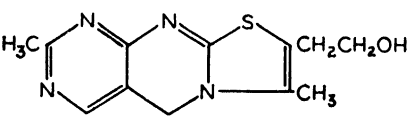
XI



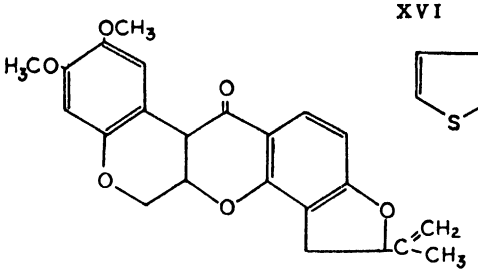
XII



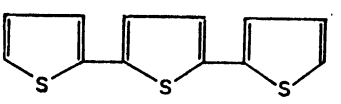
XIII



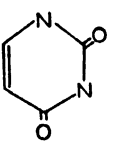
XVI



XIV



XV



XVII

(4) reported the presence of scopoletin around necrotic areas in potato tubers infected with leaf roll virus. Celery plants suffering from boron deficiency accumulate a blue fluorescent material in cells around the lesions and also produce a yellow fluorescent exudate [Spurr (185a)].

The gross fluorescence of organs and tissues is usually produced by an assortment of fluorescent compounds. These may be mixed together in the cytoplasm or vacuoles, but may frequently be morphologically separated within the cells, a fact which can be determined with the fluorescence microscope. The field of fluorescence microscopy has been thoroughly reviewed [Bräutigam & Grabner (19); Ellinger (53); Haitinger (84); Haitinger & Linsbauer (85); Richards (160); Strugger (190)]. In general, Klein & Linser (106) found characteristic fluorescent colors for various cell structures: cuticle, whitish; epidermis, light blue; cortical parenchyma, green or brown; phloem bundles, blue; xylem, violet or blue green; pith and leaf parenchyma, violet; chloroplasts, red; cytoplasm and vacuoles, various colors depending

upon their chemical composition. Metzner (144) correlated the absorption of ultraviolet radiation by the various cell components with their fluorescence.

The violet fluorescence of cellulose walls is enhanced by the deposition of lignin and other substances, and the fluorescence turns greenish upon the addition of alkali [Eichler (51); Künemund (121)]. Turschner (194) has shown that xylem, delignified by certain woodrot fungi, can be readily distinguished by its lessened fluorescence, and Kisser & Wittmann (105) have recently succeeded in extracting the blue fluorescent material from lignified walls with hot water.

Under the fluorescence microscope it is often possible to study cytological and biochemical structure which might otherwise be very difficult or impossible to observe. Thus, Loui (133), in a study of the origin of plastids was able to show that the first trace of chlorophyll is found in dumbbell shaped proplastids, and Goodwin, Koski & Owens (82) discovered uroporphyrin-containing proteinaceous bodies in epidermal cells adjacent to the stomatal apparatus of several species of *Vicia*.

The use of fluorochromes (fluorescent dyes which often have specific chemical affinity for cellular constituents) in fluorescence microscopy has been covered in the general references cited above. Höfler (90) has published some recent observations on this subject. It is interesting to note in passing that Brues (23) has found some of the most useful of these dyes to be plant alkaloids, such as berberine and sanguinarine.

Isolation and Identification

Fluorescence is often the key to the isolation and identification of biochemical constituents. Thus, the most suitable tissue can be specifically selected for extraction, and the movement of the material followed step by step under ultraviolet light during the purification procedure.

Chromatography.—The technique of separating fluorescent substances on Tswett adsorption columns under ultraviolet light has been called ultrachromatography (99, 211) and has had wide application in the isolation of such compounds as scopoletin [Andreae (4)]; flavins [Karrer & Schöpp (99)]; porphyrins [Klüver (110)]; phytofluene [Zechmeister & Sandoval (215)]; terthienyl [Zechmeister & Sease (216)]; and unknown constituents [Goodwin & Kavanagh (78); Lewis & Doty (126)]. For a recent treatment of general methods the reader is referred to the book on chromatography by Cassidy (26).

The use of filter paper chromatography in isolating and identifying traces of fluorescent materials is of particular interest. Just as in adsorption columns, the position of the fluorescent substances may be determined either on wet or dry chromatograms under ultraviolet light. This has great advantages, where subsequent elution of the unaltered material is desired. The rate of movement of a compound on the chromatogram relative to that of the solvent front (the R_F value) is characteristic and reproducible for a

given solvent mixture, when the technique is good, and gives information concerning the nature of the compound. Thus, R_F values for porphyrins depend upon the number of carboxyl groups in the molecule [Bogorad & Granick (18); Nicholas & Rimington (147)]; for various glycosides and aglycones, upon the number of hydroxyls [Bate-Smith & Westall (9)]; etc. This makes it possible to separate such closely related substances as anthocyanins [Forsyth (65)], coumarin derivatives [Swain (192)], and tryptophan metabolites [Mason & Berg (136)]. A comparison of R_F values of an unknown with those of known compounds may be helpful in the identification of the unknown, as in the isolation of anthranilic acid from a corn mutant by Teas & Anderson (193). This type of evidence can be highly significant when mixtures of the known and unknown move as a single spot on chromatograms developed with various solvent mixtures. Other diagnostic aids in the identification of compounds are the color and brilliance of the fluorescence both before and after spraying the chromatogram with suitable sprays which may enhance or quench the fluorescence or may produce specific fluorogenic reactions. Comparative data of this sort on an extensive series of flavones have been reported by Gage *et al.* (70).

Various groups of compounds not normally fluorescent in solution may be detected by their fluorescence on filter paper chromatograms. Amino acids, for instance, may be detected in microgram quantities on acid-treated cellulose [Woiwod (213)], particularly if the developed chromatograms have been subjected to heat [Fowden (66); Patton, Foreman & Wilson (150); Phillips (151)]. Advantage may be taken of specific fluorogenic reactions [Feigl (57, 58)] which convert nonfluorescent compounds into fluorescent ones. Thus, many sugars condense with *m*-phenylenediamine on the developed chromatogram to give characteristic fluorescent colors [Chargaff, Levine & Green (27)]. The reverse of the above technique has been used by Magasanik & Umbarger (135), who treated their chromatograms with a semicarbazide reagent. The fluorescence of this reagent is quenched in the presence of keto acids, which appear as dark shadows on the chromatograms.

Fluorescence spectra.—The color of the fluorescence exhibited by a compound is characteristic under a given set of conditions [Dhéré (44, 45)]. The fluorescence spectra of a great many compounds, such as quinine, thiochrome, and esculin, have broad diffuse maxima; but those of certain groups, notably the tetrapyrrole pigments, exhibit sharp structure. Analysis of the spectral distribution of the fluorescent radiation from pigments of the latter type may, in conjunction with other evidence, permit a definite identification of the compound. Visual observations of the fluorescence may be made directly through a spectroscope, and those constructed for use with a microscope, such as the Zeiss microspectroscope, may be particularly helpful in identifying fluorescent pigments *in vivo*. It is sometimes possible to determine fluorescence maxima for a single vacuole or plastid. Using microspectrographic methods, Sjöstrand (183) has been able to locate riboflavin and thiamin by their fluorescence in animal tissues.

Fluorescence spectra may be photographed, as in the extensive studies by Dhéré and his collaborators (43), but the determination of absolute intensity values from photographic plates poses some difficult problems. Spectrophotometric methods are preferable. Solutions of photolabile compounds, such as chlorophyll, must be continuously renewed during the scanning of the spectrum. Zscheile & Harris (217) designed an excellent setup for the determination of chlorophyll fluorescence spectra and give data demonstrating the importance of using very dilute solutions to minimize errors due to self-absorption of the fluorescent light. French has designed an automatic recording spectrophotometer, which has been used to determine the fluorescence spectra of various red fluorescent compounds and tissues, including phycoerythrin, suspensions of red algae, green leaves [French & Young (68)], and porphyrins [Goodwin, Koski & Owens (82)].

Action spectra.—When compounds are present in tissues in minute amounts, their extraction and purification to the point where adequate absorption spectra can be determined may be impractical, if not impossible. In such cases, action spectra, if they can be obtained, may give an indication of the position of absorption maxima. A fluorescence action spectrum (fluorescence intensity divided by the quantal content of the exciting radiation plotted against the wave length of the incident beam) was determined by Goodwin, Koski & Owens for the Soret band of a porphyrin present in *Vicia* leaves (82).

pH-fluorescence curves.—The fluorescence of compounds in solution is affected in a characteristic way by the pH of the solvent. Factors which influence the shape of the pH-fluorescence curve are: quenching by dissolved salts and oxygen, efficiency of the fluorescent process, changes in the absorption spectrum and other physical and chemical properties of the compound with changes in pH, wave length of the exciting radiation and of the fluorescent light, and characteristics of the measuring instrument. A pH-fluorescence curve may be determined by measuring the fluorescence of aliquots of a solution to which a series of buffers has been added and computing the relative fluorescence in terms of the maximum value obtained for the series. For comparative purposes, it is important that curves be made under strictly comparable conditions using the same set of buffers saturated with oxygen, the same quality of exciting radiation, and the same kind of photocell and photocell filters to measure the fluorescent light. Using these precautions, the shapes of pH-fluorescence curves can be highly reproducible and characteristic. Quenching, if not excessive, may be disregarded, as it should always be the same for a given compound at a particular pH.

Fink and his collaborators have distinguished numerous porphyrin isomers by means of pH-fluorescence curves. These curves show minimum fluorescence at the isoelectric point (60). This work has been summarized by Fisher & Orth (61). Goodwin, Koski & Owens determined the isoelectric point visually on a plant porphyrin within epidermal cells mounted in buffers

under an ultraviolet microscope (82). This was used as evidence for the identity of the compound.

Goodwin & Kavanagh have determined the pH-fluorescence curves for a large number of coumarin derivatives (80, 81). Matching the curve for one of these derivatives with that of a fluorescent fraction isolated from oat roots led to the positive identification of the unknown substance as scopoletin (6-methoxy-7-hydroxy-coumarin) (79). Leininger & Katz (123) describe a fluorometric method for the determination of malic acid and 2-naphthol based upon the reaction of these two compounds in concentrated sulfuric acid. The fluorescent reaction product was thought to be 5,6-benzocoumarin, and the shape of the pH-fluorescence curve confirms this structure [Goodwin & Kavanagh (80)]. Minute traces of fluorescent contaminants can be detected by fluorometry and their identity can sometimes be predicted from the shape of the pH-fluorescence curves [Goodwin & Kavanagh (81)].

Linser (130) has used quinine and umbelliferone as pH indicators, by observing their fluorescence in solution, and Volmar (200) has employed these compounds to determine end points in titrations. Pringsheim & Vogel (153) list a number of fluorescent indicators.

Quantitative Analysis

Fluorometric methods may be over one hundred times as sensitive as spectrophotometric [Loofbourow (132)], and hence may be of great interest to the biochemist. A helpful guide to fluorometric methods has been put out by the Klett Manufacturing Company (109). Although written for use with the Klett fluorimeter, designed and described by Kavanagh (101), the procedures described therein are of general application. Fluorescent compounds such as quinine, riboflavin, and others listed in Table I of this review may be analyzed directly. In addition, nonfluorescent substances may often be specifically converted into fluorescent compounds and then measured by fluorometry. A number of such compounds, together with references to published methods, are given in Table II.

Measurements are best carried out on compounds in solution. The solvent should be carefully defined and the temperature standardized. The exciting radiation must be absorbed by the substance to be measured; hence the wave length must fall within one of the absorption peaks of the compound, although not necessarily coinciding with the absorption maximum.

The relationship between concentration and fluorescence, as measured by the fluorometer has been discussed by Kavanagh (101) and Ellinger & Holden (54). Under suitable conditions this relationship is essentially linear and it is important that the concentration of the fluorescent material be low enough to fall within the proportionality range. At higher concentrations reabsorption of the fluorescent light may become pronounced [Watson & Livingston (205)] and may cause an apparent change in the color of the fluorescence [Zscheile & Harris (217); French & Young (68)].

TABLE II

SUBSTANCES OF BOTANICAL ORIGIN ASSAYED BY FLUORESCENT METHODS

Compound	Reaction	References
Aureomycin	Fluorescence measured in phosphate buffer	(124)
	Fluorescence measured in 5 per cent sodium carbonate	(173)
Coumarin	Irradiate with sunlight in 0.025 *N* sodium hydroxide to form a green fluorogen	(184)
Folic acid (see pteroylglutamic acid)		
Hydrastine	Oxidize to hydrastinine	(20)
Malic acid	React with orcinol to form 4-methyl umbelliferone	(92)
	React with 2-naphthol to form 5,6-benzocoumarin	(123)
	React with resorcinol to form umbelliferone	(7)
Morin	React with aluminum	(175)
	React with other ions	(11)
2-methyl-1,4-naphthoquinone	Condense with *o*-phenylenediamine	(115)
N^1-methyl nicotinamide	Condense with acetone	(91)
	React with methyl ethyl ketone	(24)
Nicotinamide	React with cyanogen bromide	(30, 181)
Nicotinic acid	React with *p*-aminoacetophenone	(69)
Penicillin	Condense with 2-methoxy-6-chloro-9-(2-aminoethyl) aminoacridine	(182)
Pteroylglutamic acid	Oxidize with permanganate to 2-amino-4-hydroxy-pteridine-6-carboxylic acid	(1, 5, 197)
Pyridine nucleotides (coenzymes I and II)	Condense with acetone	(125)
	Condense with methyl ethyl ketone	(24)
Rutin and its aglycon, quercitin	React with boric acid reagent	(75)
Streptomycin	React with hydrazino-acridine reagent	(93)
Succinic acid	React with resorcinol to form a fluorescein derivative	(7)
Thiamine	Oxidize to thiochrome	(32, 33, 39, 87, 109)
Vitamin E (tocopherols)	Oxidize to tocopherol red and condense with *o*-phenylenediamine	(114, 116)
Vitamin K (phylloquinones) (see 2-methyl-1,4-naphthoquinone)		

The most serious sources of error in fluorometry are likely to be due to the presence of quenching substances and of fluorescent impurities. Among the agents responsible for quenching are buffer salts [Kuhn *et al.* (118, 119)], other dissolved ions [Ellinger & Holden (55); Rollefson & Stoughton (168)], purines [Weil-Malherbe (207)], oxygen [Weil-Malherbe & Weiss (208)], especially in organic solvents [Chéchan (31)], and other oxidizing agents [Livingston & Ke (131)]. It is important, therefore, to standardize the amount of salts in test solutions and to select buffers which have a minimal quenching effect. If oxygen quenching is not serious, the solution may be saturated with this gas by shaking just before measuring the fluorescence.

One of the advantages of fluorometry is that excellent data can often be obtained from very impure preparations. However, the presence of materials which absorb either the exciting or the fluorescent radiation must be avoided. Fluorescent contaminants may be very serious, if they fluoresce at the same wave length and under the same conditions as the substance to be tested. Under these conditions they must be separated or destroyed before running the test. Filter paper chromatography is a useful tool in determining the fluorescent components of a solution to be tested. By the proper use of photocell filters the fluorescence of a particular substance may sometimes be measured in the presence of others. Thus, the red chlorophyll fluorescence may be measured independently of blue, green, or yellow fluorescent substances as in the case of crude fruit extracts [Kramer & Smith (117)].

The shape of the pH-fluorescence curve of the compound being measured may be very important from the point of view of quantitative fluorometry, since it will permit the investigator to select the optimal conditions for the fluorescence measurements. Thus, for maximum sensitivity quinine must be assayed in strong acid solutions, riboflavin between pH 4 and 7, umbelliferone at pH values above 8, esculetin at pH 8, anthranilic acid at pH 3.5, etc. (80, 103). Hummel (92) reports a method for the determination of malic acid based upon its reaction with orcinol to form 7-hydroxy-5-methyl coumarin. The fluorescence is measured in concentrated sulfuric acid. Judging from the pH-fluorescence curve of this coumarin derivative, the sensitivity of the method might be increased nearly fourfold by adjusting the acidity to pH 10 or above. Furthermore, errors due to fluorescent contaminants may sometimes be minimized by selecting a pH which will reduce or eliminate the fluorescence of the contaminant. Barr (7) has measured two compounds simultaneously in mixtures, one fluorescing only in the alkaline range, the other only in acid.

Physiological Investigations

Probably the most extensive exploitation of fluorescence in physiological investigations has been in connection with the process of photosynthesis. Radiant energy absorbed by chlorophyll in the living cell may be transferred to the photosynthetic (or other) acceptor as chemical energy, converted into heat, or reradiated as fluorescence. A more or less reciprocal relationship

exists between rate of photosynthetic activity and chlorophyll fluorescence. Thus, changes in the intensity of chlorophyll fluorescence in photosynthesizing plants provides an instantaneous measure of the probability of energy transfer from the chlorophyll-protein complex to energy acceptors. The voluminous literature on this subject has been recently reviewed by J. Franck (67), Kautsky & U. Franck (100), Rabinowitch (154), and Wassink (203), and will not be covered here.

A most ingenious application of fluorescence has been in the establishment of the role of accessory pigments in photosynthesis. Each pigment has distinct absorption and fluorescence spectra which can be determined separately. Irradiation of a pigment at its absorption maximum results in the excitation of its characteristic fluorescence. A plant may now be irradiated with wave lengths close to the absorption maximum of the accessory pigment present, and the spectrum of the resulting fluorescence analyzed. If a chlorophyll fluorescence is observed, energy transfer from the pigment to the chlorophyll is demonstrated. The following pigments have been shown to participate in this kind of energy transfer: fucoxanthin in *Nitzschia*, by Dutton, Manning & Duggar (49) and Wassink & Kersten (204); carotenoids in *Chromatium* and phycocyanin in *Porphyra*, by Duysens (50); phycoerythrin and phycocyanin in *Porphyridium* by French & Young (68).

Under the fluorescence microscope Gicklhorn (74) has observed a marked increase in chlorophyll fluorescence of living chloroplasts in response to a sudden rise of temperature. He attributes this to a reversible dissociation of the pigment from the plastid lipids, which becomes irreversible upon the death of the cell.

In respiratory studies Warburg & Christian have shown that coenzymes I and II (di- and triphosphopyridine nucleotides) exhibit a whitish fluorescence in ultraviolet light, but only when in the reduced (dihydro) form. They have used this fluorescence to measure the rate of oxidation and reduction of these compounds under various conditions (202).

Vital staining with fluorescent compounds has proved a useful technique in the study of the absorption, translocation, and deposition of these compounds by the plant. The distribution of the fluorescence may be followed in the living cell with the aid of the fluorescence microscope [Döring (48); Rouschal & Strugger (169); Schumacher (178); Strugger (188, 189)].

Miscellaneous Uses of Fluorescence

Fluorescence has been used for years by seed analysts to distinguish the seeds of closely related species of rye grass. The roots of germinating *Lolium multiflorum* seeds have the property of producing a brilliant blue fluorescence in the filter paper substratum. It has been suggested that the blue fluorescent substance may be a partial degradation product of cellulose. The roots of *L. perenne* and *L. rigidum* do not exhibit this property. The literature on this subject is well reviewed by Justice (96). It is interesting to note that in interspecific hybrids between *L. multiflorum* and *L. perenne* the ability to produce

the fluorescence is inherited as a simple Mendelian dominant [Corkhill (34); Linehan & Mercer (127)].

The discovery that fluorescent substances accumulate in the tissues of virus-infected plants [Andreae (4); Best (15, 16)] has led to the practice of examining seed potatoes under ultraviolet light. Discarding fluorescent tubers may prove useful in reducing the incidence of infection by leaf roll and other types of virus [McLean & Kreutzer (139)].

Vegetable drugs are frequently fluorescent or contain fluorescent contaminants. Use has been made of this property in identifying drugs, in distinguishing between synthetic and natural products, and in detecting adulterations [Casparis & Manella (25), Danckwortt (35), Radley & Grant (155)]. Chase & Pratt (29) have recently published a key by which 151 powdered drugs may be identified by their fluorescence after treatment with three different reagents.

McFadden (138) investigated the distribution of berberin and various unidentified fluorescent substances in the roots of members of the *Berberidaceae* and *Ranunculaceae* and used the presence of these compounds as biochemical characters in working out taxonomic affinities. Teas & Anderson (193) report a mutant gene in corn responsible for the production of anthranilic acid in the seedling and in the anthers.

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THE BIOGENESIS OF TERPENES[1]

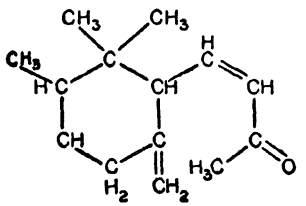
By A. J. Haagen-Smit

Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, California

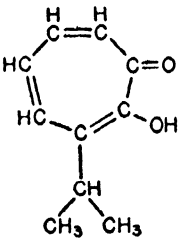
The name "terpenes" has been applied to a group of compounds distinguished by a singular chemical composition. Their empirical formula contains a multiple of five carbon atoms, and their chemical structure can be described as consisting of isopentane units. In the volatile plant materials, the essential oils, occur substances containing five, 10, 15 and small amounts of compounds with 20 carbon atoms. These have been called hemiterpenes, mono-, sesqui- and diterpenes, respectively. Still larger terpene homologues are not volatile enough to be found in the steam distillate but are obtained from the plants by solvent extraction. These include the resin acids, saponins, carotenoids, and rubber, which belong, respectively, to the di-, tri-, tetra- and polyterpenes. In addition, there are found structures which do not contain multiples of C_5 units, but are related to the terpenoid group since part of the molecule contains isopentane units (1). These compounds are sometimes indicated as isoprenoids. To this class belong the irones (I), ketones with 14 carbon atoms. Recent studies by Ruzicka *et al.* (2) and by Naves *et al.* (3) have shown that this fragrant principle of orris root cannot be constructed entirely of isopentane units.

One of the interesting discoveries of the last few years is the isolation of seven-membered ring compounds in the volatile oil of the heart wood from trees belonging to the genera *Thuja, Thujopsis, Chamaecyparis* of the Coniferae (4, 5, 6). These thujaplicins are represented by formulae II, III and IV. The 7-membered ring system, which received the name, tropolone, has since been found to occur in several natural products: colchicin; purpurogallin; and penicillin (7). The carbon skeleton of the thujaplicins cannot be constructed from two isopentane units. However, a benzil rearrangement transforms them into derivatives of methyl isopropylbenzoic acid. A related product, dehydroperillic acid (V), has been found to occur in the thuja oils (4, 8). Also distinctly related to the terpene series are the ionones (VI), leptospermone (VII), Baeckeol (VIII), angustione (IX) and dehydroangustione, all of which have been found in essential oils. In vitamin K and the tocopherols, cannabidiol and some coumarins, the terpene part of the structures can be easily detected, and the laboratory synthesis of these compounds makes use of this fact. The isoprenoids include further the large class of compounds containing the pentanophenanthrene ring structure, the steroids. It is possible to divide the carbon skeleton of cholesterol into five isopentane chains, leaving seven carbon atoms unaccounted for.

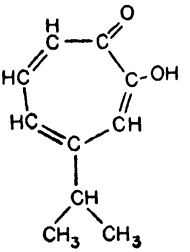
[1] The survey of the literature pertaining to this review was concluded in September, 1952.



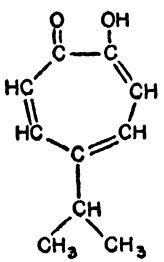
γ-Irone
(I)



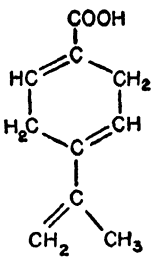
α-Thujaplicin
(II)



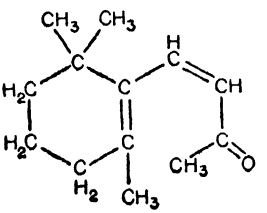
β-Thujaplicin
(III)



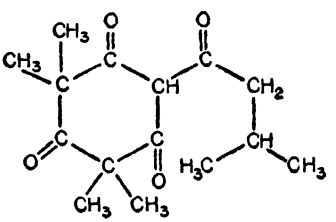
γ-Thujaplicin
(IV)



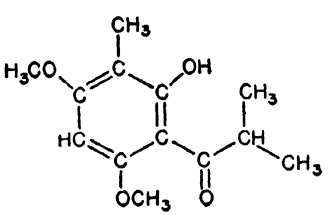
Dehydroperillic acid
(V)



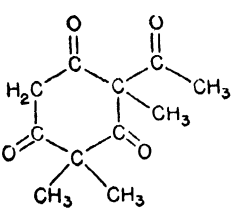
β-Ionone
(VI)



Leptospermone
(VII)



Baeckeol
(VIII)



Angustione
(IX)

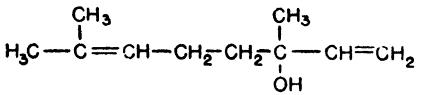
There is probably no living organism which does not contain at least one isoprenoid type compound, such as carotenoids, phytol or steroids, belonging, respectively, to the tetra-, di- and mixed terpene groups. The occurrence of mono-, sesqui- and polyterpenes seems to be confined to the plant world, and only to a limited number of species. Their distribution with respect to taxonomic classification can be found in Klein's *Handbuch der Pflanzenanalyse* (9) and in Guenther's *Essentials Oils* (10). The mono- and sesquiterpenes are most abundant in the family of *Pinaceae*, *Labiatae*, *Umbelliferae*, *Rutaceae*, *Lauraceae*, *Myrtaceae*, and *Compositae* but occur in at least 50 other families. We have to remember, however, that of the quarter of a million seed bearing plants, less than 10,000, representing only one-sixth of all the plant families, have found their way to the laboratory. As an illustration of our limited knowledge of the occurrence of these compounds, one can mention the family of the *Labiatae*. This family, well-known for its large number of essential oil containing members such as peppermint, lavender, and rosemary, consists of 3,000 species, and only 10 per cent of these have been investigated.

The tremendous task of obtaining a more complete picture of the occurrence and type of terpene present in one plant group was undertaken some 50 years ago by the Australian investigators Baker and Smith and has been carried on by Penfold & Morrison (11). Inspired by the abundance of Eucalyptus species in their home land, these workers have analyzed 231 species of the genus *Eucalyptus*, or one-third of the total number of species in this genus. A similar investigation is being carried out in the pine genus. Through the efforts of many workers, Schorger, Simonsen, Mirov (12), and others, nearly 50 per cent of this genus containing *circa* 80 species has been thoroughly investigated.

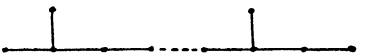
The individual members of the terpene groups have been listed by Guenther (13) and by Simonsen (14). At present, 116 monoterpenes and 124 sesquiterpenes have been isolated from plant material. Whereas nearly all of the structures of the monoterpenes have been established, only one-third of the sesquiterpenes have been fully investigated.

The large group of monoterpenes consists of hydrocarbons and their derivatives containing 10 carbon atoms. They differ in their content of hydrogen and oxygen. Hydrocarbons ranging from $C_{10}H_{12}$ to $C_{10}H_{20}$ and oxygen derivatives from $C_{10}H_{18}O$ to $C_{10}H_{12}O_4$ have been found. We find among these compounds alcohols, aldehydes, ketones, acids, esters, lactones, oxides and even peroxides.

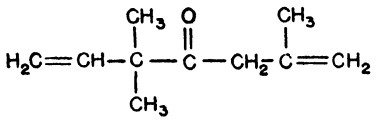
The majority of the structural formulas of the monoterpenes can be constructed from two isopentane units in head-to-tail union (X). This carbon chain is found in geraniol, linaloöl (XI) and other noncyclic terpenes. By coiling up this chain and connecting the proper carbon atoms, the majority of the cyclic terpenes can be described. There are, however, a few exceptions to this rule. Artemisia ketone (XII, XIII), lavandulol (XIV,



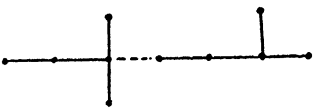
(XI) Linaloöl



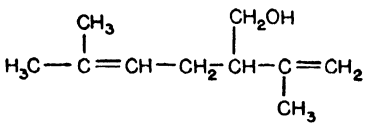
(X)



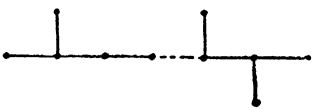
(XIII) Artemisia ketone



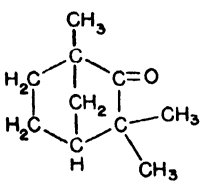
(XII)



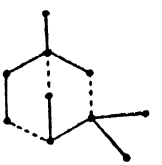
(XV) Lavandulol



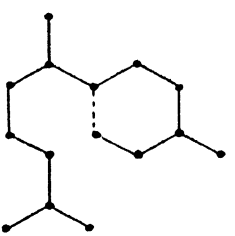
(XIV)



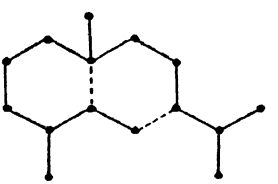
(XVII) Fenchone



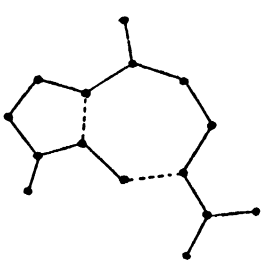
(XVI)



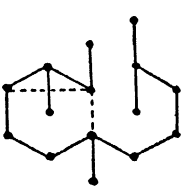
(XVIII) Bisabolene type



(XIX) Eudesmol type



(XX) Guajol type



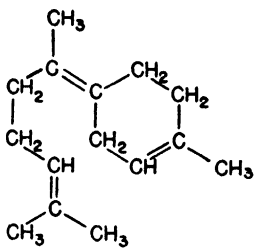
(XXI) Santalene type

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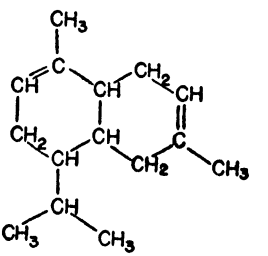
XV), and fenchone (XVI, XVII) still can be divided into isopentane units, but do not follow the head-to-tail rule.

The carbon skeletons found in the sesquiterpene series follow the same building principle as those of the monoterpenes. Farnesol, built of three isopentane units in head-to-tail union, is the terpene homologue of geraniol. Most of the cyclic terpenes can be constructed from this chain, and include mono-, di- and tricyclic systems (XVIII–XXI).

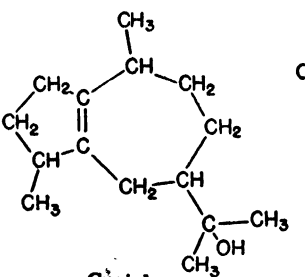
The most frequently occurring structural types are those corresponding to bisabolene (XXII), cadinene (XXIII), guajol (XXIV), and caryophyllene (XXV). Guajol is of interest since it consists of a fused five- and seven-



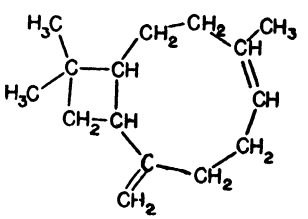
γ-Bisabolene
(XXII)



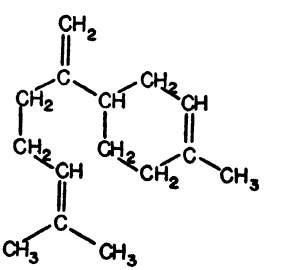
Cadinene
(XXIII)



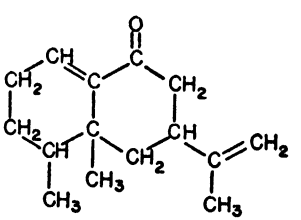
Guajol
(XXIV)



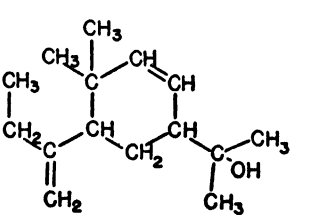
Caryophyllene
(XXV)



Lanceol
(XXVI)



Eremophilone
(XXVII)



Elemol
(XXVIII)

membered ring. Upon dehydrogenation of this type of sesquiterpene with sulfur or selenium, blue hydrocarbons, azulenes, are produced (15). One of these, lactarazulene, is formed upon cutting of the fungus *Lactarius deliciosus* (16).

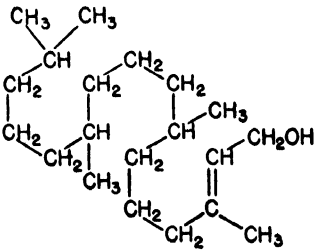
Because of the more difficult problem of accounting for 15 carbon atoms, as compared with the 10 carbon atoms of the monoterpenes, complete structure determination for more than half of the well characterized sesquiterpenes is still going on, and recently new types of structure in this group have been added. For example, caryophyllene is now represented by a fused four- and nine-membered ring system (XXV) (17, 18). On the other hand, revisions of old formula are made, and lanceol (XXVI), formerly considered as a separate type of terpene alcohol, has now been recognized as having a bisabolene type of ring system (19). Deviating from the head-to-tail union of isopentane units are eremophilone (XXVII) (20) and elemol (XXVIII) (21).

Diterpenes which contain four isopentane units arranged in head-to-tail union are phytol (XXIX), vitamin A (XXX), and the bicyclic sclareol (XXXI). Irregular arrangement is found in abietic acid (XXXII), pimaric acid (XXXIII), and α-camphorene (XXXVI). The latter can be described as consisting of two regular monoterpene chains. This doubling of regular chains is also found in the triterpene, squalene, and in all of the known tetraterpenes. In the higher terpenes of the C_{20}, C_{30} and C_{40} types, a ring structure has become prevalent which is found only in the ionones (VI), and in the sesquiterpenes with eudesmol-type structure (XIX).

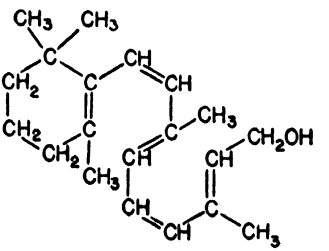
Studies on the biogenesis of the terpenes will have to explain the occurrence of the repeating C_5 units, their regular and sometimes irregular arrangement, the formation of terpene homologues, their cyclic and noncyclic structures, and the presence of a variety of oxidation and reduction products. The occurrence of isoprenoids partly built of isopentane units presents additional problems.

The irregular arrangement of the isopentane units in several terpenes gives support to the hypothesis that a C_5 unit acts as a precursor of the terpenes. We may expect that this reaction unit is subject to oxidation and reduction reactions, water addition, and dehydration, until a more stable product is evolved. The presence of several of the isopentane derivatives, isoamyl alcohol, isovaleraldehyde, and isovalerates (XXXV–XXXIX) in numerous volatile oils seems to support such a speculation.

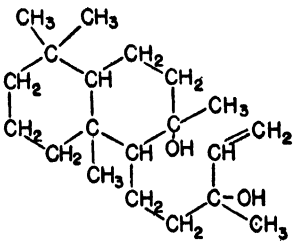
The chances of finding the C_5 units are greater when they are combined with other molecules which are soluble only with difficulty in aqueous medium. Because of their low solubility, we find isovalerates often combined with terpene alcohols as water-insoluble esters. A natural *Abfang* technique was perhaps at work when C_5 units combined with phenol ethers, coumarin and furanocoumarin. A typical coumarin structure serves in this way as a marker for the reactive compounds or radicals which are present in the reaction medium. The furane coumarins are probably derived from the coumarins by the addition of a reactive two-carbon fragment, and it is



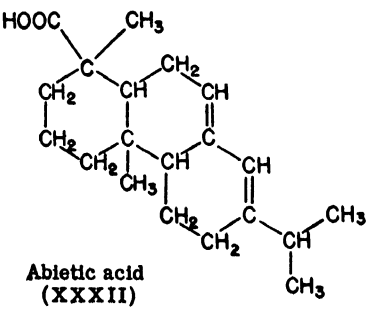
Phytol
(XXIX)



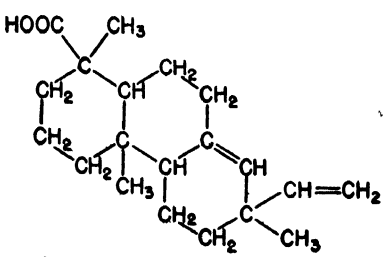
Vitamin A
(XXX)



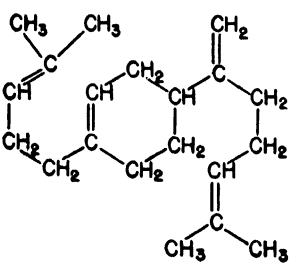
Sclareol
(XXXI)



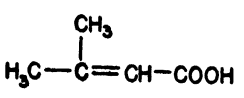
Abietic acid
(XXXII)



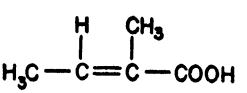
d-Pimaric acid
(XXXIII)



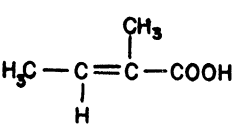
α-Camphorene
(XXXIV)



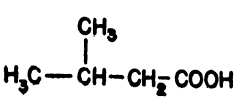
Isopropylidene acetic acid
(XXXV)



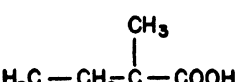
Tiglic acid
(XXXVI)



Angelic acid
(XXXVII)



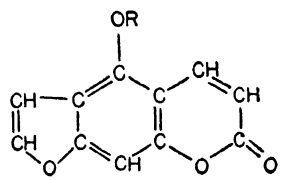
Isovaleric acid
(XXXVIII)



α-Methyl-n-butyric acid
(XXXIX)

possible that these fragments also played a role in the formation of the terpene radicals which we find incorporated in the furane coumarine molecules.

In imperatorin, the unsaturated isopentanol, *prenol*, is bound in phenol-ether linkage to bergaptol in the same way as geraniol is coupled to bergaptol (XL) and farnesol with umbelliferone (XLI). A similar case of etherification with prenol is found in feniculin, which is a *p*-anol prenyl ether (XLII). Similar relationship exists between the coumarins umbelliferone and its hemiterpene and terpene homologues, osthole, isoauraptene, auraptene, and osthrutin (XLIII, XLIV).



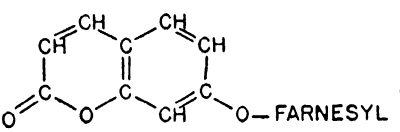
(XL)

R=H Bergaptol

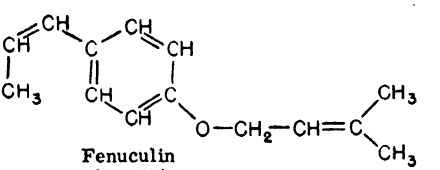
R=CH_3 Bergaptene

R=CH_2-CH=C(CH_3)(CH_3) Iso-imperatorin

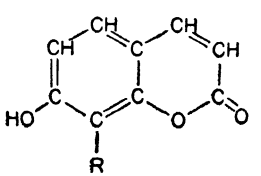
R= Geranyl ($C_{10}H_{15}$) Bergamottin



Umbelliprenin
(XLI)



Fenuculin
(XLII)



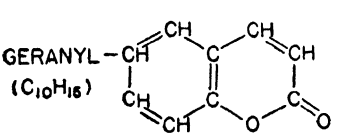
(XLIII)

R= -H Umbelliferone

R= -CH_2-CH=C(CH_3)(CH_3) Osthole

R= -CH_2-C(=O)-CH(CH_3)$_2$ Iso-auraptene

R= -CH_2-CH(O)-C(CH_3)$_2$ Auraptene



Osthrutin
(XLIV)

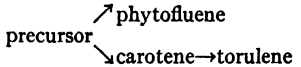
The origin of the unit has been attributed to a condensation of acetone and acetaldehyde or acetone and pyruvic acid, to an oxidative degradation of sugars and to a conversion of the amino acids, valine and leucine. Chemical literature on this subject offers a choice of the postulated terpene precursors

in isoprene, isovaleraldehyde, methylbutenal, isoamyl alcohol, leucine, and valine.

It is customary to follow speculations on the formation of the typical branched carbon skeleton by a number of condensations, hydrations, dehydrations, etc. to show their usefulness in explaining the variety of terpenes. It is obvious that once the branched chain has been produced all the terpenes can be formed by suitable reactions, at least on paper. Only in a few instances have attempts been made to verify in the laboratory the possibility of producing terpenes from their postulated precursors. Wagner-Jauregg (22) condensed isoprene under the influence of sulfuric acid and acetic acid to form some geraniol. Midgley and co-workers (23, 24) united two molecules of isoprene in hydrogenation, producing a dimethyl-octane of terpene structure. Fischer & Löwenberg (25) condensed 3-methylbutenal to dehydrocitral.

These interesting experiments do not throw light on the actual synthesis of the terpenes in the plants. The only information which, at the present time, might give some lead to the biosynthesis of the monoterpenes we find in experiments conducted in the groups of tetra- and polyterpenes and mixed isoprenoids: the carotenoids, rubber, and the steroids.

In all known carotenoids the arrangement of the C_5 units is a head-to-tail union which becomes reversed in the center. The carotenoids are therefore most probably formed by condensation of two diterpene structures with open chain. It is possible that phytofluene, a $C_{40}H_{46}$ tetraterpene, functions as an intermediate in the synthesis of the carotenoids. This compound has seven double bonds, and a dehydration is postulated by Bonner *et al.* (26) and Zechmeister & Sandoval (27) to account for the formation of the conjugated system in the carotenoids. These conclusions are based on studies on carotenoids of several mutant strains of the yeast *Rhodotorula rubra.* In addition to the original strain producing the xanthophyll, torulene, strains were isolated which produced (*a*) large amounts of carotenes, (*b*) phytofluene and small amounts of carotene, and (*c*) no carotenoids at all. These results do not exclude the more likely independent synthesis of phytofluene and the carotenoids from a common terpene precursor, as follows:

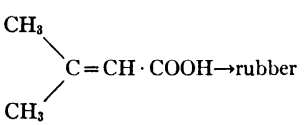


Goodwin, Garton, & Lijinski (28, 29) studying carotene production in *Phycomyces blakesleeanus* have shown that, using a medium containing 3 per cent (weight to volume) glucose, carotene synthesis was independent of the amino acid used as the nitrogen source, with the exception that glycine enhanced carotene production. Under these conditions the branched amino acids, valine and leucine, from which a hypothetical five carbon building unit could be formed, were ineffective. However, under conditions where the sugar concentration was reduced to 1 per cent, valine and leucine would stimulate carotenogenesis, sometimes as much as fourfold. The amino acids would provide the repeating isopentane unit, possibly through the inter-

mediate formation of isovaleraldehyde. Whether dehydrogenation of this unit occurs before or after polymerization has not been established, and it is not known if the conjugated double bond structure is of primary origin or whether a stepwise dehydrogenation as proposed by Porter & Lincoln (30) produces this structure. Mackinney (31) makes a suggestion that in the condensation process initiating and terminating groups are not required to be identical. On the basis of experiments on carotenoid synthesis on a strain of *P. blakesleeanus* he observed that the addition of β-ionone to a medium containing leucine increased the carotene formation considerably.

Bonner, Galston & Arreguin (32, 33), studying the formation of rubber and terpenes in the guayule plant, came to the conclusion that β-methylcrotonic acid has to be regarded as the repeating unit. Evidence was obtained that the common precursor is formed in the leaves and transformed into rubber in the stem. These conclusions are supported by experiments in which excised guayule stems received extracts of the leaves, labelled acetate, acetone or β-methylcrotonic acid. The path of rubber formation indicated by these studies is as follows:

$$2CH_3\cdot COOH \rightarrow \underset{\text{acetate}}{CH_3\cdot CO\cdot CH_2\cdot COOH} \rightarrow \underset{\text{acetoacetate}}{CH_3\cdot CO\cdot CH_3} \xrightarrow[\text{acetone}]{+CH_3\cdot COOH} \underset{\text{acetate}}{\qquad}$$



β—methylcrotonic acid

Since our information on the biogenesis of the terpenoids is still inconclusive it is interesting to look at the results obtained in the related class of isoprenoids, the steroids.

Both in yeast and in liver slices, as well as in feeding experiments, labelled acetate is incorporated into the steroids. Bloch & Rittenberg (34, 35, 36) tested a variety of small molecular substances in the formation of cholesterol in feeding experiments with rats, and they concluded that the effects of butyrate, ethanol, leucine and normal- and isovalerates parallel closely their ability to form acetyl groups. The superiority of leucine and isovaleric acid may be apparent only because these two compounds could yield more than one mole of acetic acid during their metabolism (37). From evidence obtained in liver slice experiments, whereby acetaldehyde or ethanol are more readily incorporated into the steroids than acetic acid, it is concluded that acetic acid itself is not the reactant in the condensation process, but that the acetyl group is linked to coenzyme A. As evidence for this view, Samuels & Reich (38) mention the work of Klein (39), who found that yeast deficient in coenzyme A fails to synthesize both ergosterol and other lipids. It is further suggested that several of the acetylcoenzyme A units may react to form acetoacetate and other condensation products.

It is probable that the same agents found to promote the formation of carotenoids, rubber, and steroids will be active in the synthesis of the lower terpenes. An investigation of this kind should therefore not be satisfied with the over-all incorporation of the tracer material, but should be directed toward finding the position of the tracer atom in the final product. One of the drawbacks in this kind of study was the necessity of working with larger amounts of plants, since most terpenes are liquids and have to be separated by fractionation, followed by the preparation of a characteristic derivative. The application of chromatographic techniques for terpenes, recently developed by Kirchner and Miller (40) will make such studies possible on a micro scale.

From cytological investigations on oil glands in plants there is evidence that the elementary units combine to form a prototerpene which subsequently is transformed into the oils deposited in oil sacs. In a careful study on the glandular cells of several plant species it was shown that often strongly refractive droplets appear in the protoplast. From a minute outgrowth of the cellulose cell membrane a sac is formed. The droplets then disappear while oil is secreted in the sac. Lehmann (41) suspects that the initial droplets are resorbed by the inner cellulose layer, which converts them into the secreted oil. The cell nucleus probably plays a role in these transformations, since it is always in contact with the oil sac and a diminution in the size of the nucleus with progressive formation of oil makes it possible that substances are given off which add to the contribution of the plasma in the oil formation. During this process the protoplast changes in appearance and becomes opaque and granular. The initial droplets, often yellowish or greenish, are different from the oil in the sac, and are not stained with Sudan reagent. The droplets do not, therefore, simply coalesce and form directly a larger oil globule. A chemical process is apparently involved in which the droplets, not sensitive to Sudan stain, are converted into the final oil. Their water insolubility points to a stage in oil formation beyond that of the postulated 2, 3, or 5 carbon skeleton. From the difference in affinity towards the staining reagent we may conclude that these prototerpenes in the initial small droplets are of a more hydrophilic character than the terpenes. They present, therefore, a stage of terpene formation before that of dehydrocitral, linaloöl or geraniol, which have often been postulated as intermediates or starting points in the formation of the variety of terpenes present in the individual oils.

The arguments in favor of regarding the open chain terpenes as precursors are largely based on the ease with which these compounds are converted into other terpenes *in vitro*, and the fact that most terpenes can be constructed from a regular isopentane chain by coiling up the chain and connecting the proper carbon atoms to form the different ring systems found in the terpene groups. Further support for this idea is supplied by a study of the composition of the oils. For example, Kremers (42) postulates the formation of dehydrocitral from two moles of methylbutenal to explain the for-

mation of the terpene families in *Mentha piperita* and *M. spicata*. Through hydrogen addition citral is formed, which in turn is subjected to two types of reduction processes, one eliminating the double bond, the other, the aldehyde group. The double bond hydrogenation gives rise to the terpenes found in peppermint oil, whereas the reduction of the carbonyl group would lead to the terpenes found in spearmint oil (Fig. 1).

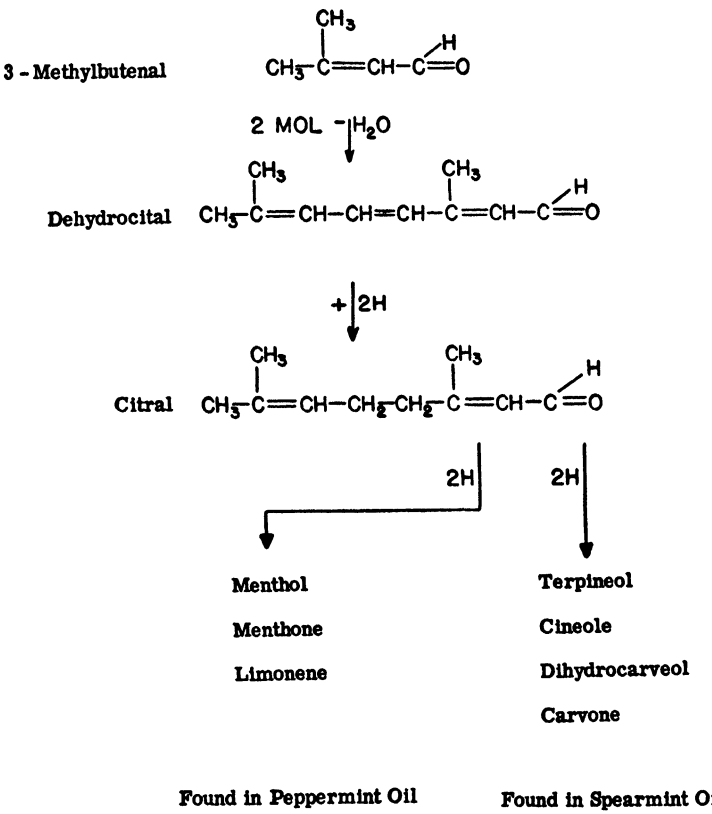


FIG. 1. BIOGENESIS OF TERPENES IN OILS OF PEPPERMINT AND SPEARMINT.

From a study of the terpenes occurring in Eucalyptus globulus we can conclude that α-pinene may be considered as parent terpene for a considerable number of other terpenes. The *E. globulus* oil contains mono- and bicyclic terpenes, many of which can be converted into each other by hydration, oxidation and rearrangements which are readily performed *in vitro*. These relations are presented in Fig. 2. One of the most frequently occurring terpenes, α-pinene, is here considered as starting compound for this terpene family, since it is easily converted into monocyclic and other bicyclic systems.

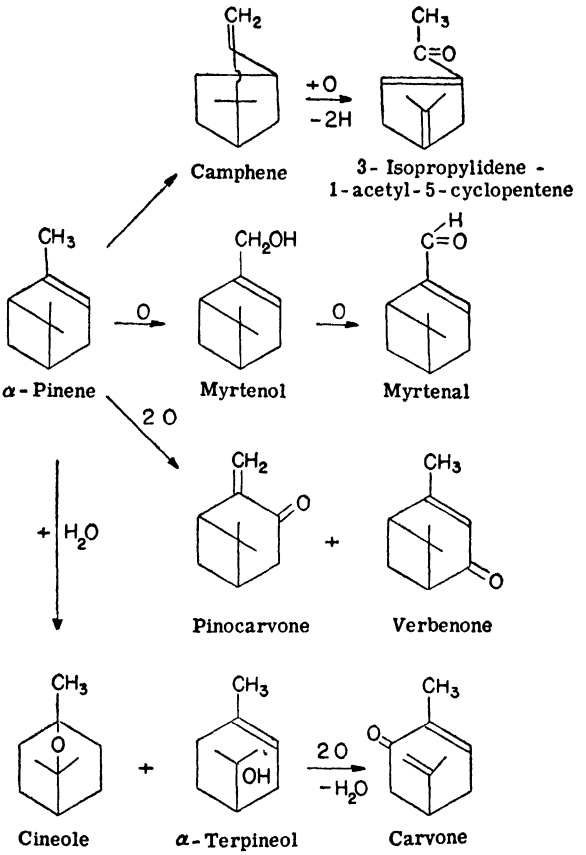


FIG. 2. INTERRELATIONS OF TERPENES OCCURRING IN EUCALYPTUS GLOBULUS.

The formation of families of related terpenes in a plant is especially noticeable when we examine terpenes which are distinguished by a rare chemical structure such as the tricyclic terpenes found in sandalwood oil. The occurrence of the different components can be explained by an oxidative degradation starting with santalene, forming santalal, noreka santalal, and teresantalic acid. A decarboxylation of teresantalic acid, followed by a breaking of the three ring structures accounts readily for the occurrence of the C_9 terpenes, santene, santenone and santenol (Fig. 3).

Injection experiments with terpenes lend support to these postulated secondary transformations of initially secreted terpenes. Fujita (43), injecting linaloöl, citronellal, citral and geraniol into *Ficus retusa L.*, was able to isolate several terpenes, and his results are summarized as follows:

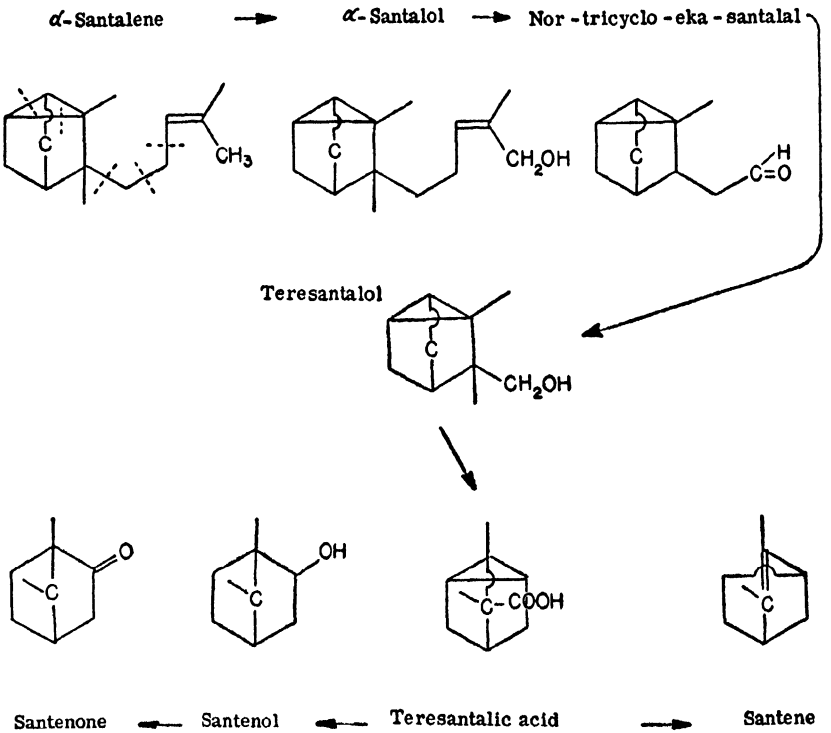


FIG. 3. COMPONENTS OF SANDALWOOD OIL.

Injection of linaloöl→geraniol→*d*-α-terpineol
Injection of *d*-citronellal→*d*-citronellol→geraniol→*l*-limonene
↘ nerol ↗
Injection of citral→geraniol→citronellol
Injection of geraniol→citral
↘citronellol

We see rearrangement, oxidation and reduction and ring closures have taken place, with the production of compounds which are frequently found together in the essential oil. These experiments strongly support the conclusion reached earlier: that a few terpene types serve as parent compounds for the diversity of terpenes present in the oils.

The isolation of the monocyclic α-terpineol in these experiments shows that ring closures take place from open chain terpenes. This ring closure is accomplished quite readily *in vitro*. The formation of bicyclic and tricyclic systems is, however, much more difficult, and their tendency is to undergo ring fission with the formation of monocyclic systems. Subsequent oxidation, reduction, hydration and dehydration and Wagner rearrangements are further causes of the formation of terpene families in the individual species of plants, as shown in Fig. 4.

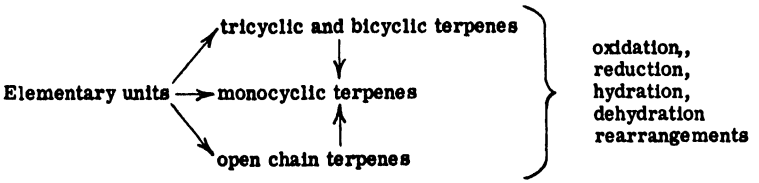


FIG. 4. BIOSYNTHESIS OF TERPENE FAMILIES.

Many oils contain several of these terpene families, but it is often possible to find within a genus species which have oils of much simpler composition. This observation also holds for the benzene derivatives and straight chain compounds often accompanying the terpenes in essential oils.

The complexity of some of the oils is explained by hybridization of the species with oil of simple composition. The several phases of terpene formation are gene controlled, and it is not surprising that in the study of a single species large differences in oil composition are found. Some of these differences may be climatological, the growing conditions influencing yield and composition to some extent; however, radical differences have been found in the systematic investigation of the genus Eucalyptus and of the pine genus. The Australian workers Penfold & Morrison (44) found that in a group of *E. dives* trees growing but a few feet apart some contained an oil rich in cineole, whereas the others were rich in piperitone. The currently known variants of the *E. dives* species with the chemical composition of their essential oils are listed in Table I.

TABLE I

EUCALYPTUS DIVES
CHEMICAL COMPOSITION OF TYPE AND VARIETIES A, B, AND C

	Type	Var. A.	Var. B.	Var. C.
piperitone content	45–53%	5%	10–20%	under 5%
cineole content	nil	nil	25–45%	68–75%
phellandrene content	20–30%	60–8%	present in quantity	absent

The systematic study of the genus Eucalyptus has furnished many more of these cases, and Penfold and Morrison list these as follows: *Eucalyptus dives* (type and three forms); *E. australiana* (type and three forms); *E. numerosa* (type and two forms); *E. piperita* (type and one form); *E. micrantha* (type and two forms); *E. citriodora* (type and one form). Similar observations have been made on the family of the *Myrtaceae* on the oils of *Leptospermum citratum*, which occurs in three forms. The normal Tea tree oil consists largely of the aldehydes citral and citronellal, whereas Var. A contains mainly the terpenes γ-terpinene, *d*-α-pinene and *p*-cymene and Var. B. citral, geraniol and citronellol (45).

In a review on the chemical composition of oils occurring in species of the genus Pine, Mirov (46) gives several examples of variations in the analytical data of turpentines in morphologically identical species. A systematic survey of the chemical composition of plants will undoubtedly uncover many such cases.

The composition of the volatile oil from different species belonging to one genus may differ greatly. Penfold & Morrison (44) recognize four main tendencies of terpene formation in the 231 investigated members of of the genus Eucalyptus: (*a*) formation of cineole; (*b*) piperitone; (*c*) phellandrene; (*d*) geraniol and other open chain terpenes.

In other cases, for example in the genus *Camphora*, we can distinguish three main tendencies: (*a*) formation of terpenes directly related to linaloöl; (*b*) formation of related complex ring compounds, pinene, camphor and borneol; and, finally (*c*) synthesis of benzene derivatives of the propylbenzene series, safrol, eugenol, elemicin, cinnamic aldehyde and alcohol, benzoic acid, and benzyl alcohol. Among the 23 investigated species of the genus *Cinnamomum* we find some which contain representatives of only one of the three groups; the majority, however, contain mitxures of all three.

It is likely that the formation of the two groups of terpenes and the propylbenzene derivatives result from independent genetic factors, and that through hybridization the intermediate forms have appeared. Such a hybrid from plants with radically different essential oil composition has been produced by Mirov (46) by crossing *Pinus ponderosa* (limonene as major component) with *P. jeffreys* (consisting nearly completely of *n*-heptane). The hybrid contains both groups, the terpenes as well as the heptane from the parent trees.

Fujita (47) has listed the occurrence of the oil constituents in a number of genera and arranged the species of the following plant genera according to their chemical composition: genus *Cinnamomum* (*Lauraceae*), *Ocimum*, *Thymus*, *Origanum*, *Monarda*, *Salvia*, *Saturcia*, *Lavendula*, *Mentha*, *Orthodon* (*Labiatae*), *Artemisia* (*Compositae*), *Asarum* (*Aristolochiaceae*), *Piper* (*Piperacae*), *Boronia* (*Rutaceae*) *Leptospermum*, *Baeckea*, *Backhousia*, *Metaleuca*, *Pimenta*, *Eugenia*, *Eucalyptus* (*Myrtaceae*). From such a tabulation, Fujita has attempted to find the genetic relationship of the species based on the assumption that plants containing the parent compounds of a terpene family are from the point of view of evolution of an earlier origin than those containing terpenes which can be regarded as secondary transformation products. It is to be expected that the oil obtained from hybrids is more complicated than that of either parent. This principle, coupled with assumptions on the biochemical relationship of the terpenes, has been used to find the species of origin in a number of plant genera. These relationships are presented in three dimensional graphs based on the relative concentration of substances present in the essential oils.

The observations on the genus *Monarda* (*Labiatae*) may serve as an

example of such a treatment. The investigated species of this genus contain thymol and carvacrol as major components, accompanied by smaller quantities of the phenol oxidation products, thymohydroquinone, thymoquinone, dioxythymoquinone. Other compounds found are isovaleraldehyde, linaloöl, geraniol, citral, limonene, α-thujene, α-pinene and dihydrocuminaldehyde. Adopting the scheme presented in Fig. 5 for the biogenetic relationship of some of these terpenes, Fujita considers *M. didyma*, containing linaloöl and none of the phenols, as the original species and arranges the other species according to their phenol content expressed in percentages as follows: *M. didyma*, 0; *M. punctata*, 24 to 66; *M. fistulosa*, 52 to 72; *M. citriodora*, 65 to 80; *M. menthaefolia*, 82; *M. pectinata*, 77 to 97.

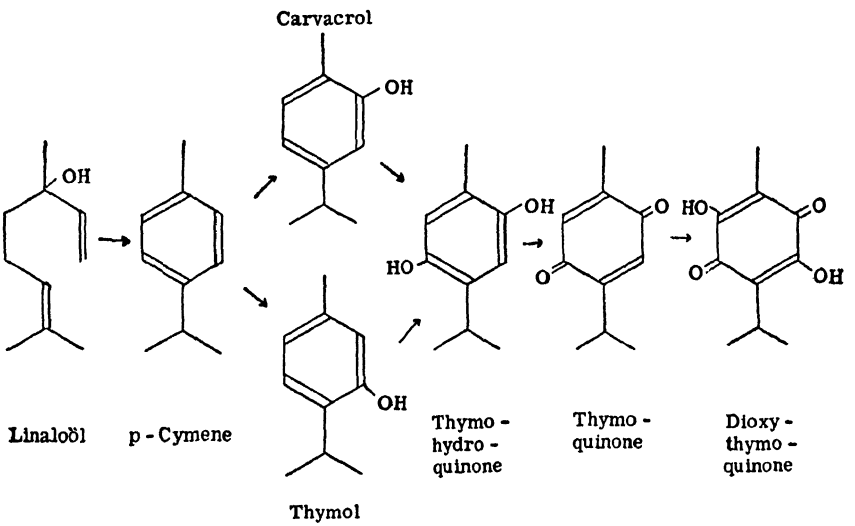


FIG. 5. BIOGENETIC RELATIONSHIP OF TERPENES IN THE GENUS MONARDUS.

Our meager knowledge on the biogenesis of the terpenes, the incomplete data on the occurrence and nature of the terpenes in plant groups, and, finally, our lack of data on the variation in oil composition in relation to growing conditions make deductions on phylogenetic relationships rather speculative. Nevertheless, the successful application of biochemical studies in a few isolated cases hold promise that this type of analysis may become a valuable tool in many taxonomic problems. The present status of these attempts has been clearly expressed by Mirov (46) who on the basis of extensive studies on the pine genus came to the following conclusion: it would be a futile task even to attempt to devise a biochemical classification of pines that would replace the existing botanical classification, but at the same time the biochemical characters of a pine may well be used to establish or clarify relationships that are not discernible by morphological characters

alone. This cautious attitude on studies of a genus from which nearly 50 per cent of the species have been analysed for its oil components is a distinct warning against overoptimism in a similar treatment of lesser known, plant families and genera.

Through chemical genetic studies it should be possible to describe the formation of the terpenes as a series of independent gene controlled reactions. These reactions include the formation of prototerpenes, subsequent oxidations and reductions, dehydrations and water additions, ring formation, esterification, etc. With the introduction of spectroscopic methods and the application of chromatography to the characterization of the terpenes, the main obstacle in these studies is being overcome.

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PHYSIOLOGY OF MYCORRHIZAL RELATIONS IN PLANTS[1]

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For a deeper insight into the physiology of mycorrhizal relations it is necessary to investigate not only the mycorrhizal associations but also their associate partners. Although mycorrhizae occur in most higher plants, their physiology has been studied extensively only in orchids and some forest trees. Since 1936, when Burgeff (15a) published his last work on the mycorrhizal relationships in orchids, only a few contributions concerning this subject have appeared. Recent investigations have been limited largely to the ectotrophic and ectendotrophic mycorrhizae in forest trees.

The earlier literature concerned with studies on mycorrhizal relations in plants has been reviewed comprehensively by Rayner (106) and Hatch (43). A general review of these problems was recently written by Kelley (48) while Harley has reviewed some aspects of ectotrophic mycorrhizae (38) and of endotrophic mycorrhizae (39), respectively.

Nature of Mycorrhizal Fungi

Kelley (48) has claimed that there are no mycorrhizal fungi but that there is only a mycorrhizal state. According to him "almost any fungus" apparently can be a mycorrhiza producer and, therefore, the identity of the fungus should be a relatively inconsequential thing. There is, however, no evidence supporting this view since little or nothing is known about the nature of the fungal partners of most mycorrhizae. Early statements in this respect are not reliable since they were based mainly on presumed hyphal connections between sporophores and the endophytic mycelia. It has been emphasized by Melin (75), Modess (96), and others that only synthesis experiments under controlled conditions can furnish the conclusive proof concerning the mycorrhiza-forming ability of a given fungus. If, then, the occurrence of the fungus in nature is correlated with the higher plant concerned, the evidence is conclusive that it produces mycorrhizae under natural conditions also. According to Kelley, however, the synthesis method is not reliable because of the unnatural experimental conditions. As an example of this he noted that *Lactarius deliciosus*, which was proved by Melin and others to be a mycorrhiza-former with spruce as well as with pine, has been observed in nature by Romell (115) only in association with spruce. The discrepancy in this case, however, has been caused by an undue generalization of the report of Romell. It is now well known that this species is common also in pure pine stands in Sweden as well as in other parts of Europe (138).

[1] This review covers the period from approximately 1937 through 1951.

For a deeper understanding of the nature of mycorrhizae, it is of fundamental importance to know the fungi which are mycorrhizal in nature. Although this side of the mycorrhiza problem may fall outside the scope of this review, some comments may be made concerning the recent progress along this line. Modess (96), Rayner & Levisohn (112), Santos (123), Norkrans (103), and Hacskaylo (35, 36) presented added evidence which indicates that the fungi producing ectotrophic and ectendotrophic mycorrhizae with forest trees are mainly Hymenomycetes and Gasteromycetes. Fries (26) showed that the haploid stages also produce mycorrhizae. Hitherto, about fifty fungal species have been experimentally proved to be mycorrhiza-formers with forest trees. Undoubtedly this number will be greatly increased as a result of future investigations. Probably it will be found that several genera of forest Hymenomycetes, such as *Amanita*, *Boletus*, *Cortinarius*, *Lactarius*, and *Russula*, exclusively or predominantly contain mycorrhizal fungi (78). Others such as *Clitocybe*, *Collybia*, and *Mycena* perhaps contain exclusively saprophytic species. The hitherto examined representatives of these genera in Sweden decompose celluloses and lignins of the litter and in that way play a great part in soil ecology (54, 55). Still others, such as *Tricholoma*, contain, besides tree mycorrhizal fungi, numerous saprophytic litter-decomposing species (103, 104). Norkrans, studying comprehensively these two ecological fungus types, showed that a cellulose-decomposing species, *T. fumosum*, is also capable of forming ectotrophic and ectendotrophic mycorrhizae. Gosselin (34) noted that the wood-destroying fungus *Polystictus circinnatus* forms mycorrhizae with spruce. Since the synthesis experiment, however, in this case was not performed under aseptic conditions, these results remain to be verified. Occurrence of known or presumed mycorrhizal fungi (Hymenomycetes) with conifers have been noted in field studies by Birch (5), Young (142, 143), Thomas (135), Vassilkov (138), and Gilli (33).

Whether certain Ascomycetes are also able to form mycorrhizae with trees has not yet been established with certainty. Lihnell (52) has shown that *Cenococcum graniforme*, probably belonging to the Ascomycetes, forms ectotrophic and ectendotrophic mycorrhizae with certain conifers as well as with deciduous trees. It also produces ectotrophic mycorrhizae with juniper (51) but it is not involved within the vesicular-arbuscular mycorrhizae in this conifer. Sappa (124) noted that *Tuber magnatum* lives in mycorrhizal association with oak. This statement was based on inoculation experiments in pot cultures and, therefore, needs further investigation under controlled conditions. However, there seems to be some difficulties in growing this fungus in pure culture (125).

The mycorrhizal fungi isolated from green orchids have been referred by Bernard to the genus *Rhizoctonia*. A great number of species and strains have been described, particularly by Burgeff (15, 15a). *Rhizoctonia* was also found to be associated with *Neottia* (141) and sometimes with *Corallorhiza* (21). Porter (105) and Vermeulen (139) obtained added evidence

which indicates that there is a close and specific relationship between orchids and their mycorrhizal fungi. A single *Rhizoctonia* species or a limited number of species appear to be constantly associated with any given orchid species in nature. Stahl (134) recently showed that the endophyte of a tolypophagous liverwort, *Aneuria pinguis*, is a *Rhizoctonia*. Cappelletti (17), Sprau (133), and von Malmborg (72) presented added evidence which indicates that the *Rhizoctonia* endophytes concerned belong to the Basidiomycetes. von Malmborg isolated from *Aneura pinguis* a *Rhizoctonia* mycelium, which fruited in culture and so was identified as a *Corticium* species. Most carbon-heterotrophic orchids studied were found to be associated with true Hymenomycetes such as *Armillaria mellea*, *Marasmius coniatus*, and *Xerotus javanicus* (15, 37).

Observations made by Francke (32), Lück (58), and Lihnell (53) indicate that the fungal associates of *Monotropa* and *Pyrola* belong to the Basidiomycetes. However, further investigations in this matter are needed. Freisleben (24) made successful isolations of the endophyte from some *Vaccinium* species. The taxonomic position of the isolates could not be determined.

Attempts to isolate the endophytes of vesicular-arbuscular mycorrhizae have been reported by Butler (16), Elisei (23), Lihnell (51), Magrou (66, 69), Magrou & Magrou (71), Neill (100), and Stahl (134), but, in these cases, slight growth was obtained only in the presence of host tissues. Stahl (134), studying the mycorrhizae of liverworts, obtained synthesis with nondisinfected plants. Cross-inoculations indicated that most thamniscophagous mycorrhizae of liverworts may be caused by the same fungus. Recently Barrett (4) succeeded, for the first time, in culturing without host tissues a strain of *Rhizophagus* isolated from roots of garden pea. He has since made successful isolations of *Rhizophagus* from roots of several other plants. In unpublished experiments six isolates from as many hosts showed in pure culture a close similarity in morphology and cultural habit.

Nutritional Requirements of Mycorrhizal Fungi in Pure Culture

Growth requirements of tree mycorrhizal fungi have been studied extensively in the last few years and many important results have been obtained. The effects of oxygen supply, hydrogen-ion concentration, and certain metallic ions on the growth were investigated by several writers (46, 93, 96, 104). Most studies, however, have been devoted to the nitrogen nutrition, carbon utilization, and requirements for vitamins and other metabolites.

Carbon utilization.—How (46) studied the utilization of different carbon sources by *Boletus elegans*, a mycorrhizal fungus with larch. She found glucose to be the best source of carbon, although some other sugars such as xylose, fructose, sucrose, and maltose also yielded good growth. Pectin was readily utilized. Melin (77) reported that *Boletus variegatus* did not develop on ground up forest litter previously leached with water but grew very well when glucose was added. A similar result was obtained when forest humus

was used as substrate instead of litter (79). Norkrans (104) has reported the behavior of eight species of *Tricholoma* in pure culture, special attention being paid to their utilization of cellobiose, lichenin, and cellulose. Five of the species studied had been proved to form mycorrhizae with either pine or spruce or both while the others were found to be saprophytic litter-decomposers. Glucose and mannose were readily utilized by all of the species. Fructose was equally effective in the nutrition except for two mycorrhiza-formers, while galactose was a poor source of carbon. Cellulose, as well as lichenin and cellobiose, was readily decomposed by the saprophytic species. The same was true of *T. fumosum*, which is able to form mycorrhizae with pine. Since the cellulase production was measurable only in the presence of cellulose, it was concluded that the cellulolytic enzymes were adaptive. If both glucose and cellulose were present in the medium, most of the glucose was consumed first. The other mycorrhiza-forming species examined did not utilize cellulose as a sole source of carbon, but two of them (*T. imbricatum* and *T. vaccinum*) showed slight cellulose decomposition in the presence of a small amount of starter glucose. This behavior suggests the formation of an adaptive cellulolytic enzyme which was not formed when these species were cultured on cellulose alone. Cellobiose was a good or fairly good source of carbon for three obligate mycorrhiza-formers, but it was utilized by *T. flavobrunneum* only in the presence of glucose. Two species (*T. imbricatum* and *T. vaccinum*) were capable of decomposing lichenin without adaptation.

Lindeberg (56) found that an isolate of *Boletus subtomentosus* (fungal associate of pine) behaved as a litter-decomposing fungus, causing a great decrease in dry matter when grown on sterilized litter. He also showed that this isolate, as well as the mycorrhiza-forming *Lactarius deliciosus*, was capable of forming polyphenol oxidase abundantly, as did all the litter-decomposing Hymenomycetes tested. On the other hand, most of the tree-mycorrhizal fungi studied produced none, or just small amounts, of this enzyme.

The utilization of different carbon sources by *Cenococcum graniforme* was investigated in detail by Keller (47). He found glucose, mannose, trehalose and cellobiose to be equally effective in the nutrition of this species, while sorbitol, galactose, dextrin, starch, and inulin were poor sources of carbon. Cellulose was not utilized.

The results mentioned above show that ectotrophic and ectendotrophic tree-mycorrhizal fungi differ in their ability to produce cellulase. Most of them seem to lack this ability or have only a slight capacity to form adaptive enzymes for cellulose decomposition. On the other hand, some species, such as *Tricholoma fumosum*, are strong cellulose decomposers. These may be assumed to be facultative mycorrhizal fungi in nature. In fact, Romell (117) found sporophores of *Boletus subtomentosus* (cf. above) in areas which had been isolated from the living tree roots by trenching. When living in mycorrhizal association with trees, the cellulose-decomposing species may not

produce cellulase as long as soluble carbohydrates are available in the roots. The cell wall, however, constitutes a cellulose substrate which will induce an increase in cellulase production when the roots no longer offer any excess of sugars (104). According to Norkrans, this may furnish an explanation of the ectendotrophic tree mycorrhiza.

Most orchid mycorrhizal fungi studied are capable of utilizing celluloses and lignins as sources of carbon (15a, 37, 45). Only *Corticium catonii* and *Rhizoctonia neottiae* were found to lack this ability. Some species, such as *Armillaria mellea* and the associate of *Galeola hydra*, are very strong wood-destroyers. According to Francke (32) the fungal associate of *Monotropa* is able to utilize, besides sugars, certain complex organic compounds such as humic acid as a source of carbon. On the other hand, cellulose was found to be useless.

Nitrogen nutrition.—According to Norkrans (104) nitrate is a poor source of nitrogen for the *Tricholoma* species tested, whereas inorganic as well as organic ammonium salts are easily utilized. Mikola (93) reported that *Cenococcum graniforme* is able to utilize both kinds of nitrogen although it uses ammonium nitrogen in preference to nitrate when both are supplied in the medium.

The value of amino acids as sources of nitrogen for mycorrhizal fungi of trees has been studied extensively in the last few years (79, 81, 87, 93). Melin & Mikola (81) and Mikola (93) reported that casein hydrolysate is a better source of nitrogen than is ammonium nitrogen for *Cenococcum graniforme*. Melin & Norkrans (87) showed that a mixture of 18 amino acids furnished in proportions equal to those found in hydrolyzed casein strongly favored the growth of *Lactarius deliciosus*.

Amino acids were found to greatly increase the growth rate of most investigated mycorrhizal associates of trees in nutrient media also containing ammonium nitrogen. The beneficial effect may be due to an inability of the fungi to synthesize some indispensable amino acids from ammonium nitrogen as rapidly as needed. Partial heterotrophies, with regard to one or more amino acids, have been found in *Cenococcum graniforme* (81, 93), and in species of *Tricholoma* (104), *Amanita*, *Boletus*, *Cortinarius*, *Lactarius*, and *Rhizopogon* (79). Different fungal species, however, have different demands for amino acids. Norkrans, comparing the growth of various *Tricholoma* species under the same conditions, showed that the mycelial yields of *T. flavobrunneum* and *T. imbricatum* were doubled when casein hydrolysate was added to the medium. No single amino acid, however, was capable of increasing the growth of *T. flavobrunneum*, whereas glutamic and aspartic acid, as well as proline, increased the growth of *T. imbricatum* about 50 per cent. *T. pessundatum* and *T. vaccinum* were not affected by hydrolyzed casein, but glutamic acid had a favorable effect on their growth, as had aspartic acid in the case of *T. vaccinum*. The mycelial production of *T. fumosum* was nearly quadrupled by an addition of casein hydrolysate and nearly doubled by tryptophane while other single amino acids did not influence the growth

rate to any great extent. Concerning *Cenococcum graniforme*, it was shown that an addition of arginine, methionine, lysine, or histidine produced a great increase in the growth of an isolate tested (81, 93). Melin, comparing various mycorrhiza-forming Hymenomycetes and Gasteromycetes, also found great differences in their demands for amino acids. Most species studied were strongly favored by an addition of glutamic acid, but some others were not affected by this amino acid (79).

Some amino acids were found to exercise an inhibitory effect on the growth rate of the fungus mycelia when added singly to the nutrient media in adequate concentrations (104). This inhibition may be counteracted by the presence of other amino acids. Norkrans further demonstrated that the proportions of the amino acids present in the medium exert a profound influence upon their action, as is the case with many other fungi investigated. Present information indicates that purines have no growth-promoting influence on mycorrhizal fungi of trees (77, 93, 104).

According to Burgeff (15a), Holländer (45), and Reitsma (114), the orchid mycorrhizal fungi studied are able to utilize both inorganic and organic nitrogen compounds. In several cases the latter were better sources of nitrogen than the former. Francke (32) showed that ammonium compounds as well as proteins are very good nitrogen sources for the mycorrhizal fungus isolated from *Monotropa*. Norkrans (104) and Keller (47) made some studies on the nitrogen content of the mycelia. A particularly interesting finding is that of Keller who demonstrated that the mycelia of *Cenococcum* grown in nutrient solutions containing ammonium nitrogen exuded great quantities of organic nitrogen into the media.

Vitamins.—A number of studies on the effect of vitamins on mycorrhizal fungi of trees have been made. The vitamin requirements for 15 species forming mycorrhizae with trees were reported by Melin and collaborators (80, 86, 88, 89), Mikola (93), and Norkrans (104). In addition, several other species have been studied in this respect in my institute (79). All species tested have been proved to be deficient for thiamine when cultured in synthetic nutrient solutions in pure culture. Most of them show only partial deficiency for thiamine, while a few may be totally, or nearly totally, deficient. The degree of partial thiamine-heterotrophy varies widely for various species; different isolates of the same species may have different demands for thiamine (89); and the partial deficiency for thiamine may be influenced by certain environmental conditions, such as supply of oxygen and composition of the medium (93). The mycorrhizal fungi studied differ also in their ability to utilize or synthesize the moieties of thiamine. Most species are heterotrophic for both thiamine moieties, although some are more deficient for pyrimidine than for thiazole.

Besides thiamine many mycorrhizal fungi have one or more additional vitamin requirements. Norkrans (104) reported that *Tricholoma imbricatum* is partially heterotrophic for pantothenic acid and *T. fumosum* partially heterotrophic for nicotinic acid. Melin found *Lactarius deliciosus* to be defi-

cient for nicotinic acid (79). Deficiencies for other vitamins, such as biotin for certain isolates of *Cenococcum* (88, 93) and inositol for *Rhizopogon roseolus* (80), have also been reported.

Other mycorrhizal fungi may have more complex requirements. In the experiments of Modess (96) many Hymenomycetes, several of which were assumed to produce mycorrhizae with trees, could not be made to grow, or developed only very poorly, in pure culture. The same experience has been repeatedly encountered in our laboratory. This failure of growth may be, at least in part, due to complex vitamin deficiences.

The requirements of orchid mycorrhizal fungi for vitamins have not been studied in detail. Verveulen (139) found *Rhizoctonia repens* to be partially deficient for thiamine. Mariat (73) showed that the mycelium of this fungus, when grown in a nutrient solution containing no vitamins, exuded in the medium great amounts of nicotinamide and riboflavin, which indicates that it may be autotrophic for these vitamins.

Unidentified organic supplements.—Melin (77) demonstrated that water extracts of litter of forest trees, such as aspen, beech, birch, and pine, exert a strong growth-promoting effect on mycorrhiza-forming Hymenomycetes and Gasteromycetes grown in a nutrient medium containing sugar, salts, and thiamine. In addition to growth-promoting ash constituents, studied particularly by Lindeberg (54) and Norkrans (104), the extracts were concluded to contain one or several organic substances which exercised fairly general growth-promoting effects on the fungi tested. A similar effect was caused by water extracts of forest humus from various types of Swedish forests (79) and also by extracts of sporophores of various Hymenomycetes (87). It may be assumed that the action of the extracts mentioned was in part due to amino acids. The compost effect on fungal activities described by Rayner (108, 110, 112) may partly be explained in the same way.

Melin found that tree-mycorrhizal fungi were greatly stimulated in their growth by pine roots growing aseptically in media containing sugar, salts, vitamins, and amino acids (79). Even species which had previously developed poorly or not at all showed a marked stimulation from contact with the living roots. It appears probable that unidentified growth-promoting metabolites not included in the known B vitamins, purine and pyrimidine bases, and the amino acids in casein hydrolysate, are present in the rootlets concerned.

Fries (25, 27) studied the spore germination of a number of soil inhabiting Hymenomycetes, several of which had been previously proved or assumed to be mycorrhiza producers. In many cases germination was obtained on agar containing malt extract or in a synthetic nutrient solution, only in the presence of activators such as living colonies of *Torulopsis sanguinea*. The germination-promoting principle, though less active, was also produced by living colonies of other fungi. The effective material could not be replaced by any defined chemical compound tested. Its nature was not identified. However, Khudiakov & Vozniakovskaia (49) obtained germination of

spores of some *Boletus* species in a nutrient solution containing casein hydrolysate and vitamins.

Conditions for the Formation of Ectotrophic and Ectendotrophic Mycorrhizae

Host nutrition.—The effect of host nutrition in relation to mycorrhizal infection and mycorrhiza frequency has been extensively studied, particularly by Hatch (43), Gast (28), and Björkman (6, 7, 8). Hatch studied the relation between mycorrhizal development in pine and the nutrient environment, using soil culture experiments. He found that mycorrhizae were produced in abundance only under conditions of a lack of balance or a low availability of any one or more than one of the elements: nitrogen, phosphorus, potassium, and calcium. He concluded that the susceptibility of the short roots to infection by mycorrhizal fungi is controlled, in some unknown way, by their internal concentration of these elements. Gast (28) showed that low radiation intensities greatly reduced formation of mycorrhizae in conifers and assumed that this effect was due to a reduced carbohydrate production.

Björkman, investigating pine and spruce seedlings grown in various types of soil under various conditions, confirmed the finding that light intensity as well as deficiences of certain nutrients in the soil, favor mycorrhiza production. According to Björkman, however, only three external factors exert important influences on the formation of ectotrophic and ectendotrophic mycorrhizae in trees, viz., light and the quantities of inorganic nitrogen and phosphorus available in the soil. In the dark or in weak light up to about 10 per cent of full daylight no mycorrhizae developed, as a rule, in his experiments. If the intensity of light was increased from 10 to 25 per cent, a great increase in mycorrhiza formation resulted. With a further increase in the light intensity, there was but a slight increase in the frequency of mycorrhizae. In pure cultures, *Rhizopogon roseolus* did not produce mycorrhizae with pine at 10 per cent of full daylight, but at 14 per cent, mycorrhizae were formed, the frequency increasing with greater light intensities. However, Mikola (93), studying birch seedlings in pure culture at different light intensities, found that mycorrhizae were rather abundantly formed by *Cenococcum graniforme* at 10 per cent of full daylight. According to Björkman (7), moderate additions of glucose favored mycorrhizal infection in pure cultures, particularly in weak light. Björkman assumed that glucose could, in part, replace light as a condition for mycorrhiza development. He further demonstrated that ammonium nitrate, when supplied to pine seedlings grown in forest soil deficient in inorganic nitrogen but rich in phosphorus, caused a certain decrease in mycorrhiza formation. On the other hand, phosphoric acid when added instead of ammonium nitrate, increased the frequency of mycorrhiza. In experiments with a bog soil rich in nitrogen but deficient in phosphorus, a supply of ammonium nitrate did not affect the mycorrhiza production in pine seedlings. Even very large

additions of ammonium nitrate to this soil did not decrease the frequency of mycorrhiza. When phosphoric acid was added to this peat, a certain decrease in mycorrhiza frequency resulted. Additions of both nitrogen and phosphorus greatly reduced the formation of mycorrhizae in both types of soil. The amounts of potassium and calcium did not affect the mycorrhiza frequency in Björkman's experiments.

Amounts of carbohydrates in the roots.—Having found, in some experiments, evidence for a correlation between the amounts of soluble carbohydrates in pine roots and mycorrhiza frequency, Björkman concluded that the infection by mycorrhizal fungi may be conditioned by the excess of soluble carbohydrates present in the roots of the trees. A surplus of sugar will promote the entrance of fungi into the roots producing mycorrhizae with them. On the other hand, a deficiency of soluble carbohydrates in the roots strongly suppresses the development of mycorrhizae. Therefore, factors influencing carbon assimilation and the amount of soluble carbohydrates in the roots, particularly light, nitrogen, and phosphorus, indirectly influence the mycorrhiza frequency. Certain experiments with three-year-old pines that had been strangulated for one summer seem to favor Björkman's conclusion (8). In strangulated plants the formation of new mycorrhizae was inhibited almost completely, whereas the control plants had very well-developed mycorrhizae. Chemical analyses showed that the amount of reducing substance in the roots of strangulated plants was lower than in the control plants.

Björkman assumes the amount of sugar in the roots to be the only important factor directly influencing the ability of the mycorrhizal fungi to invade the roots, this factor therefore regulating the formation of mycorrhizae in nature. According to him, this theory will explain very simply the different frequency of mycorrhizae in different forest types and probably also the varying development of nodules in alder and legumes under different conditions. The theory remains to be experimentally evaluated. This will be difficult, however, since it may be impossible to vary the sugar concentrations in the roots without influencing metabolic processes and the formation of metabolic products which may affect the susceptibility of the roots to infection by mycorrhizal fungi. According to the theory, sugar should be the most suitable source of energy and carbon for the fungal associates. As mentioned above, however, this is not always the case.

Diener (18), studying the conditions for production of root nodules in *Pisum sativum*, found that root nodule formation depends mainly on light intensity and on the supplies of nitrogen and phosphorus. He showed that the carbohydrate theory of Björkman is not valid in this case. The same may be true of holomycotrophic plants or seedlings.

Metabolites in the roots.—The theory of Björkman is opposed by observations made by MacDougal & Dufrenoy (64, 65). They demonstrated that isolated segments of mycorrhizal pine roots could survive and live for many seasons like chlorophyll-less plants. These roots also produced new normal

appearing mycorrhizae. The carbohydrates found in them had been obtained from the soil by means of the fungal associate. This demonstrated that carbon material suitable for the fungus occurred in the soil used in the experiment. Despite this the hyphae invaded the roots. In my laboratory, excised pine roots grown in pure culture in nutrient solution containing glucose (4 per cent) and vitamins (solution changed every 15 days) were inoculated with mycorrhizal fungi, such as *Boletus variegatus* and *Cenococcum graniforme*. The hyphae rapidly invaded most of the short roots and mycorrhiza-like structures developed (90). This may have been related to certain unknown growth stimulating substances formed by the roots (cf. above).

The fungal associates of ectotrophic and ectendotrophic mycorrhizae gain entrance into the roots by means of pectolytic enzymes which dissolve the middle lamellae of the cortical cells. It is not yet fully understood why intercellular infection is caused only by certain fungal species, although both mycorrhiza-forming and purely saprophytic fungi are capable of producing pectin-splitting enzymes. It seems reasonable to assume that the resistance of trees to many soil fungi may be due to substances which are excreted from the roots and which exercise an inhibiting effect specific for certain species. The ability of several mycorrhiza-forming Hymenomycetes to associate with only one or a few genera of trees (74) may be explained in the same way (78). However, there is no experimental evidence for this assumption. The conditions for development of ectendotrophic mycorrhizae of trees have been discussed by Björkman (7, 10) and Norkrans (104).

Soil effects on the fungus.—For a successful establishment of a mycorrhizal relationship the fungal associate must find the environmental conditions favorable for development. This fact has been overlooked by most students of mycorrhizae and, therefore, direct observations concerning the influence of soil conditions on the mycorrhizal fungi are scarce. As mentioned above, pure culture experiments with tree mycorrhizal Hymenomycetes and Gasteromycetes have shown that these are heterotrophic for certain B vitamins and amino acids. There is evidence that the vitamins concerned are usually available in the soil in sufficient amounts for maximum growth of mycorrhizal fungi. It is well known that B vitamins are formed by numerous microorganisms and the presence of these vitamins in the soil has been recently demonstrated by Schmidt (127), Schmidt & Starkey (128), Roulet (120), and Roulet & Schopfer (121). According to Schmidt & Starkey (128) organic layers and litter of forest soils contain greater amounts of vitamins than agricultural soils. Maximum production seems to occur during periods of most rapid decomposition of organic matter. Besides, many purely saprophytic Basidiomycetes heterotrophic for these metabolities occur in various soils (54, 55a 137). Occurrence of amino acids in adequate proportions and concentrations in the soil may be a factor of great importance for the distribution and activities of different mycorrhizal fungi. The amounts available in the soil may greatly depend on the degree of nitrogen mobilization and

the proportions of the various amino acids may depend on the nature of the proteins under decomposition.

All tree mycorrhizal fungi investigated with respect to pH have been found to be acidophilic, although different species require different hydrogen-ion concentrations for maximum growth (93, 96, 104). It, therefore, seems reasonable to assume that in nature the soil reaction may affect the occurrence of different fungal associates and consequently the formation of different mycorrhizal associations. Thus, the tree mycorrhizae found in neutral or slightly acid soils may be constituted by fungal species other than those in more acid soils.

The possible occurrence in certain soils of substances inhibitory to mycelial growth and mycorrhizal production has been studied by Rayner (107, 110, 112) and Neilson-Jones (101). Rayner showed that toxic substances obstruct or even prevent mycorrhiza formation in pine and other conifers in certain heath soils in southern England. By using a "nutrient-agar-film" test method, Neilson-Jones demonstrated the presence of antifungal compounds in these soils. A pine mycorrhizal fungus, *Boletus bovinus*, was found to be very susceptible. The inhibitory effect was removed by autoclaving, steaming, or air-drying of the soil. A number of observations supported his view that H_2S-producing bacteria were responsible for the inhibitory action. Brian, Hemming & McGowan (14) isolated from the same soil saprophytic fungal strains which produced antifungal substances such as gliotoxin and griseofulvin when grown in pure culture (11, 12, 13). It may be possible that these and other antibiotic substances are produced in sufficient quantities to affect mycorrhizal fungi in the local micro-environment of the soil mentioned, although this has not yet been proved. Certain observations made by the author, when studying the effects of filtrates of certain soils, indicate that in forest soils substances may also occur that inhibit growth of mycorrhizal fungi (79). Under what conditions they are formed and what inhibitory influence they have on the formation of mycorrhizae remains to be elucidated.

Melin (76, 77) demonstrated that leaves and needles which are shed during the autumn in the Swedish forests contain water-soluble, thermostable substances that exercise a strong antibiotic effect on tree mycorrhiza fungi. This was the case with leaf litter of aspen, beech, birch, maple, oak, and pine. The experimental method employed was to extract the ground up leaves with water, whereupon the filtrates were autoclaved or passed through a Seitz filter. In low concentrations the extracts had a stimulating effect, as mentioned above, whereas in higher concentrations they prevented the growth of mycorrhizal fungi. On the other hand, Hymenomycetes naturally inhabiting litter and causing its decomposition proved to be insensitive. These findings were confirmed by Mikola (93) and Norkrans (104). The toxic substances concerned were not identified. The presence of inhibitory substances may, therefore, be the reason that tree mycorrhizae do not develop in forest litter. In the course of time, however, these disappear

as a result of leaching or decomposition. Probably, therefore, any antibiotic substances present in forest humus are newly formed by microorganisms.

Rayner and associates (108, 110 to 113) studied in extensive experiments the effects of various composts on the development of seedlings and mycorrhizae in the heath soil mentioned above. The composted materials were derived from sawdust, straw, and other organic wastes. They found that the inhibiting influence of the soil was counteracted by the addition of compost. The growth of conifer seedlings as well as the formation of mycorrhizae were enormously favored. They concluded that the compost effects were directly associated with qualitative changes in the humus constituents and with microbiological activities related to these changes. This conclusion has been rejected by Crowther who also made manuring experiments in the same soil using inorganic material instead of compost. According to him the favorable effect of compost on mycorrhizal production is due only to its inorganic constituents, especially phosphoric acid (10). It is particularly interesting that, in Rayner's experiments, plants showing maximum size showed also maximum development of mycorrhizae, whereas in the manuring experiments of Hatch and Björkman the maximum seedlings had poorly developed mycorrhizae. This seems to indicate that the causative factor in compost was not simply its content of phosphoric acid. It appears probable that, besides the inorganic constituents, organic material also had a favorable influence.

Morphological Influence of Mycorrhizal Fungi on the Higher Partner

MacDougal & Dufrenoy (64) demonstrated that auxin occurs abundantly in the fungal hyphae of pine mycorrhizae and assumed that the coralloid branching of the short roots was due to the action of auxin translocated from the hyphae. Slankis, studying the influence of mycorrhizal mycelia on the development of pine roots in pure culture, inoculated excised pine roots with various mycorrhizal fungi such as *Boletus luteus* and *B. variegatus* (129). He found that the mycelia caused profuse dichotomous branching of the roots similar to coralloid pine mycorrhizae found in nature. This kind of branching was also induced in roots which were not yet in contact with the hyphae. Even cell-free filtrates of cultured mycelia brought about the same effect. As a result of these observations, Slankis investigated the influence of various concentrations of auxins, such as β-indoleacetic acid, β-indolepropionic acid, β-indolebutyric acid, and α-naphthaleneacetic acid, on the development of isolated as well as attached pine roots in pure culture (130, 131, 132). In the experiments it was found necessary to renew the nutrient solutions containing auxin every 15 days. He showed that additions of auxins very markedly affected the pine roots. Low concentrations increased formation and elongation of roots; this effect was especially pronounced in the case of long roots. Higher concentrations inhibited growth of long roots but caused rich formation and also profuse dichotomous branching of root-

lets. The length and thickness of the short roots as well as the degree of their coralloid branching was controlled by the amounts of auxin added to the solution. Thus, as the concentration increased, the rootlets became shorter, thicker, and more branched. The formation of root hairs was totally inhibited by those concentrations inducing dichotomous branching.

The observations of Slankis lead to the conclusion that auxins formed by mycorrhizal fungi may be the agents causing the production of freely branched ectotrophic and ectendotrophic mycorrhizae in nature. The various degrees of branching may, at least in part, be due to various amounts of auxins produced by different mycorrhizal mycelia.

Observations made by MacDougal & Dufrenoy (64) indicate that the coralloid branching of roots and rhizomes of certain orchids and other mycotrophic plants may also be induced by auxins translocated from the fungus associate. Other changes of form in mycotrophic plants consequent upon fungus infection, such as the tuberization of orchid seedlings, may at least in part have the same cause (73). The influence of mycorrhizal fungi on formation of potato tubers has been reinvestigated by Magrou and associates (67, 68, 70). They found that asymbiotic tuberization, both in orchids and potatoes, can be affected by adjustment of the osmotic concentration in the medium through addition of glycerine or sugar. In nature the formation of tubers in cultivated potatoes lacking mycorrhizae was attributed to the action of the osmotic pressure of the medium surrounding the roots.

Nutritive Relationships

Benefit for the higher partner.—During the last two decades facts have accumulated which confirm the view that the ectotrophic and ectendotrophic mycorrhizae of trees are nutrient-absorbing structures and that the mycorrhizal fungi in certain habitats are beneficial or even necessary for the trees. In the early as well as in the recent literature, observations in various treeless regions in the world have been frequently reported concerning the favorable effect on the growth of tree seedlings of inoculation of seed-beds and pot cultures with soil containing mycorrhizal fungi or with pure cultures of appropriate fungi (42, 43, 109, 119, 140, 142). Beneficial results of inoculations in nurseries or pot cultures have also been observed elsewhere (9, 31, 59, 94, 95, 112).

Special attention should be given to the experiments reported by Hatch (42) who cultured seedlings of *Pinus strobus* in containers with soil from a forestless area in Wyoming. Half of the pots were inoculated with pure cultures of known mycorrhizal fungi. The uninoculated seedlings developed feebly and soon assumed a yellowish tint, while those inoculated grew vigorously and gradually developed green needles. The inoculated plants had well-developed mycorrhizae, whereas the feeble ones were entirely destitute of such. Chemical analyses showed that mycorrhiza-bearing plants contained 86 per cent more nitrogen, 234 per cent more phosphorus,

and 75 per cent more potassium than the plants without mycorrhizae. Hatch concluded that the roots of pine seedlings in this soil were incapable of bringing any significant quantities of nitrogen, phosphorus, or potassium into the seedlings. The content of nitrogen and phosphorus in the nonmycorrhizal seedlings was less than had ever been recorded for pine seedlings grown under any soil conditions. The results of Hatch were supported by Young (142), who demonstrated that several *Pinus* species were unable to grow in certain Australian soils lacking mycorrhizal fungi. However, seedlings inoculated with a known fungal associate (*Boletus granulatus*) became healthy and developed normally. Experiments recently made by Björkman (9) also showed that infection with mycorrhizal fungi greatly improved the growth of pine seedlings. Mitchell, Finn & Rosendahl (95), who inoculated nursery beds (infertile clay and well decomposed sawdust) with soil containing mycorrhizal fungi, studied extensively the nutrient absorption by mycorrhizal and nonmycorrhizal seedlings of white pine and red spruce. The two-year plants were analyzed for nitrogen, phosphorus, and potassium. The differences between the two types of seedlings with respect to these elements were considerably greater than in the experiment of Hatch.

Regarding the mechanism of the superiority of mycorrhizal roots in nutrient absorption, there are still different opinions. Hatch (43) strongly maintained the mineral salt theory of Stahl and accordingly assumed that mycorrhizae are more efficient than nonmycorrhizal roots in absorbing mineral salts from infertile soils. This superior efficacy was considered to be due to a highly increased surface area of short roots resulting from infection by mycorrhizal fungi, followed by an increased absorption of soil nutrients by the fungal associates. Hatch claimed the mycorrhizal relationship to be a "physical" one. The hypothesis of Hatch was accepted by Mitchell, Finn & Rosendahl (95), and Finn (31).

Routien & Dawson (122) found the carbon dioxide production of mycorrhizal roots to be two to four times that of nonmycorrhizal roots. They, therefore, concluded that mycorrhizae increase the salt-absorbing capacity of the roots chiefly by increasing the supply of exchangeable hydrogen ions derived, at least in part, from respiratory carbonic acid. McComb & Griffith (60) also showed that mycorrhizal seedlings absorbed more inorganic elements than nonmycorrhizal ones and believed the higher metabolic activity of mycorrhizal roots to be the cause of their greater capacity for absorption.

Kramer & Wilbur (50) sought to verify the last mentioned theory by using the isotope technique. They studied the absorption of radioactive phosphorus by mycorrhizal and nonmycorrhizal portions of pine roots (*P. taeda* and *P. resinosa*). Detached pieces of roots, obtained from potted seedlings, were exposed to solutions containing P^{32} in varying amounts. The subsequent measurements of the radioactivity seemed to show that mycorrhizal short roots accumulated larger quantities of phosphorus than nonmycorrhizal ones. Similar results were obtained by Harley & McCready (40) studying excised root tips in beech. In their experiments the mean

ratio between the contents of labelled phosphorus of mycorrhizal and non-mycorrhizal tips was 4.4:1.0. In a subsequent paper (41) they stated that the main accumulation of P^{32} in excised mycorrhizal tips (ca. 0.5 cm.), after having been exposed to radioactive phosphorus, was in the fungal sheath and not in the host tissue. Although these experiments are very interesting, it may be impossible to estimate at present to what extent the physiological activities in the root pieces had been affected by their excision and by breaking the hyphae connecting the mycorrhizae with the soil.

In order to ascertain whether mycorrhizal fungi are capable of transferring inorganic nutrients to the roots Melin and Nilsson (82, 83) studied intact pine seedlings growing in pure culture (purified sand) which had been inoculated with the known mycorrhizal fungus, *Boletus variegatus*. When mycorrhizal relations had become well established, labelled phosphorus or nitrogen was added. By a special arrangement only the fungal associate was exposed to the isotope and then the amounts of the isotope were measured in different parts of the seedlings. In the first series of experiments (82) it was demonstrated that *B. variegatus*, as fungal associate, is able to transfer phosphorus from a phosphate solution to the pine roots. The isotope was also found to be translocated from the roots to the stems and needles. This result was confirmed in other similar experiments in which *Cortinarius glaucopus* was the fungal partner (85). In subsequent experiments labelled nitrogen was added as ammonium nitrate containing the stable isotope N^{15} in the ammonium group (83). The measurements showed that the *Boletus* hyphae are able to transport nitrogen from a solution of ammonium salt to the roots from where it is translocated to the stems and needles.

Melin (74), having accepted as a principle the theory of Stahl, assumed that in addition to inorganic nutrients, organic nitrogen compounds may also be utilized by the mycorrhizal fungi. Hatch (43) rejected this assumption. However, he produced no experimental evidence to explain why the mycorrhizal hyphae should produce proteolytic enzymes only in saprophytic stages but fail to do so in mycorrhizal associations.

MacDougal & Dufrenoy (61 to 65) made extensive cytochemical studies of ectendotrophic pine mycorrhizae (*P. radiata*) under natural conditions and in experimental cultures of isolated segments of mycorrhizal pine roots which were capable of extended existence. They obtained evidence which indicates that the mycorrhizal hyphae act as absorbing organs capable of taking up from the soil all necessary nutritive elements. These elements are metabolized in the hyphae and then translocated to the cortex of the higher partner. Nitrogen was concluded to be absorbed not only in inorganic but also in organic form. The hyphae in pine mycorrhizae were found to be rich in proteids, polypeptides, and amino acids, the latter being assumed to be translocated to the root cells.

There is also experimental evidence which indicates that the fungal associate absorbs not only inorganic nitrogen but also organic nitrogen from

the soil. All tree mycorrhizal fungi studied are capable of utilizing organic nitrogen in pure culture. According to the findings of Melin *et al.* (79, 81, 86, 93), mixtures of amino acids are even better sources of nitrogen than ammonium nitrogen, the fungi being partially heterotrophic for some amino acids. It seems reasonable to assume that the same situation exists in nature. Recently Melin & Nilsson (84) demonstrated, by using the isotope technique mentioned above, that hyphae of *Boletus variegatus* in mycorrhizal connection with pine seedlings in pure culture, were able to transfer nitrogen from N^{15}-labelled glutamic acid solution to pine seedlings. Since the evidence indicates that glutamic acid is not deaminated by this fungus, the acid may have been translocated from the mycelium to the root cells in organic form.

MacDougal & Dufrenoy (64, 65) stated that in their cultures of isolated mycorrhizal pine roots carbohydrates were also absorbed from the soil by the fungal associate. They assumed the same to be true of the attached pine mycorrhizae in nature. Since glycogen was the only carbohydrate recognizable in the hyphae concerned, phosphorylated glycogen was regarded as the source of carbohydrate which was transformed to glucose and starch in the cells of the cortex. A carbohydrate supply to pine by its fungal associates was also concluded by Young (144, 145).

The observations of MacDougal & Dufrenoy seem to show that under certain conditions carbohydrates could be supplied to plants with ectotrophic and ectendotrophic mycorrhizae by their fungal associates. It seems reasonable to believe that in this case the mycorrhizal partner must be capable of utilizing cellulose in the soil as a source of carbon. However, since most tree mycorrhizal fungi appear to lack this ability, it may be considered likely that in nature their demands for carbonaceous material usually cannot be fully satisfied from the soil. In order to answer this question, however, further investigations are needed.

Concerning the nutritive relationships in orchids, Burgeff (15, 15a) obtained clear evidence that the fungal associates, as a rule, are essential for the higher partners, at least in holomycotrophic orchids and in holomycotrophic stages of green orchids. This view has been recently supported by Downie (19 to 22), MacDougal & Dufrenoy (64, 65), Mollison (98), and Vermeulen (139). In some cases it has been convincingly shown that nutrients are conveyed to the hosts by their mycorrhizal fungi, although different fungal species appear to be unequally effective. Hamada (37) demonstrated that rhizomorphs of *Armillaria mellea* are extremely efficient conveyors of food material, even from a distant source, to the tissues of *Galeola septentrionalis*. Burgeff concluded that all essential nutrients are transferred to the host by its fungal partner, and MacDougal & Dufrenoy by their microchemical studies also obtained evidence for this conclusion. In adult green orchids with well developed leaves, however, the supply of carbohydrates by the fungus may be of minor importance (99). It seems likely that the dependence of such adult orchids on the endophyte is correlated with certain environmental conditions. In some cases, particularly in rich soils, adult orchids were reported to be either free or deficient in fungal endophytes.

Partial deficiencies of vitamins and auxin in orchid seeds and young seedlings have been recently reported by Schaffstein (126), Noggle & Wynd (102), Meyer (91), Meyer & Pelloux (92), Bahme (3), Henriksson (44), and Mariat (73). Good germination and subsequent development of the seedlings occurred when nicotinic acid was supplied in the nutrient medium. In some cases the inability of the seeds to germinate was overcome by addition of pyridoxine, but then subsequent development was poor (44, 102). The growth of the seedlings of some orchids was also found to be promoted by thiamine (44, 73, 91). According to Mariat (73) pyrimidine has the same effect as thiamine. Meyer & Pelloux (92) and Mariat (73) demonstrated that auxins also are capable of stimulating germination and early growth. This observation seems to indicate that auxin deficiencies occurring in orchid seeds and seedlings may be overcome by, at least in part, the presence of the vitamins mentioned. There is evidence that in nature the necessary vitamins and auxin are obtained mainly from the invading fungal associate (73), although several nonmycorrhizal fungi, which probably produce these substances, also have been shown to induce good germination of the seeds under laboratory conditions.

Asai (1, 2), studying particularly the vesicular-arbuscular mycorrhizae in different plants, provided data suggesting that the host plant may be supplied with growth-promoting substances by its fungal associate. However, the experimental evidence on which this conclusion was based was rather meagre. MacDougal & Dufrenoy (64) concluded from their cytochemical investigations that auxin is produced in the fungal associate and translocated from there to the roots. In the case of pine mycorrhizae Slankis (130, 131, 132) obtained experimental evidence for this assumption. Although pine roots were capable of synthesizing auxin, a supply of various auxins in adequate amounts markedly affected them and caused a coralloid branching of their short roots. It seems evident that a profuse corralloid branching of pine mycorrhizae in nature indicates a relatively high transfer of auxin from the mycorrhizal fungus. It seems reasonable to assume that this supply of fungal auxin influences the metabolic acitivites in the root cells. Lindquist (57) and White (140) assumed that "growth substances" translocated from mycorrhizal associates favor the growth of the higher partner (conifers), whereas Björkman (7) found that single additions of 0.1 to 1 μg indoleacetic acid did not affect the growth of pine seedlings in pure culture.

Benefit for the fungal associate.—Möller (97) recently suggested that tree mycorrhizal fungi are mainly epiphytes on the roots. There is, however, no evidence for this assumption. Since the mycorrhizal fungus invades the host, it may be considered primarily as a parasite, although, contrary to many real parasites, it does not seem, as a rule, to produce substances toxic to the host. A mycorrhizal equilibrium will be established by defensive reactions of the host, mostly confining the fungus to definite parts of the infected organ. According to MacDougal & Dufrenoy (64) a barrier to the extension of the fungal associate is formed by layers or islands of cells containing antifungal substances such as oxidized catechol.

The defensive mechanism of the higher partner has recently been reinvestigated by Gäumann & Jaag (30) and Gäumann, Braun & Bazzigher (29). They confirmed the early finding of Bernard and others that the tissues of orchid tubers contain an unidentified substance which prevents the growth of the fungus. It was also shown experimentally that the antifungal substance concerned originates in the tubers as a true defensive reaction to metabolic products produced by the mycorrhizal hyphae. It was assumed that the same mechanism of defense occurs in a mycorrhizal root, preventing penetration of the whole root by the fungus. Mikola (93), demonstrated that living birch leaves and pine needles contain antifungal substances retarding growth of tree mycorrhizal fungi.

It is generally believed that either the growth or reproduction of the fungus is favored by the mycorrhizal association. Romell (116, 117, 118) presented added evidence for the view that the obligate mycorrhizal Basidiomycetes of conifers are able to produce sporophores in nature only when living in connection with their higher partners. In agreement with Frank and others he assumed that tree mycorrhizal fungi absorb soluble carbohydrates from the roots. They were, therefore, considered as energetically parasitic on their host trees. This idea has recently been strongly supported by Björkmann (7, 10). This hypothesis may be true in the case of ectotrophic and ectendotrophic mycorrhizae of trees. However, it cannot be valid for carbon-heterotrophic plants such as *Monotropa*.

Concerning plants with endotrophic mycorrhizae such as orchids, there is no evidence that the fungal associates obtain carbohydrates from their hosts. At least in the case of holomycotrophic plants and holomycotrophic seedlings of green plants, it seems reasonable to assume that the mycorrhizal fungi absorb the necessary carbonaceous material from the soil (15).

As mentioned above, the fungi of ectotrophic and ectendotrophic mycorrhizae are mostly deficient for certain B vitamins, just as are many purely saprophytic soil fungi. However, different species and even different fungal associates of the same host species have different demands in these respects. This indicates that in nature the main source of these metabolites for the mycorrhizal fungi may not be the host but the soil in which qualitative and quantitative differences in this respect may occur. The above mentioned results of cultural experiments with tree mycorrhizal fungi using excised pine roots as substratum, suggest the possibility that those associates obtain some unknown growth-stimulating metabolites other than B vitamins from the roots. More extensive investigations concerning this matter should be undertaken. In the case of orchid mycorrhizae it is assumed that the fungus obtains one or more unknown nutrilites from the root cells (139). The experiments of Francke (32) also seem to indicate that the fungal associate of *Monotropa* might obtain accessories stimulating the rate of metabolism from its host.

Conclusion

Summarizing the data so far there is clear evidence that mycotrophy, in

orchids as well as in forest trees with ectotrophic and ectendotrophic mycorrhizae, normally represents a state of equilibrium, constituting a functional unity in the sense of Gregory (136). Recent investigations have conclusively confirmed that the tree mycorrhizae concerned are nutrient-absorbing structures, which in certain soils are more effective than unaided roots. The multiple mycorrhizal habit in trees suggests the possibility that different species of fungal associates may be unequally effective in their contribution to the nourishment of the higher partner. From a practical point of view it may be of great value to get precise information on the influence of different species of mycorrhizal fungi upon the growth of seedlings and trees under different environmental conditions. Possible differences in this respect are still to be discovered.

Orchid mycorrhizae have, no doubt, a similar function. Comparing these with pine mycorrhizae, MacDougal & Dufrenoy (64, 65) produced added evidence that there are great similarities in the nutritive relations between the associates in both cases although their morphological engagements are widely different. Concerning other types of endotrophic mycorrhizae such as the vesicular-arbuscular ones, there is still little experimental evidence bearing on the nature of the physiological relations between the associates. It is hoped that recent successful experiments mentioned above will lead to a deeper understanding of this widespread root infection.

Many problems regarding physiological relations in mycorrhizae remain to be investagated, and much detailed work on various mycorrhizal associations and their partners is required before a clear picture will be obtained of the metabolic processes in these structures. Other aspects in need of investigation are those concerned with mycorrhizal activities in relation to activities of saprophytic soil microorganisms and with the survival and the life of mycorrhizal fungi outside their hosts. Studies are also required concerning the nature and causes of possible disturbances of mycorrhizal equilibria under natural conditions.

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THE EFFECT OF TEMPERATURE ON PLANT GROWTH[1]

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In spite of the extensive work on the effect of temperature on plants in general, there are very few reviews on this subject as a whole, the most extensive being that of Belehradek (7). The ecological effects of temperature are discussed by Daubenmire (35), Whyte (153), and Walter (139). Temperature, along with light, humidity, etc., belongs to the important factors in the environment of plants. It is also an insufficiently appreciated tool for research, for in many cases the processes of growth can be differentiated by their temperature responses. A good example of this fact is the photosynthetic process, in which temperature affects a chemical process at high light intensities and a diffusion process at low light intensities when the photochemical process becomes limiting.

The temperatures at which most physiological processes go on normally in plants range from approximately 0°C. to 40°C. Very high and very low temperatures cause injury effects, which are a different matter, have already been discussed in previous volumes (64, 75), and need no further elaboration (see also 81). We can distinguish between direct temperature effects on physiological partial processes, which allow us to draw conclusions about the physico-chemical processes involved, and the different effects of temperature on the organism as a whole. There are direct and delayed effects, and effects of fluctuation in temperature. For practical purposes we can subdivide the subject still further to effects of temperature on *a*) the early stages of growth (germination, vernalization), *b*) during the plant's maturity, and *c*) on the dormant stages.

Almost half of all published papers in plant physiology could be quoted in this review, for all contain references to temperature, if only to describe experimental conditions. Actually, only the few in which temperature response is treated as a major problem will be discussed; this should give a bird's-eye view of the whole.

The effects of temperature on the individual life and growth processes must be known in order to understand the effect of temperature on plants as a whole. Cell growth, especially cell elongation, has a high Q_{10} which indicates that this is a chemically rather than physically controlled phenomenon (26). The growth of excised plant parts has been studied at different temperatures. The optimal growth temperature for tomato roots was found to be 30°C. (150); for cotton roots, it was 25°; for corn and

[1] The survey of literature pertaining to this review was concluded in November, 1952.

sunflower roots, 20°; and for pea roots, 10°C. (43). For callus tissue, these values were 24° to 28° for sunflower, 32° for tobacco (58, 110). Since no other plant parts grow well when excised, we have no data for, say, the effect of temperature on excised leaf growth; however, 25° is the optimal temperature for leaf expansion in *Cucumis* (47). The optimal night temperature for stem elongation of tomato plants is 18° when the whole plant is subjected to this temperature, but the growing zones elongate much faster when they by themselves are exposed to 26°C. (142). These data show that optimal temperatures for growth of plant parts are, in general, fairly high and usually lie at or above 25°.

Respiration is affected much the same way as any other chemical reaction with a Q_{10} of from two to three, up to a limit of 30°; at 32 to 35° the rate decreases, with a marked time factor, due to denaturation of the protoplasmic proteins. This denaturation has a Q_{10} of about seven. Went (142) had attributed the low optimal night temperature, which we find in so many intact plants, to a Q_{10} of less than one for the process of translocation of products of photosynthesis within the plant. Evidence from ringing experiments and determinations of the sugar content of leaves in the morning (142, 148) showed that translocation of sucrose decreased with increasing temperature above 10°C. This was confirmed in further experiments in which the effects of temperature on the translocation of sugar in tomato stems was measured by the effects on the bleeding activity of the roots. These experiments also indicated that translocation decreases linearly with increase in temperature from 2° upward (149). Hull (61) was able to demonstrate this phenomenon in several other ways. Translocation of radioactive sugar at 2° was as fast as or faster than at higher temperatures. Hewitt & Curtis (57) did not confirm this conclusion. When whole plants of tomatoes were subjected to different temperatures, a decrease in translocation rate with increasing temperature was found only above 25°C.

The effects of temperature on the plant are largely mediated by their effects on chemical reactions. In only few instances has the biochemistry of the plant been studied in relation to temperature although in many cases biochemical differences have been attributed to temperature and other climatic factors. The content of aromatic compounds in the tea plant is, e.g., different according to the altitude at which it is grown, and the lower the growing temperature, the more aromatic the tea is (84a). The same was observed in the Earhart Laboratory for strawberry fruits: at lower growing temperatures the berries were more aromatic.

In many cases plants grown at a low temperature have a higher sugar content; in fact, frost hardiness is correlated with high sugar (75). In the case of the potato, the biochemical background of this phenomenon was investigated by Meeuwse (82) and Arreguin-Lozano & Bonner (3). The latter found that hydrolysis of starch at low temperatures was correlated with high phosphorylase activity; at higher temperatures an inhibitor is

formed for this phosphorylase. Meeuwse (82), on the other hand, found a powerful phosphorylase stimulator in potato juice.

In tomatoes it was found that the sucrose content of leaves was high after both cool (8°C.) and warm (26°C.) nights, largely because growth is reduced at those temperatures (148). Algera (2) made a very detailed study of the carbohydrate metabolism of tulip and hyacinth in relation to temperature treatment of the bulbs. He found a direct effect, which was reversible, of low temperature on the conversion of starch into sucrose, and therefore the promotive effect of lower temperatures on subsequent growth could not be explained by an increase in nonreducing sugars. There was a good correlation between growth and reducing sugars:

> . . . the velocity of the development of the plant at different temperature treatment is almost exactly parallel to the changes in the concentration of the reducing sugars induced by the temperatures.

Yet it should be recognized that these changes may well have been a result of growth, since the vacuoles of the elongating cells contain largely reducing sugars (137). Algera (2) leaves open the possibility that temperature treatments induce the formation of specific growth factors.

Rubber synthesis in the guayule plant (*Parthenium argentatum*) occurs optimally at 4°–7° night temperature, and above 15° very little synthesis takes place (16). Those same conditions favor the formation of seeds of good quality (151).

There are many studies in which differential effects of temperature on respiration and photosynthesis are found; whereas at lower temperatures the ratio of photosynthesis to respiration is over 10, at higher temperatures respiration is increased relatively more and thus low P/R ratios are found (38, 113, 138). This might explain, at least partly, why many plants grow better in temperate regions than in the tropics.

The effects of temperature on the growth of plants can be assessed to a limited degree by interpretation of field data. For this purpose correlation coefficients are calculated for the relationship between yield and average temperatures at different growth stages of the plant in question for a series of years and a number of localities with sufficiently differing climates. As an example can be quoted a study on the effects of rain and temperature on seed production of carrots (96). It was found that whereas temperature around May first and June tenth was positively correlated with seed production, in the middle of March and the end of August it was negatively correlated. By sowing plants at different dates the effects of temperature could be calculated by Geslin (45) for wheat. Similar correlations were calculated by others (89). The effects of temperature have also been represented in terms of "heat sums" or degree-days. This procedure, which was used extensively by de Candolle in 1854 (25), is now being used, e.g., by Nuttonson (88) to compare climates. The average temperatures for all days of the

growing season of a crop are added and thus give the heat sum in degree-days. Usually only the temperatures above a certain minimum (either 0° or 5°C.) are added (135). This procedure presupposes a linear relationship between average temperature and growth, which definitely does not exist. Over a limited range of temperatures such a relationship may hold by approximation, and thus in fairly even climates the heat sum may express the growing conditions over a whole growing season for certain plants, such as peas and wheat, but for plants like tomato and potato, where predominantly night temperatures control fruit and tuber formation, heat sums as used currently are useless to describe their optimal climate.

Still another approach to the analysis of field data is the use of the crop-log, as introduced by Nightingale for pineapple (33) and Clements (31) for sugar cane. Any character which can be measured currently, such as growth rate, nitrogen content of leaves, rate of leaf emergence, etc., is correlated through the multiple regression method with any of the meterological conditions prevailing between successive measurements. Thus strong correlations were found with both maximum and minimum temperatures for growth rate and rate of leaf emergence in sugar cane.

Went & Cosper (147) have reversed the procedures described above and, by using the laboratory response of tomato varieties to temperature as a basis, showed that their field behavior, as far as fruit set and fruit production was concerned, actually paralleled their greenhouse responses. It was shown that a maximum in fruit production was reached in Stone and Beefsteak tomatoes following, always after about 40 days, a period of five successive minimal night temperatures above 15°. This relationship did not hold for the Earliana tomato, which can set fruit at somewhat lower night temperatures. Since then several other investigators (152) have confirmed the controlling effect of night temperature on fruit set of tomatoes in the field.

The most extensive work of the effects of temperature on plant growth was carried out by Blaauw and collaborators. This work has been partially summarized (97, 98, 145). Their basic work is found in (13a, 52, 79). In these investigations it was shown that throughout the development of hyacinth and tulip, different optimal temperatures follow each other in succession. This is due to a succession of morphological and physiological processes, each with its own optimal temperature. Thus flower and leaf initiation in the tulip has an optimal temperature of 20°, whereas stem elongation is optimal at 13°. Before the actual elongation occurs there is a so-called delayed optimum of 8° to 9°C. which prepares the bulbs for most rapid development during the actual period of stem elongation which follows one or two months later. In the hyacinth, leaf and flower initiation occur optimally at 34° and 25°C., whereas stem elongation occurs best at 13°. Root growth in these same plants is optimal at 27°C. Since then, much more work has been carried out on other bulbous plants. The growth of narcissus

was followed in relation to temperature, but here it was found that flower initiation already has occurred by the time the foliage of the previous year dies (15, 40, 119). When the bulbs are treated at too high a temperature during storage, the flower primordia will die. In irises a more complicated temperature response was found (13a).

In the case of onions, their practical growing requires that they do not flower, because otherwise the bulbs are useless. When onion bulbs are stored 23° to 28°C. no flowering occurs at all, whereas with storage at 9° to 13°C. flowers are initiated (14). Heath (53, 54) confirmed these results and, in addition, found an interaction of day length and temperature in the life cycle of the onion. Emsweller & Pryor (41) are working on the flowering of Easter lilies and have shown that when the lilies are stored at low temperatures, flower initiation occurs.

Whereas, in these bulbous plants, temperature is a major factor which controls their flowering, in the so-called long- and short-day plants, which are mostly annuals, flowering is controlled largely by daylength, according to a very large number of investigators (71). However, in practically every case, temperature has a modifying effect on the photoperiod response, and in a number of cases temperature seems to be even more critical than daylength. Roberts & Struckmeyer (103, 104, 118) have shown that these temperature effects on the photoperiodic response are very widespread. These effects were investigated in greater detail by Parker and co-workers (91, 92, 93) for the Biloxi soybean.

Bünning's theory of photoperiodic response assumes that plants have an innate or autonomous rhythm of approximately 24 hr. During one cycle the plants pass through a photophylic and a skotophylic phase, in which light has or does not have an influence on flowering. An attempt was made to tie this rhythm in with the well-known 24 hr. cycle in cell division, which occurs especially in plant parts containing chlorophyll (22). Brown & Rickless (21) did not find an effect of temperature on the rate of mitosis in Cucurbita roots, but later Brown (20) found a very strong temperature effect on the length of all mitotic phases in the roots of peas, about halving their duration for 10° rise in temperature. This would argue against Bünning's theory, but these temperature effects should be investigated in shoot apices before they are considered to be conclusive in connection with this theory, since also Rotta (105) found no rhythm in mitoses in *Vicia faba* roots.

Since 1933, Thompson and collaborators (67, 107, 108, 121, 122, 123, 125, 126) have been investigating the interrelationships between temperature and length of day in growth and flowering of biennial vegetables. In all of these cases, they found that an initial treatment with fairly low temperature was necessary to make plants respond to a long photoperiod afterwards (celery, carrots, spinach, and different kinds of *Brassica*). This is now being developed in further detail by Wellensiek & Verkerk (140) and

Stokes (116). These relationships were worked out in great detail for *Hyoscyamus* and for beets. Most of this work has been reviewed by Lang (71).

The effects of temperature on the development of a plant as a whole were studied first under constant temperatures. In higher plants most work was carried out with seedlings developing from large seeds because they do not need much light for their early growth. Sachs (106) found very high optimal temperatures, for maize, beans, and squash 34°C, for wheat and barley 29°C. This work was confirmed by many later investigators. This led to the conclusion that most plants were adapted to much higher temperatures than they are usually exposed to in nature, and that, except in the tropics, there was no close correlation between normally occurring temperatures in nature and optimal temperatures of the organisms living under those conditions. This view is still persisting (127).

Among microorganisms we find a very wide range of optimal temperatures, from 15° or lower (*Phacidium infestans*, the snow-blight fungus) (13), named hypothermal, to thermophilic bacteria (62) and CYANOPHYCEAE, with optimal temperatures as high as 80°, named hyperthermal (*Synechococcum ecimius*, *Phormidium geysericola*) (136). It may be significant that all hyperthermal organisms are without typical nuclei, and they must have rather remarkable proteins which are not denatured at those high temperatures. More recent work on the temperature effects in higher plants has not been confined to constant temperatures, and this has led to new developments.

Thermoperiodicity is the term used for responses of plants to cyclic temperature variations. Chouard (29) in a most interesting review has distinguished these responses as annual and diurnal thermoperiodicity. Annual thermoperiodicity is found in deciduous trees and shrubs and in most plants with underground storage organs in temperate climates, such as bulbous and rhizomatous plants. In all of these plants, development is possible only when periods of high temperature (during summer) are alternating with periods of low temperature (winter). In general, these cycles are approximately of a year's duration, but can be shortened or lengthened about 50 per cent by proper succession of temperature. When peaches, pears, and similar deciduous plants are not subjected to low temperatures near the freezing point during their dormant season, then they will not come out of dormancy. Recent work by Bennett (11) has shown that this low temperature treatment does not necessarily have to be given continuously, but that daily fluctuations at which temperatures near freezing are reached every night are also effective, although much less so than the continuous low temperature.

The annual thermoperiodicity in tulips and other bulbous plants has as its primary cause the succession of different developmental stages, each with its optimal temperature. There is no stage of real dormancy in the tulip or hyacinth. But in deciduous trees the annual thermoperiodicity is

brought about by a stage of dormancy, during which no visible morphological changes occur, and from which plants can be awakened only by a period of cold treatment. The biochemical basis of the breaking of dormancy is still very obscure and is attributed to either starch hydrolysis at low temperatures, removal of inhibitors from the buds or branches (48, 55, 56, 83), or development of growth-promoting substances in the buds (12, 49).

The actual temperatures and length of chilling required to break the dormancy of buds are not accurately known in most plants. In the literature we find some data on grapes (80), on *Helianthus tuberosus* (114), on pears (10), and on deciduous trees (59). These temperatures have been determined in minute detail by Vegis (133, 134) for winter buds of Stratiotes.

In other nondeciduous plants there still may exist a yearly cycle. Howland (60), for instance, found that in *Daphne cneorum* flower buds are formed at high temperatures but require a low temperature for their further development. This low temperature is about 10° to 13°C. Similar behavior was found by Bonner (17) in the camellia. He found that flower buds were initiated at high temperatures, around 25°C. The latter part of their development and the opening of their flowers occurs optimally around 10° to 15°. This behavior synchronizes camellias with the yearly climatic cycle, causing them to flower in late winter. Vegetative growth occurs best at higher temperatures.

Diurnal thermoperiodicity concerns the adaptation of plants to the daily cycle of higher day and lower night temperatures. The response of plants to these daily cycles in temperature was known by greenhouse growers, and was investigated in detail in the air-conditioned greenhouses of the California Institute of Technology. Diurnal thermoperiodicity was studied in greatest detail in the tomato plant (141, 142, 143, 147). Because during daytime photosynthesis is the predominant process, and has a fairly high optimal temperature, the optimal day temperature is approximately 25°C. Most of the growth of tomato plants occurs during night and, consequently, the night temperature is correlated with the growth rate. In young plants, the optimal night temperature lies above 25°C., but as the plants grow older, the optimum shifts to below 20°C. This same phenomenon was found for *Capsicum* (39), where it actually comes below 10°C., and for tobacco (24). This gradual lowering of the optimal night temperature makes it possible to synchronize the growing of these plants with the climate by letting the latter part of their development coincide with the lowering night temperature in autumn. In many plants the optimal temperatures during early growth are so high that they can be obtained only in greenhouses, and this explains the general practice in temperate climates of starting seedlings in the greenhouse and transplanting them into a garden or field when the outside temperatures are sufficiently high to enable the plants to grow there as they have reached a lower optimal temperature.

This difference in optimum day and night temperature already explains

why heat sums cannot be used to express the relationship between climate and plant growth, because the heat sum integrates the temperature throughout the day and night without any differentiation. Besides, it does not take into account the shift in optimal temperature during the development of the plant. Finally, it also does not take into account that the optimal day and night temperatures are strongly dependent upon the light intensity. It was found that at lower light intensities, the optimal temperature is decreased. In young tomato plants, the optimal night temperature shifted from 26° to 8° when the light intensity was decreased from full daylight to 200 foot-candles (143). This same behavior was found in sugar cane (72).

Not only vegetative growth, but also fruit production is thermoperiodic in the tomato plant, with the optimum lying at about the same temperature. Different varieties reach different optimal temperatures. Whereas the optimal night temperature for Western tomato varieties are about 18°C., greenhouse varieties grow optimally at about 15°, and Eastern varieties reach an optimum in between these two values (143). Excised tomato fruits have a slightly higher optimal temperature (20°C. for the John Baer variety) (74). In the case of tobacco, very big differences were found in the optimal temperatures for different varieties (24).

The thermoperiodic behavior of a number of wild plants was investigated too (76). It was found that many of these such as *Baeria chrysostoma* and *Phacelia parryi* would die at night temperatures above 25°C. The latter plants are spring annuals, and, therefore, in nature they are never subjected to high night temperatures. Work now in press (32) adds a number of plants which are strongly thermoperiodic in their behavior. *Bellis perennis*, which is a weed in lawns during the spring months in southern California and in summer in more temperate climates, grows best at night temperatures below 10°C. and will die when the night temperatures are above 15°C. *Matthiola incana* will not flower when either day or night temperature is above 18°C., but its vegetative growth is unimpaired by the higher temperatures. A long photoperiod can offset the low temperature requirement for flower induction. Thus in summer, the plant will behave like a long-day plant, whereas in winter, it is indeterminate.

Effects of temperature on growth and sugar content of sugar beets were studied under controlled growing conditions by Ulrich (131, 132). He found a very pronounced optimum for root growth around 20°C, which varied with variety. This agrees with Pickett (95), who concluded this from field tests. The G. W. 304, developed for the cooler Colorado and Wyoming climate, was much more efficient at the lower temperatures, whereas the U. S. 22/3, produced in warmer Utah and California, was relatively more efficient at higher temperatures. The effect of temperature on the sugar content of the beets was very different: instead of showing an optimum, the sugar content is negatively correlated with temperature, so that at the lowest temperatures the sugar percentage is highest. In this respect other

studies should be mentioned (90, 117) in which the effects of low temperature on "bolting" (flower stalk production) in sugar beets were studied. A treatment of the beets at 6° to 9°C. was optimal for this process. Peas and beans seem to be very sensitive to temperature, but little is known about them in this respect. High day temperatures influence the set of pods more than any other single factor: for every degree of maximum temperature above 24°C. there is a 4 per cent reduction in set (37). A number of investigations (9, 78, 111, 120, 154) deal with temperature responses of grasses. Whereas *Bouteloua gracilis*, *Paspalum*, *Cynodon*, and *Sorghum vulgare* grow well at high temperatures, *Dactylis*, *Festuca*, *Agrostic*, *Poa*, and *Phleum*, all northern pasture grasses, grow best at lower temperatures (21° to 13° alternation). Wheat (85, 94) and oats (50) were found to respond well to cool temperatures.

In many cases, the effect of temperature on plant growth was attributed to effects of the soil temperature (101). It was found that for roses the optimal soil temperature was 16° to 18°C. (68, 109). Soil temperature affected primarily root growth and had relatively less effect on top development. In apples and peaches Nightingale (86) found that at the higher temperatures the newly-formed roots were very deficient in carbohydrates, whereas the older roots contained high concentrations of starch at 32° to 35°C. Daubenmire (34) found that in the Rocky Mountains the altitudinal distribution of plants was not primarily controlled by the high temperature tolerance of their roots, but rather by their resistance to the length of drought period.

Temperature has been investigated extensively in connection with germination. Three distinct temperature effects have to be distinguished: (*a*) the direct effect on germination, (*b*) the effects on dormancy, and (*c*) the indirect effects on further development (vernalization). It is impossible to give a complete summary of the direct effects of temperature on germination within the limits of this review. Only a few general points will be brought out. In many plants germination occurs only within a rather narrow range of temperatures, which have to be known if successful germination tests are to be carried out. Cholnoky (27, 28) has published a list of 389 species with the optimal germination temperature of their seed. This varies from 12° for winter germinators, like *Linum*, *Linaria* and *Delphinium*, to 20° for summer germinators (*Perilla*, *Phlox*, *Tropaeolum*), or even higher for tropical plants (*Ageratum*, *Amaranthus*, *Coleus*), whereas many weeds and grasses need a very wide daily range of germination temperature (12° to 33° for *Achillea*, *Physalis*, *Specularia*, *Viola*). In nature the establishment of plant communities can also be determined by the temperature of germination. In the desert, for instance, winter annuals germinate only at low temperatures, whereas summer annuals require much higher temperatures (4, 144, 146).

The optimal temperature for germination is markedly affected by previ-

ous treatment of seed (23) or its age (129). It was found that in several Brassica species the optimal temperature for germination increased from 5 or 10°C. to 20 to 25° in the course of seven months storage. In Digitaria it was found that the speed of germination at all temperatures was increased by pre-chilling at 3°C. (130). Experiments of Borriss (18) give some indications as to the mechanism of the temperature dependence of germination. He showed that many seeds germinate better when leached with water or when placed on charcoal or in soil (both inactivating or absorbing inhibitors). Seeds of, e.g., *Vaccaria* do not germinate at 20° unless placed on charcoal, but do germinate at 15° on filter paper. As they grow older they become less temperature-dependent. Therefore it seems as if at unfavorable germination temperatures more inhibitors are present and that this gradually disappears with age. It was shown that lettuce seeds can germinate at temperatures outside of their normal range when treated with thiourea (124). In this connection it should be pointed out that Lehmann (73) long ago found that dark-germinating seeds, like *Nemophila*, *Phacelia* and *Phlox*, will germinate both in dark and light at cool temperatures, are typical dark germinators at 21 to 24°C., and at 31°C. germinate neither in light nor in darkness. In light-germinating seeds like *Epilobium* at 31° germination occurs both in dark and light, but at 21° germination is much better in light.

This brings us to the effects of temperature on the dormancy of seeds. Peach seeds will grow without chilling treatment when embryocultured, whereas, when left intact they do not germinate at all without a low temperature treatment. But the seedlings then do not elongate unless they are subjected for some weeks to 5°C. or are kept at 21 to 24° in continuous light (70). Barton (6) found in many LILIACEAE seeds a dormancy for germination, which could be overcome by low temperature treatment, and also an epicotyl dormancy. When *Polygonatum* or *Trillium* seeds, e.g., have germinated, then only their roots develop, until the seedlings have been subjected to another period of low temperature, when the epicotyls will start to grow. This cold requirement was variously explained by the removal of inhibitors by the low temperature treatment (99) or was attributed to mobilization of carbohydrates in the endosperm at the low temperatures (115).

Temperature also has a very strong influence on the longevity of seeds. When dry they can be stored at a very wide range of temperature without losing viability (5, 8, 65, 102, 128), but when their moisture content is higher, viability disappears within a few months at room temperature or higher, whereas at 0°C. they may remain viable for years. In many cases seeds do not germinate or germinate poorly when subjected to a constant temperature. In such seeds best germination occurs when for seven hr. daily they are subjected to a much higher temperature than during the night (27, 30, 51, 69, 84).

Temperature influences color intensity in many flowers. In roses, e.g.,

the red or pink color becomes more pronounced at lower temperatures. In Briarcliff roses color fades when growing at 35°C., at 27° they are still somewhat pale, but at 18° color and vigor of plants is normal (100). As in the anthocyanin color development in leaves in autumn, this is attributed to the higher sugar content at low temperatures. In other flowers the color pattern is temperature-dependent. Spots and markings in flowers of *Calceolaria hybrida* and *Viola tricolor* are suppressed when the buds are subjected to 30°C. or higher while they are 4 to 15 mm. long, but in other flowers, like Gloxinia, the markings are not influenced by temperatures as high as 40°C. (42).

The literature on low- and high-temperature damage to plants was discussed in previous volumes (64, 75); it was shown that this was largely explainable on the basis of either freezing or coagulation damage. We have to consider now for a moment death caused by low temperatures, well above the freezing point. This occurs in a number of tropical plants (*Coleus*, *Saintpaulia*, *Palisota*, etc.) and cannot be attributed to ice formation. It is associated with a loss of sugar and other changes in tissues. By growing these plants for a long period (two months) at 12° they could be hardened against the above-freezing damage (112). Cold resistance of tropical plants was observed as result of heating failure (46) or war damage.

Many plants, especially spring annuals, die when they are subjected to night temperatures of 25° or over, in spite of the fact that they can well support day temperatures of 25° or over (*Baeria*, *Phacelia*) (76). Therefore death cannot be attributed to heat damage to the protoplasm, the explanation used for disappearance of luminescence in bacteria even at fairly low temperatures (below 30° for *Photobacterium phosphoreum*) (63). Nor is death due to pests or diseases, for Loo (77) found that *Baeria* died at 26° night temperature when grown under completely sterile conditions, even in the presence of sugar.

There are numerous miscellaneous effects of temperature reported. It influences greatly susceptibility of plants to diseases. Toxicity of 2,4-D was found to be greater at high temperatures (66), but when 2,4-D was applied to young bean plants their leaf expansion was inhibited to the same proportion at all temperatures (19). Guayule was more susceptible to oil applications for the killing of weeds at 10° and 37° than at 21° and 27°C. (9). Salt tolerance of plants is affected by temperature, too (1). Whereas tree ring growth is usually correlated with rainfall, it was shown that temperature also affects their width (36, 44). The sexuality of the flowers of CUCURBITACEAE was found to be strongly influenced by temperature (87): high temperatures keep plants in the male phase, whereas low temperatures cause a very rapid transition of the plants to the female phase.

Zurzycki (157) has investigated anew the effects of temperature on protoplasmic streaming in Elodea leaf cells, in which he confirmed Belehradeks formulation that the velocity V depends on temperature t as fol-

lows: $V=(a)(t^b)$ in which $a=0.51$ and $b=0.81$. But van't Hoff's law did hold when the streaming rate in these leaf cells was measured in darkness. Another protoplasmic response, the phototactic movement of chloroplasts, was investigated in relation to temperature (156). Movement from epistrophe (all chloroplasts facing light) to parastrophe (chloroplasts along walls parallel to light), brought about by increasing light intensity, had a Q_{10} of 1.57, whereas the reverse reaction, as a result of lowering light intensity, had a Q_{10} of 1.00. In a subsequent investigation Zurzycka (155) found that the response to increasing light intensity seemed to be mediated by light absorbed by chlorophyll, whereas the reverse reaction was related to carotene-absorbed light.

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PHYSICAL PROPERTIES OF PROTOPLASM[1]

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Introduction

This subject has been reviewed previously by Taylor (79) in 1943 and Seifriz (70) in 1945. An excellent review of protoplasmic flow and related subjects by Seifriz (69) has also appeared in a recently published symposium.

This review considers primarily the literature of the last three years following the 1948 English edition of Frey-Wyssling's book (32). The problems are discussed mainly from a botanical point of view; however, some purely zoological works will be referred to because of the similar properties of animal and of plant protroplasm.

The main difference between the animal and the plant cell is the existence of a large vacuole in the latter. In the mature plant cell there is only a very thin layer of cytoplasm covering the cell walls and the vacuole comprises most of the cell contents. In a treatment of the physical attributes of the protoplasm, especially permeability and viscosity properties, this must be emphasized, since one can get different results by using the same experimental methods on the two kinds of cells. This is particularly obvious when viscosity measurements are made by the centrifugation and plasmolytic methods, respectively.

It is always a difficult task to determine whether or not a certain problem belongs to the subject in question. In this review I have included some investigations of plasmolytic phenomena and related subjects. Although these are not direct examples of the physical properties of protoplasm, I have given an account of the most important facts, because the course of plasmolysis is often intimately connected with protoplasmic consistency.

The Consistency of the Protoplasm

Viscosity under normal conditions.—By centrifuging cells of *Nitella* in a microscope-centrifuge, Breckheimer-Beyrich (13) was able to study the course of the displacement of the cell contents. The cytoplasmic layer slides down along the cell walls to the distal ends of the cells leaving but a thin layer of cytoplasm in which the chloroplasts are located. This appears to demonstrate the looser consistency of the inner parts of the cytoplasm compared with those lying close to the cell walls. Under the influence of centrifugal forces of about $625 \times g$, the chloroplasts move only in cells showing necrotic symptoms. The inner parts of the cytoplasm were moved by means of much lower forces ($30 \times g$). As shown by Breckheimer-Beyrich (13) and Küster (49), the cytoplasm in the centrifugal ends of the cells separates

[1] The survey of literature pertaining to this review was completed in May, 1952.

into two layers, the most distal part of which is as transparent as glass. The other layer, containing the microsomes and other particles of the cytoplasm, is heterogeneous. At the end of centrifugation, the cell contents begin to move back again (4, 5, 48, 85). With prolonged centrifugation, the displacement is accelerated indicating a decrease of protoplasmic viscosity as a consequence of mechanical stimulation (13). A similar decrease in protoplasmic viscosity can be shown following the shaking of various species of plants (41).

Another type of displacement occurs wherein all the chloroplasts are immediately assembled in the centrifugal ends of the cells upon the application of low centrifugal forces (26, 74, 88). This indicates a rather low protoplasmic viscosity of the layer close to the cell walls. By treating the cells with agents which alter the viscosity it is also possible to obtain very high viscosity values. Under normal conditions the protoplasm of terrestrial plants has a greater viscosity than has the protoplasm of submerged species.

Viscosity as influenced by various agents.—Protoplasm is very sensitive to light as manifested by changes of protoplasmic viscosity [Virgin (87)]. Most of the experiments have been performed with *Helodea densa* protoplasm which responds markedly to light. The response is localized in the illuminated areas of the cells (87, 88); no transmission of stimulus has thus been established. Response is greatest to blue light; red phototropically inactive light has little or no effect. The viscosity of the protoplasm under natural conditions is never constant but fluctuates, showing a maximum during the night and a minimum during the day (73, 85, 88). These fluctuations cease after three days in complete darkness, but begin immediately upon reillumination of the cells. Strong illumination (>2200 lux) decreases the viscosity, medium intensities (2200–25 lux) cause an increase, and weaker intensities induce a decrease again. These changes resemble the positive and negative phototropic movements which are also dependent on the intensity of the light. Maybe such light-induced changes in protoplasmic viscosity are one of the primary reactions preceding the plant movements. Support for this idea is the fact that the absolute threshold value for the intensity during four hours illumination with white incandescent light is less than 0.001 lux. Also, the threshold value for the quantity of high intensity (22,000 lux) light for a short period during the autumn is about 6 lux seconds. The product law seems to be valid for these viscosity changes in the case of strong light intensities.

Seifriz & Pollack (72) showed that all substances having anesthetical properties also increase protoplasmic viscosity, while stimulating agents such as caffein, etc., cause the opposite effect. Protoplasmic streaming is stimulated by small amounts of caffein [Waris (89)]. Accompanying the increase in protoplasmic viscosity (gelatinisation) is a diminished respiration, electrical conductance, and redox potential as well as a lower metabolism in general [Seifriz (71)]. Previously, it was assumed that the effect of anesthetics is connected with their solubility in the lipoids of the plasma membranes.

There are, however, many substances which exhibit anesthetic effects although they are insoluble in lipoids. This makes the mechanism behind the anesthetic reaction, resulting in a reversible gelatinisation of the protoplasm, more puzzling. It may be viewed as gelatinisation, incipient coagulation, thixotropy, coacervation, or reversible protein denaturation. The only statement which can be made with a fair degree of certainty, however, is that the physical changes observed are caused by chemical reorientation. According to Diannelidis (26), an example of the effect of anesthetics in increasing viscosity is the decreasing rate of redistribution under the influence of a centrifugal force shown by cells treated with a solution of ethyl ether in water.

Various physiologically active substances have specific effects on protoplasmic consistency. According to Stålfelt (74), protoplasmic viscosity is maintained at some equilibrium value by means of chemically unknown substances occurring in all waters having had contact with the soil. The substances can be obtained from leaves, litter, and dead and living plant materials of various kinds by soaking them with distilled water. The distilled water itself markedly decreases the viscosity of the plasma.

Since the protoplasm consists partly of protein colloids, the viscosity of these constituents will contribute to the overall viscosity of the protoplasm. All factors having any influence on the viscosity of the protoplasmic proteins will also act on the protoplasm as a whole. Colchicine (55, 76) and β-indoleacetic acid (IAA) (35, 76) have a large effect on protoplasmic viscosity. According to Stålfelt (76) the viscosity increases in proportion to the concentration of auxin during the first hours of treatment and is later followed by a decrease. The reaction thus shows a certain reversibility. The highest viscosity values are obtained by using the weakest effective concentrations and only after a rather long period of treatment. In brief experiments the highest viscosity values are obtained earlier and are caused by higher concentrations of IAA. Evidently the optimum concentration of IAA varies with the duration of treatment. Consequently, an optimum concentration cannot be specified nor is there any fixed connection between the concentration of the agent in question and its physiological effect. Changes in the protoplasmic viscosity of *Helodea densa* could be obtained with concentrations of IAA lower than $10^{-12}M$ using the centrifugation method. Guttenberg & Beythien (35) found that the time for deplasmolysis is decreased when cells are treated with $10^{-7}M$ IAA. If increased over 10^{-4} M, the time is increased, too. The plasma in this case thus shows the same behaviour as in the experiment of Stålfelt just mentioned. The changes in time for deplasmolysis are interpreted as an effect of IAA on the permeability of the plasmatic membrane to water. It is thought that other growth-promoting substances might affect the protoplasm similarly. Growth inhibitors like coumarin and parasorbic acid have an opposite effect (35).

It is of interest to compare the influence of IAA, colchicine, and other active substances upon viscosity with their influence upon growth and es-

pecially upon the complex mechanism connected with mitosis. For these processes to follow a normal course the physical state of protoplasm, i.e., the viscosity, must not diverge too much from the normal state. If the divergence is excessive serious disturbances result in the mitotic figure. Simultaneous measurement of protoplasmic viscosity and the formation of c-tumours in the roots of *Allium cepa* shows a strong parallelism between the two phenomena (55). According to Northen (55) the decrease in viscosity is caused by a dissociation of proteins which are an integral part of the cytoplasm and of the nucleus. The dissociation of proteins of the spindle fibres is considered to be the primary cause of the cessation of mitosis in metaphase. The effect of oxychinoline on protoplasmic viscosity as well as on the mitotic figure is another illustration of the connection between the two phenomena [Stålfelt (76); Tjio & Levan (80)]. To this should also be added the long list of substances affecting normal mitoses (2, 22, 23, 24, 33, 46, 93). By means of the electron microscope, Sedar & Wilson (67) have succeeded in distinguishing the change in consistency of colchicine-treated chromosomes compared with untreated chromosomes. It is beyond the scope of this review to discuss the influence of different chemicals on the course of mitosis. A few studies may be mentioned, however, because they help to show the nature of the relation between the state of the protoplasm and the physiological processes in the cell. Connected with the suggested correlation between protoplasmic consistency and the course of mitosis is the c-mitotic action of certain narcotics [Ferguson *et al.* (30)]. The colchicine-induced formation of c-tumours during mitosis is an example of the so-called narcotic cell reactions common to a number of substances.

Toxic substances having an influence on mitosis can be divided into different groups according to their specific effects on different parts of the cell contents. Geissler (34) observed three types of poisons affecting different parts of the chromosome mechanism and came to the conclusion that there is one continuous chain of reactions which can be modified in different ways depending on the poison used, on its concentration, and on the physiological state of the cell. Since protoplasmic consistency changes very rapidly under natural conditions during mitosis [Katô (45)], it is quite natural that the same substances can have different effects at different times. The effect depends on the state of the cytoplasm. The strong poisonous effect shown by many herbicides seems to be partly caused by their influence on the protoplasmic state (Currier, 21) and by their influence on mitotic processes [Doxey & Rhodes (29); Nygren (57)]. Currier (21) studied the influence of 4,5-dinitro-2-secondary butyl phenol (DNSBP), pentachlorophenol (BCP), 2,4-dichlorophenoxyacetic acid (2,4-D), and *o*-isopropyl-N-phenyl carbamate on cells of *Anacharis* and *Allium* and other sensitive tissues, such as roothairs and the staminal hairs of *Tradescantia*, etc. All these substances have a pronounced effect on protoplasmic streaming as well as on protoplasmic viscosity, as indicated by plasmolysis. The effect can be divided into different types, namely, stimulation, reversible and irreversible injury,

and death. The first type, i.e., stimulation, is caused by concentrations between about 10^{-4} to 10^{-7} *M* (the highest for NH_4-DNSBP and the lowest for Na-2,4-D).

The action is manifested by an increase of the rate of streaming as well as by a general decrease in the firmness of the protoplasmic membrane, a decrease in the time for plasmolysis, and a more convex shape of the plasmolysed cells as compared to the control. The protoplasmic firmness increases when concentrations causing reversible injury are used. Irreversible injury is associated with a lack of protoplasmic streaming, very rapid or no plasmolysis, a marked leakage of vacuole pigments, and sometimes a shrinkage of the whole protoplast indicating a marked rigidity of the ectoplast. All these these departures from the normal behaviour of cells to plasmolytic agents give the impression that herbicides have an extremely pronounced effect on the state of the protoplasm. The effect of such substances [4-chloro-2-methylphenoxyacetic acid (Methoxone), 2,4-D, and others] on root tips of *Allium* are generally the same as those produced by colchicine (29, 52, 57, 64). Northen (56) earlier showed an effect of the vital stains methylene blue and neutral red on viscosity, using the centrifugation method. Cholnoky (14) found the same to be the case for the stain light green using the plasmolytic method. Effects of various dyes on the chromosome mechanism are reported by d'Amato (23).

Elasticity and thixotropic properties.—Further evidence for the elasticity of cytoplasm has been shown by Beams (4) and Virgin (88) by treating cells with varying centrifugal forces. If cytoplasm, with its contents of chloroplasts etc., is to be displaced under the influence of a centrifugal force, the latter must exceed a certain minimum value, its magnitude depending on the state of the protoplasm (88). If a cell is subjected to a centrifugal force below this minimum value, no change can be observed however long centrifugation is prolonged. Thus, the centrifugal force must exceed the elasticity of the maximally extended ectoplasm if the cell contents are to be displaced. The same is true of animal protoplasm. Small magnetic particles were distributed in the plasma of chick fibroblasts in tissue cultures. The rate of reorientation of the particles to an electromagnetic field of variable direction was dependent on the application of pressure to the cytoplasm, because of the latter's thixotropic properties [Crick (19, 20)].

Hysteresis and resistance to low temperatures.—The importance of the past history of the plasmatic colloidal system on its present behaviour—the phenomenon of hysteresis—may be illustrated by an experiment reported by Höfler (39). By cultivating different species of mosses in the Jungermanniales over sulphuric acid of increasing concentration, the hardiness of their protoplasm can be increased. A change in the protoplasm is brought about, which is unaffected by a subsequent shorter cultivation under more humid conditions. The protoplasm of all species, however, does not behave in the same manner. Some species have less resistance to violent desiccation than others. According to Kramer (47) the permeability seems to be de-

creased after desiccation. It is probable that desiccation brings about a shrinkage of the whole plasma gel and the decrease in permeability may be the result of a diminution of the micellar net work.

The protoplasm of some mosses also shows a very marked resistance to low temperatures. If the water content is less than 30 per cent, the mosses are able to survive immersion in liquid nitrogen for one week. With a water content of 60 to 70 per cent, all the cells die rapidly in spite of a fairly rapid heating to about 30°C. [Bequerel (6)]. A necessary condition for the survival of the plant cell though such treatment is very rapid cooling and thawing of the tissue in order that the state of the protoplasm is not changed. Otherwise, the "life structure" of the protoplasm will be damaged and the cells will die.

When living cells of *Allium cepa* are cooled with liquid air for 10 min. up to 3 weeks (6), they no longer plasmolyse after thawing, but, instead, the protoplasm of the dead cells loses water and forms a new colloidal gel by syneresis. On the other hand, living cells which have been plasmolysed before the cold treatment can be deplasmolysed (6). Killing, in these cases, is not by plasmolysis. Subsequent to the death of the cells the protoplasm coagulates by syneresis. Under certain circumstances, the phenomenon of syneresis is reversible and the cells are not killed. This is the case with the mosses of low water content mentioned above.

Protoplasmic streaming.—According to Virgin (86), there seems to exist some relation between the rate of streaming and protoplasmic viscosity. Streaming is faster in protoplasm having a low viscosity as compared with that having a higher viscosity. It has been possible by means of a microscope centrifuge to measure the motive force of the plasma flow in *Helodea densa.* When the leaves of this plant are centrifuged the greater part of the cytoplasm and the chloroplasts assemble in the centrifugal ends of the cells. The looser the cytoplasm, the shorter the time for this displacement. The moment centrifugation is interrupted and the chloroplasts and the plasma are no longer pressed down towards the centrifugal ends of the cells, a plasma flow starts. This is visible in the microscope as a return movement of the chloroplasts along one of the side walls of the cells. The course of the changes in the cell can be followed continuously in the microscope centrifuge. It can be seen that the return movement of the plasma and the chloroplasts starts before the end of the centrifugation. Thus, a motive force exists in the plasma of such an order of magnitude that it is able to overcome a superimposed force (the centrifugal force) in the opposite direction. A rate of centrifugation can be obtained at which the plasma flow—visible as a passive movement of the chloroplasts—does not occur. An increase of the rate then gives rise to a displacement of the plasma and chloroplasts towards the centrifugal end of the cell and a decrease allows the plasma flow to start once more.

According to Beams (4) the protoplasmic flow will cease in *Helodea* cells without disturbance of the life processes after treatment with high centrifugal forces ($35{,}000 \times g$) for 30 min. It is assumed that the cessation of

streaming depends on a decrease in protoplasmic viscosity. This statement is, however, not proven by any experiment and seems improbable since the relationship of low viscosity–rapid plasma flow is a demonstrated general phenomenon. When streaming begins again, the initial movements are usually of an unorganized type. Organized streaming is slowly established resulting in a slow, irregular rotation of the large massed chloroplasts. In only seven per cent of the cells was the direction of streaming altered. This is of great interest and may perhaps indicate a certain degree of cellular polarity, which is unaffected by the violent centrifugation treatment. From these experiments it is evident that the rate of protoplasmic streaming is no doubt connected with the viscosity of protoplasm. It is therefore quite natural that the streaming rate is changed when the cell is treated with agents altering protoplasmic viscosity.

Electric current has a considerable effect on protoplasm. Brownian movement in a cell subjected to a potential difference between the two opposite ends soon ceases, beginning at the anode [Tobias & Solomon (81)]. This points to a differential effect of the applied potential on protoplasmic viscosity. It is not a question of an aggregation of the small microsomes—microscopic observations preclude this—but of a change in the state of the hyaline part of the cytoplasm itself. The effect is reversible, for by switching off the current, the movement begins again at the cathode and advances towards the anode. If the current acts for a sufficient time, Brownian movement will cease in the whole cell; but even this reaction is reversible. Cessation of Brownian movement is also accompanied by a cessation of protoplasmic streaming, a further proof of a change in protoplasmic consistency.

Plasma flow has often been used as an indicator of the rate of metabolism. Protoplasmic streaming is an energy-demanding process and the requisite conditions for streaming in slime moulds under anaerobic conditions have been thoroughly investigated by Allen & Price (1) and Loewy (51). The rate of streaming is decreased when the oxygen pressure is lowered, but may sometime continue for several hours without any oxygen supply. There is some evidence that carbon dioxide in low concentrations, when oxygen is absent has a slight stimulating effect on the streaming rate (51). Chemicals such as NaN_3, which inhibit the uptake of oxygen, also depress the streaming rate. An exception to this is KCN which has no obvious effect in as great a concentration as 1×10^{-3} *M*. Dinitrophenol, which stimulates respiration in low concentrations, causes a reduced rate of streaming. The effect is reversible if the treatment is not prolonged (1). It is evident from this that many factors are involved in the maintenance of protoplasmic streaming. In some cases alterations of the streaming rate are doubtless only the result of a change in protoplasmic viscosity. In others, as in the last example, it is a question of a more far-reaching influence of the agent in which the metabolism as a whole is disturbed. The mechanism behind protoplasmic streaming is still an unsolved problem. The influence of x-rays on the streaming rate is doubtless due to a direct effect on viscosity. By measuring the cytoplasmic

streaming of particularly sensitive tissues such as pollen tubes and staminal hairs, Bishop *et al.* (9) showed that diploid cells are more resistant to x-rays than are tetraploids. The threshold value for the production of a radiation effect is about 10,000 r. The pollen tubes are the most sensitive tissue, the lethal dose being about 250,000 r, while that for staminal hairs is about 750,000 r. Continuous irradiation has a more detrimental effect on the rate of cytoplasmic streaming than has radiation administered intermittently, indicating a reconstruction of the disorganized cytoplasm during the rest period.

The rhythmic flow of the protoplasm of slime moulds has been further investigated by Kamiya (43) using an ingenious method previously employed (42). The plasmodium is divided into two parts connected by a thin strand which passes through a hole in the wall between two separate chambers each of which contains one part of the protoplasm. By measuring the change in volume of the two chambers it is possible to get an idea of the mass movement associated with the flow. The flow shows rhythmic fluctuations with changes in the direction of flow at regular intervals of one to several minutes. Within one and the same streaming more than 4 $mm.^3$ of protoplasm can be moved. No relation is found, however, between the duration of the one-way directed flow and the volume of protoplasm transported. Kamiya & Abe (44) showed that these rhythmic fluctuations of protoplasmic flow are in some way connected with rhythmic potential variations. The latter, however, continue even if protoplasmic flow is hindered in some way. According to Seifriz (69), this fact demonstrates that the variations in rhythmic potential can proceed quite independently of protoplasmic flow and consequently cannot be regarded as streaming potentials resulting from the flowing protoplasm.

In a critical discussion of the structure and molecular construction of living substances, Lepeschkin (50) criticizes Frey-Wyssling's theories of the molecular structure of protoplasm. Amongst other things he disagrees with the conception of the plasma as a molecular framework consisting of a more or less rigid network of polypeptide chains connected with each other in different ways. According to Lepeschkin (50) such a construction would not permit a plasmic flow to take place in such a rapid and sweeping way as is the case. This protoplasmic framework proposed by Frey-Wyssling has been and is a critical point in his hypothesis of the structure of protoplasm. It is the only way in which we are now able to explain the behaviour of protoplasm, especially the intensive synthetic activity.

Optical Properties of the Protoplasm

Refractive index.—The refractive index of protoplasm during mitosis has been investigated by Pfeiffer (60) using a method in which microneedles of known refractive indices are introduced into the protoplasm. He found large changes in the index during mitosis. Two marked maxima of refraction were found, one at the beginning of metaphase (1.504) and another at the

end of anaphase (1.520) as well as a less pronounced maximum at a certain stage of prophase. A minimum of refraction was found at the end of metaphase (1.491) and at the very beginning of the polar movement of the chromosomes (1.499). Two other minima, less marked, were found just before the beginning and after the end of mitosis (1.466). The measurements of the refractive indices were made on four different types of plant and animal cells and in all of them nearly the same changes could be established. The plant cells were from staminal hairs of *Tradescantia* and from the stigma of *Glyceria*. In all probability these changes in protoplasmic refraction are connected with the changes in protoplasmic viscosity during mitosis as observed by Katô (45) and others. This is further evidence of the complicated and sensitive mechanism behind the mitotic processes (cf., foregoing discussion of the effect of certain chemicals on protoplasmic viscosity). Pfeiffer (62) was also able to determine the refractive index of droplets of cytoplasm by placing them between a slide and a watch glass and studying the Newtonian interference rings thus formed. Preliminary investigations of the cell sap and the cytoplasm of different plant cells have given values between 1.334 (*Nitella*) and 1.486 (*Vaucheria*). An isolated protoplast of *Tradescantia* gave the value of 1.402. These two techniques have never been used before for the determination of the optical properties of plant protoplasm and will surely be of great value. Pfeiffer (58) has further shown that it is possible to determine the refractive index of the protoplasm by means of a polarizing microscope. The protoplasmic droplets are placed on a slide and covered with a thin crystal lamina having light-retarding properties. By determining the different angles of refraction it is possible to calculate the refractive index of the protoplasm.

The three methods mentioned suffer from the disadvantage of requiring rather large amounts of protoplasm. Only the first one can be used for cells *in situ*. Here there would appear to be some difficulties in distinguishing the real cytoplasm from the cell sap, especially in mature cells.

Birefringence.—As far as the reviewer knows, no important fact about the birefringence of the cytoplasm itself has appeared, whereas the denser bodies such as the chromosomes and the plastids, etc., have been thoroughly investigated. In general, it is not possible to find any reliable indication of birefringence of chromosomes when they are placed between crossed Nicols. But after they have been treated with ethanol or stretched between microdissection needles, a birefringence of low intensity is observed (61). These experiments were performed with chromosomes from *Drosophila*, but there are no reasons why chromosomes from plant cells should not behave in the same manner. The value of Δn lies between -0.0004 and -0.0019 and is always negative to the length of the object. This implies the presence of structural units, probably nucleoproteins, disposed in a regular way. When treated with fumes of acetic acid the chromosomes will swell a little and in some cases they show a weak birefringence. Experimental stretching of the threads establishes the fact that the intensity of the positive double

refraction is largely caused by the different rates of stretching, but perhaps also to a certain extent by the increased disorder resulting from Brownian movement.

The chloroplasts and the brown colored plastids of diatoms and brown algae are optically anisotropic [Menke (53)]. The sign of the birefringence depends on the wave length of light and the axis is always perpendicular to the surface. Some chloroplasts of the desmids show negative birefringence to all colors of the spectrum, but this is to be considered a borderline case. Plastids may show increased negative or increased positive birefringence depending on the species and the actual state of the plastids. This is evidenced by a displacement of the curve for the dispersion in a positive or negative direction. The chloroplasts also show a weak dichroism in that the ordinary beam is absorbed slightly more strongly than is the extraordinary one. The birefringence is the result of three different kinds of birefringence consisting of two intrinsic ones and a form birefringence of a negative value. From these findings the conclusion may be drawn that the chloroplasts have a laminar structure with alternating layers of protein lamellae and bimolecular lamellae of lipoids. In the latter case, the lipoid molecules are in a perpendicular position to the surface, closely adjoining one another. No certain conclusions can be drawn about the structure of the protein lamellae. In the interpretation of these experiments it must be emphasized, however, that the birefringence has only been established for dead cells. Chloroplasts in the living state exhibit no such phenomena. It is possible by the plasmolytic removal of water from the tissues to induce such birefringence. With the removal of water, the birefringence of the more easily dehydrated parts of the chloroplasts show the greatest change, consisting of a decrease of the form birefringence and an increase of the intrinsic one. By using this method it is possible to find out for certain that the chloroplasts really have a negative form birefringence and a positive intrinsic birefringence, for it is unlikely that the structure should change very much during the plasmolysis of the chloroplasts.

The birefringence of the membranes of the spermatozoids of a lot of species is very obvious [Pfeiffer (59)]. The phenomenon is accentuated only to a small extent by fixation in alcohol, while other fixation media alter the chromatin and have a decreasing or quenching effect on the birefringence. The cytoplasm of the spermatozoids show a much smaller positive birefringence which is thought to be related to its particular protein content. By adding KOH, both the positive and the negative birefringences are decreased.

Permeability

The permeability of a cell can be defined from two different points of view. It may be regarded as that property of a living membrane involving the passage through it of different ions and molecules without the expenditure of energy derived from metabolic processes. This is permeability in a strict

sense and depends mainly on the physico-chemical properties of the protoplasmic membranes of the cell. On the other hand, permeability may be regarded as the regulation of the flux of all particles through the living membrane by either active or passive processes. This review will consider permeability only according to the first definition.

Permeability to lipophilic and lipophobic substances.—Among the different permeability theories, the lipoid theory of Overton and the ultrafilter theory of Ruhland are the two most generally accepted. The lipoid-filter theory of Collander and his school is a compromise between these two. During recent years the literature covering this subject has been enriched with several papers from the different schools and reflects certain differences in the conception of the mechanism of permeability. According to Wartiovaara (91), two main types of ultra-filtering are the determining factors in permeability. Lipoid solubility functions simultaneously with, or subsequent to, molecular size discrimination. Pore permeability, the first type of ultra-filter effect, is seldom pronounced but is characteristic of diatoms. The other type may be called the sieve effect. Within homologous series of permeating substances the sieve effect can be established as a relative disproportionality between lipoid solubility and penetration rate. The resistance of the protoplasm to diffusion of individual members of the series can be expressed as a product of the figures for lipoid solubility and sieve effect. It looks as if there are two layers close to one another, namely, a molecular sieve and a dense layer of lipoids without any pores. Wartiovaara (91) is of the opinion that the lipoid molecules of the protoplasmic membranes lie closely packed together. When using alcohol as a permeating medium the alcohol molecules are then taken up as rigid rods perpendicular to the position of the lipoid molecules.

The permeability of baker's yeast to different weak bases has been thoroughly investigated by Äyräpää (3). According to him lipoid solubility is the main factor controlling penetration. The smallest molecules, however, (ammonia, methylamine, and hydrazine) penetrate 10^2 to 10^3 times faster than do larger molecules of the same lipoid solubility. This shows the great sieve effect exerted by protoplasmic membranes. Collander (16, 17, 18) found the same discrepancy between the penetration rate of small and large molecules. The relations between penetration power, lipoid solubility (as indicated by the ether/water partition coefficient), and molecular weight are as follows. For medium sized molecules lipoid solubility is the decisive factor, although the smallest molecules permeate more rapidly than would be expected on the basis of their solubility alone. Accelerated penetration is particularly evident for the first or the first two members of every homologous series, that is, for substances with molecular weights of about 50 to 60 or less. The sieve effect of the protoplasmic membrane is thus rather critical and restricted to molecules of a certain size. The Q_{10} values obtained for the penetration of methanol, ethanol, n-propanol, and n-butanol are 2.6, 2.7, 2.7, and 2.1, respectively (90). The temperature coefficient seems to

be a function of the molecular pattern and independent of the rate of penetration. The high value of the temperature coefficient may be explained by assuming that the properties of the protoplasmic membrane are altered by the change of temperatures.

The penetration of urea and methyl-urea is characterized by the fact that in some organisms the permeation of methyl-urea takes place two to six times faster than does that of urea [the *Chara-Majanthemum* and *Rhoeo* types, according to Höfler (38)], while other objects show the same permeability to urea [the *Gentiana sturmiana* type]. The different types have been characterized by Biebl (7). It has since been shown by Höfler, however, that this division of the plants into two groups depends on the time at which the measurements are performed, thus showing the importance of the actual state of the protoplasm. This is particularly obvious in the case of *Gentiana sturmiana*. Here, the penetration of urea changes from a very fast to a slow rate in a reversible manner. According to Collander & Wikström (18), the greater permeation of methyl-urea may be explained by taking into consideration the fact that the distribution ratio lipoid/water for methyl-urea is about two to six times greater than that for urea. In the cases where urea shows the fastest penetration, the authors envisage a molecular sieve effect, for in a normal "homogeneous" medium the diffusion of methyl-urea would be but 10 to 20 per cent slower than that of urea itself. The authors are of the opinion, however, that the water-filled pores of the protoplasmic membrane are not permanent. It is also thought that this difference in the penetration rate may perhaps be explained by thermal movements of the lipoid molecules of the protoplasmic membrane. An explanation of the many different patterns of penetration of various substances requires, however, the postulation of plasmatic membranes of varying structures. Using the plasmometric method of Höfler (36), Bogen (10) found that the rates of penetration of malonamide, glycerol, and urea are somewhat less for the tetraploids of *Oenothera franciscana* than for the diploids, while acetamide, thiourea, and dicyanamide show the opposite behaviour. There seems to be no direct relation between penetration rate and molecular volume of the penetrating substances. On the other hand, there is a parallelism between penetration and lipoid solubility expressed in terms of the partition coefficient oil/water. The substances showing the smallest distribution ratio also show the lowest penetration rate in the case of tetraploids. The more lipoid soluble substances have the higher penetration rates. As a general rule, if a distinction is made between pore and lipoid permeability, the former is decreased and the latter is increased in the case of tetraploids. These differences in the behaviour of the two variants can be explained by the assumption of either a denser membrane system in tetraploids in conjunction with an unchanged thickness of the plasmatic layers, or an unchanged cytoplasmic density coupled with a thicker cytoplasmic layer. It is, however, difficult to choose between the two alternatives,

but measurements of the time for deplasmolysis give the value of 30 to 40 min. for diploids and 50 to 90 min. for tetraploids. This indicates that at least the outermost parts of the cytoplasmic layers have a difference in viscosity in diploid and tetraploid types, respectively.

Two papers from the Ruhland school show very clearly that the penetration rate of different substances through protoplasmic membranes is only a question of the correlation of the sizes of the pores to the molecular weights of the penetrating substances [Bogen (11); Ruhland & Heilmann (66)]. The consideration of other factors is quite unnecessary for the explanation of the data presented. By taking into account the van der Waals's forces (intermolecular forces), every deviation from the ultra-filter theory can be explained. The energy content of the forces is small in the case of lipoids, but great in the hydrophilic substances. Permeation of a soluble molecule through a plasmatic membrane can be regarded as a kind of blocked diffusion. The attraction forces between the penetrating molecules and the molecules of the membrane are the most effective factors preventing diffusion in this case, not the mechanical barrier. The van der Waals's forces of the nonelectrolytes are thus the main forces preventing diffusion. The smaller the distance between the pores of the membrane, the greater the van der Waals's forces (11). Membranes of plant tissues are characterized by extremely narrow pores. The differences in penetration rate of the easily penetrating, more or less hydrophobic substances, compared with the more slowly penetrating hydrophilic nonelectrolytes, must thus increase with decreasing distance between pore walls and thus show high values for plasmatic membranes of algae and higher plants. In the case of *Beggiatoa*, with its extremely wide pores, there is no great difference between the penetration rates of the two kinds of substances, although the transport of lipophilic substances through the membranes is somewhat promoted. It is also shown that the molecular size of the penetrating substances cannot exceed a certain value in relation to the pore size. Otherwise, penetration is prevented. If the cells are treated with primary alcohols belonging to the saturated aliphatic series, diffusion into the cell of hydrophilic as well as of hydrophobic substances is blocked. With the longer alcohol molecules, the blocking of the entry of smaller molecules is not as efficient. An interesting feature is that no difference can be found between the restricting effect on penetration of hydrophobic and hydrophilic molecules, respectively. The authors suppose that the alcohols form a monomolecular layer covering the walls of the pores completely or in part. The constriction of the pores may, however, also be explained by starting from the hypothesis that the narcosis affects the hydration state and therefore the pore size.

Influence on permeability by irradiation.—The permeability of protoplasts of *Allium cepa* is very strongly affected by ultraviolet light [Biebl (8)]. Usually the primary effect of exposure to light is an increase in permeability followed by a decrease. Sometimes only a decrease can be established. If

the method of deplasmolysis is used, however, the change in permeability depends on the plasmolytic agent as will be shown in the following. By using certain nonelectrolytes as a plasmolytic medium, cap plasmolysis is induced showing the alteration of the protoplasmic state [Toth (82)]. The time for deplasmolysis by many substances such as urea, sulfurea, ethylene glycol, and malonamide shows a strong decrease after irradiation of the cells with lethal doses of ultraviolet light. After moderate doses, however, with an exposure time of about 5 to 60 min., an increase of permeability to ethylene glycol and a decrease to glycerol is obtained, while for urea the time for deplasmolysis is nearly the same as before. After an exposure of 20 to 24 hr. there is an increase of the time for all substances. This difference in the behaviour of the *Allium* cells to the penetration of different substances shows the danger of determining permeability by the use of only one or a few penetrating substances.

Also, the permeation of water seems to be increased by irradiation with ultraviolet light [Seemann (68)]. The increased permeability is retained for some time after the irradiation. This suggests that the size of the pores is increased by the ultraviolet treatment. A rise in temperature also has an increasing effect, but in this case the change in permeability is quite reversible and reverts to the original value as soon as the temperature falls.

The lipoid contents of the plasma membranes.—It is possible to get an idea of the lipoid content of the outer plasma membrane (the plasmalemma) by adding small amounts of KOH to the KNO_3 plasmolytic medium [Cholnoky (15)]. In certain cases the cells are plasmolysed with concentrations of KOH as high as 10 per cent. This indicates a low percentage of lipoids in the semipermeable membrane. *Calceolaria* was unaffected by this concentration of KOH, while the cells of some species of *Senecio* remained unplasmolysed. These results show that the plasmalemma and probably even the tonoplast can be of a very different structure. It is possible according to these experiments, that this applies even to different tissues of the same species. It should be of great interest to do such comparative experiments with plants belonging to the different permeability types of Höfler (38) mentioned above. A better understanding might thus be obtained of the nature of the variations in permeability behaviour. According to Cholnoky (15), his experiments with KOH exclude the existence of single monomolecular plasma membranes. Such a monomolecular lipoid layer should be immediately destroyed by the penetrating molecules of KOH. It is therefore necessary to postulate the existence of a colloidal structure consisting of at least two phases. A similar division of plant cells into different groups according to the properties of their plasma membranes can be obtained by using sodium carbonate as a plasmolytic medium (40). Fresh water algae show much greater resistance to the penetration of this salt than do terrestrial types. There is also evidence that Ca is necessary in some cases to rebuild a plasmalemma destroyed by plasmolysis. Whereas this holds good for cells of *Spirogyra*, the presence of

Ca is unnecessary for the building of the dense and strongly resistant plasmatic membranes of the desmids [Höfler (40)].

Permeability of different parts of the plant.—Different layers of the tissues of herbaceous plants do not show the same permeability to nonelectrolytes. In the case of *Taraxacum officinale, Epilobium hirsutum, Gymnadenia conopea* and *Physalis alkekengi* the epidermal layers are much more permeable than are the underlying layers [Url (83, 84)]. The latter show a permeability gradient, the innermost cell-layers having the lowest permeability. The difference in penetration rate does not exceed a certain value. Deplasmolysis occurs four times faster in the epidermal layers than in those of the underlying tissues. In the special case of plants of the *Gentiana sturmiana* type, the quotient of the time for deplasmolysis of the epidermal cells to that of the underlying layers may exceed the value of 10. The quotient of the glycerol type (84) is higher for glycerol than for amides, while the urea type shows the inverse behaviour, depending on the different shape of their plasma membranes (84). It has been shown by several investigators, e.g., Diannelidis (27), that stomatal cells have a much greater permeability to urea than have the surrounding cells as well as a higher osmotic value of the cell sap. The form of plasmolysis is usually convex. But, as has been shown in an earlier work by Weber (92), this form of plasmolysis depends on the degree of opening of the stomata. Therefore, it is necessary to be very cautious in a discussion of the cause of the irregularities in the behaviour of the stomata. That the permeability of the stomatal cells is changed with the degree of opening is shown by Stöger (77), but, of course, it is difficult to decide if these changes are a result of the light itself or are a link in a more complicated chain of reaction. According to Reuter (65), the stomata of *Lemna* show the same permeability properties as do the other cells.

It has been shown by several workers that there are different physiological zones in different parts of plants [Diannelidis (28); Fischer (31); Pirson & Seidel (63)]. The cells of the lower parts of the zone of elongation of roots of *Lemna* thus show a maximum time for plasmolysis as well as a minimum value for the permeability to urea (31). Deficiency in potassium and calcium gives rise to a more even gradient of the time for plasmolysis and of the permeability. For the algae of *Halophila*, the permeability to urea is very great in the cells of the stalk. Neither glycerol nor glucose, however, can penetrate (28). These differences in permeability are, no doubt, connected with corresponding changes in protoplasmic viscosity and osmotic pressure of the cell sap in different parts of the tissues.

Plasmolysis and Other Osmotic Phenomena

Vacuolar contraction and cap plasmolysis.—Vacuolar contraction and cap plasmolysis can result from several treatments. Vacuolar contraction, as well as that kind of plasmolysis called irritation plasmolysis, often arise when a cell is stimulated. The protoplasm takes an active part in the processes,

but the vacuole is also of great importance since the water taken up by the protoplasm is supplied from the vacuole. Transference of water from vacuole to cytoplasm can, however, be induced by many agents without giving rise to a swelling of the cytoplasm, e.g., neutral red. The cause of this water transport is as yet unknown. The decrease in volume of the vacuole seems to occur as a result of exosmosis from the cell sap of osmotically active substances because of an increase in permeability. According to Bogen (12), however, this hypothesis is not sufficient to explain the increased water uptake of the protoplasm. Furthermore, it has in some cases been impossible to show any difference between the permeability of normal cells and those showing vacuolar contraction.

Cap plasmolysis, however, is not interpreted as an irritation phenomenon. It has only been observed when salts of the alkali metals have been used as plasmolytic agents. It is accompanied by the accumulation in the cytoplasm of ions which exert a swelling influence on the plasma (37). According to Bogen (12) there is no reason for distinguishing between the two processes of cap plasmolysis and vacuolar contraction, for he has found that cap plasmolysis can be produced by LiCl as well as by a solution of neutral red provided that the solutions are made hypertonic by the addition of sucrose. The cap plasmolysis obtained in this way is a temporary effect, disappearing after a short time. The type of plasmolysis obtained depends only on the concentration of the plasmolytic medium. Hypertonic solutions thus give rise to cap plasmolysis and hypotonic to vacuolar contraction. It has been possible here to give but a short account of the contents of this very interesting paper. The interpretation of the two phenomena as special cases of the same main process is a concept which helps to explain many different facts in connection with plasmolytic phenomena. Yet, it must be emphasized that in the case of vacuolar contraction there is streaming of the protoplasm, whereas this is absent in cells showing cap plasmolysis. This shows that the presence of an excess of ions, greatest in the case of cap plasmolysis, has a strong effect on the physical as well as on the physiological state of the cytoplasm. The excessive concentration of ions also results in the formation of myelin figures at the phase boundary between cytoplasm and vacuole.

Electro-osmosis.—The diffusion pressure deficit (suction force) of the plant cell has in addition to the salt component, an electric one which can be determined by plasmolysing different cells in sugar solutions to which different electrolytes have been added. It has been shown by Studener (78) that the osmotic effect of electrolytes is in many cases greater than can be calculated from their physico-chemical activity. Experiments have been performed with cells of *Helodea*, *Allium*, *Nitella*, and other plants. In all these cases plasmolysis is obtained in solutions having a composition and concentration which would not be expected to result in plasmolysis at all. Electrical measurements performed on internodal cells of *Nitella* in a 0.0004 *M* solution of sodium nitrate show that these cells have a concentration potential of ap-

proximately 18 mv. below the potential obtained with cells in distilled water. With increase of the concentration of the solution, the potential decreases proportionately. Potassium salts have a much more pronounced effect than have the sodium salts. It is clear from these experiments that the potential effect shown by the potassium ions is of great importance for the maintenance of these abnormal osmotic conditions. These electrical properties of the protoplasm seem to play an extensive rôle in the exudation of water and nectar from the nectaries of flowers and from the glands of *Lathraea* and *Drocera* [Diannelidis (25)]. The nectaries have a negative charge in relation to the potential of the surrounding cells when the nectar is thin and vice versa when the nectar is concentrated. If water is added to the nectaries, the potential becomes more positive. If, on the other hand, glucose is added at a concentration of 30 per cent, the nectaries become more negatively charged. This implies that the potentials are not the result of differences in concentration but are probably the cause. The digestive glands of *Drocera* are negatively charged in the unirritated state. After having been irritated by proteins their negative charge shows further increase.

The bladders of *Utricularia*, transporting water from the inner to the outer medium, are charged positively inside and negatively outside, but a solution of 0.1 *M* KNO_3 neutralizes the electrical potential and stops the exudation of water. Even in this case, KNO_3 alone or together with glucose shows a higher osmotic effect than can be calculated from the freezing-point depression of the solutions. It is reasonable to presume that the suction force here includes an electrical component which is neutralized by potassium nitrate, since this salt, as shown above, has a strong effect on the potential of the cell surface. Excised leaves do not show this effect of potassium nitrate, probably because the electrical potential has been neutralized. These experiments on the electrical properties of surface membranes of the protoplasm are of great general interest. Though they were performed with highly specialized cells, they give general information about the one-way transport of different substances. Such electrical forces have been shown to exist in the *Avena* coleoptile by Mills & Schrank (54).

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THE PLANT STEROLS[1]

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INTRODUCTION

During the four years which have passed since the appearance of the most recent edition of Fieser & Fieser's comprehensive text on steroids (125) the important discovery has been made that the steroid cortisone is of significant value in the treatment of arthritis and other diseases. Since then many chemists have been engaged in the arduous task of increasing the commercial production of this valuable compound through its partial synthesis from plant steroids. Among them saponins like diosgenin and hecogenin, and sterols like ergosterol and stigmasterol appear to be the most promising starting materials.

The search for suitable steroids has caused a revival of interest in their occurrence and distribution in plants. The saponins, the cardiac active principles and the steroid alkaloids have been most intensely investigated during the past decade. During the same time the study of the true sterols of plants has largely been neglected and left in a rather chaotic state. It is for this reason that the present review concerns itself exclusively with the plant sterols now known and is an attempt to furnish a useful guide through the maze of information concerning them. Discussion of the methods used in the isolation and establishment of the structure of sterols, and their standard colorimetric and gravimetric determinations has been omitted from this review, and only such material has been included which has become available since the appearance of Fieser & Fieser's monograph.

STRUCTURE OF STEROLS

In this review the term sterol is understood to refer only to those steroids whose structures show close resemblance to that of cholesterol (Fig. 1, II, R=A). They differ from cholesterol only in the number and position of cyclic and acyclic double bonds and in the structure of the alkyl side chain. Such sterols are widely distributed among plants, and the only organisms in which their absence has been demonstrated are the tubercle bacilli (12) and possibly also *Aerobacter aerogenes* (482) and certain primitive, asexual algae (*Myxophyceae*) (77). During the past decade it has been shown, principally by this reviewer, that many if not all plant sterols are also found in certain invertebrate animals. The old distinction between phyto- and zoosterols is therefore no longer of practical significance.

In spite of the ubiquity of sterols in plants, no comprehensive, comparative studies on their occurrence have so far been carried out. Data derived

[1] The survey of literature pertaining to this review was completed in July, 1952.

from numerous isolated studies have previously been compiled by Dalmer in 1932 (92) and by Schwab in 1941 (381), but their critical evaluation in the light of modern knowledge has not yet been available. It has been known for some time that the presence of certain sterols may be fairly characteristic for one or another class or order of plants. Thus, ergosterol (Fig. 1 I, R=G) may be regarded as the principal sterol of higher and lower fungi, and possibly also of the lichens. Its presence is, however, not restricted to these organisms for ergosterol also occurs as a minor component in the sterol mixtures of several other plants and of animals, and it has recently been shown to be the principal sterol of the alga, *Chlorella pyranoidosa* (223). Among the algae, fucosterol (Fig. 1, II, R=J) appears to be a characteristic component of the *Phaeophyceae* (77). The data on the sterols of green algae and of higher plants are at present in a rather chaotic state and hence difficult to interpret. As a rule the higher plants contain very complex

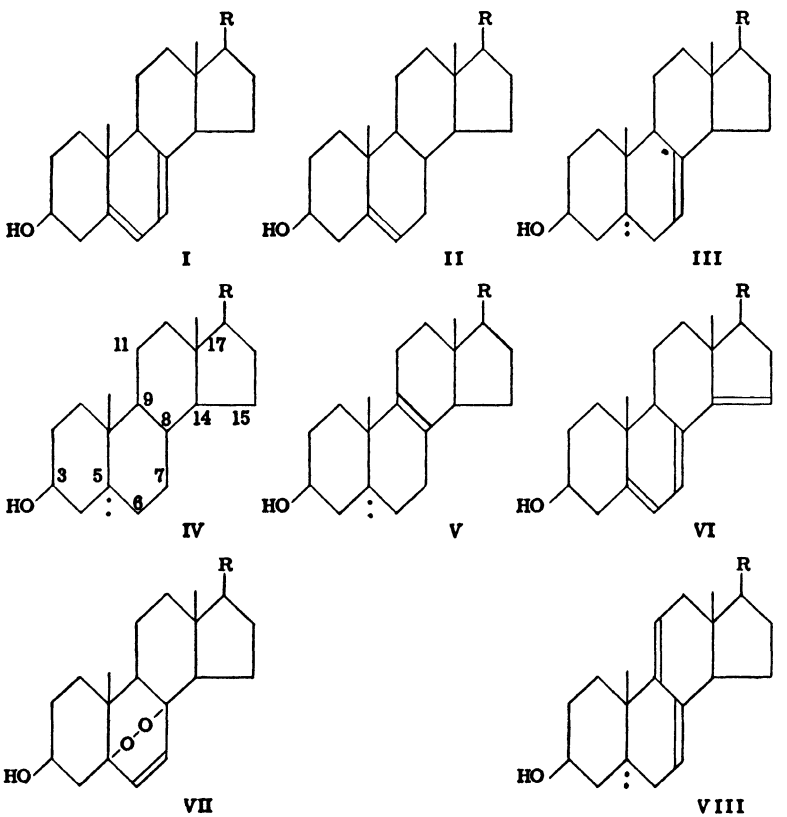


FIG. 1. Structural Formulae of Sterols.

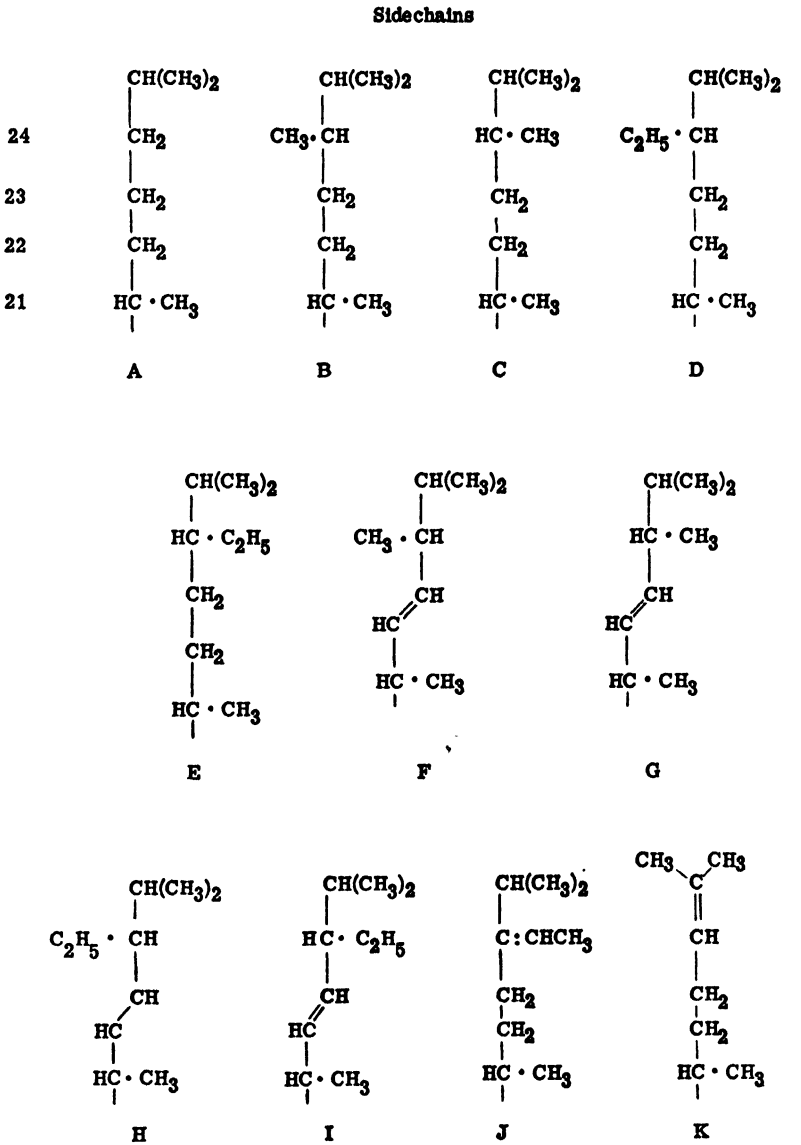


FIG. 1. (*continued*) Side Chains.

mixtures of structurally closely related sterols, whose partial separation has so far been accomplished only in a few isolated cases.

The components of sterol mixtures which are most difficult to separate are homologs and epimers. Many of the sterols of higher plants are homologs

of cholesterol differing from the latter only in the presence of a methyl or an ethyl group at the 24-position of the side chain. The presence of such groups confers asymmetry upon the C-24-atom, and two isomers, epimeric at this point, are theoretically possible. The recognition of the natural occurrence of both epimeric types has been one of the most significant advances in the chemistry of sterols during the past decades, and it has immeasurably helped to understand the often bewildering complexity of sterol mixtures. In order to differentiate between the members of a series, epimeric at C-24, Fieser & Fieser (125) have suggested to call *b* the configuration in sterols derived from ergostanol (Fig. 1, IV, R=C) and stigmastanol (Fig. 1, IV, R=E), and *a* the configuration of opposite sign which is found in sterols derived from campestanol (Fig. 1, IV, R=B) and poriferastanol (Fig. 1, IV, R=D). The proof that ergosterol and stigmasterol possess identical configuration at C-24 has been derived by Bergmann & Low (43) from comparisons of the rotations of side chain fragments in which C-24 is the only asymmetric carbon atom.

Stokes & Bergmann (407) have recently pointed out that sufficient data are now known about all asymmetric centers of the sterol molecule to permit an extension of the well-established symbols α and β from the cyclic centers to those of the side chain. Originally the configurations of the asymmetric centers in the side chain had been arrived at by making use of Fischer's convention for writing asymmetric structural formulae. Experimental and theoretical evidence, however, has been advanced by Bergström *et al.* (48) and by Stokes & Bergmann (407) which shows that the present convention for writing steroid ring structures is the reverse of Fischer's convention. Consistency therefore requires that the side chain centers of asymmetry also be written reversed.

In 1951, a committee sponsored by the CIBA corporation published a number of proposals for standardizing steroid nomenclature (447). One of these, contributed by Plattner, suggested that the side chain be written as in figure I (A to E) and the β-oriented substituents be placed on the left of the chain, and α-oriented substituents on the right. Using Plattner's proposal and our present knowledge of the side chain configurations the cholesterol side chain should be written as in A (Fig. 1) with the C-20 methyl group in the α-position, and the side chains of several other known sterols with C-24 substituents as in B to I (Fig. 1). Tentative evidence advanced by Bergmann *et al.* (40) indicates that in haliclonasterol and palysterol the C-20 methyl group is β-oriented. These sterols have so far only been isolated from marine invertebrates, but it appears quite probable that they may eventually also be found in plants.

Although the known plant sterols possess from 8 to 10 asymmetric centers, depending on the degree of unsaturation, no isomers have as yet been found which are epimeric at positions other than C-24. A possible clue to this interesting dualism of the occurrence of the C-24 epimers may be found in the structure of fucosterol (Fig. 1, Formula II, R=J), the typical sterol

of the *Phaeophyceae* (77). Fucosterol lacks asymmetry at C-24 because of the presence of a double bond at this point. Hydrogenation of the double bond establishes asymmetry, and the formation of either the C-24-α or C-24-β ethyl derivative of cholesterol (Fig. 1, Formula II, R=D, E) or both is to be expected. Assuming fucosterol and its C-24-methylene homolog, as yet unknown, to be intermediates in the biochemical transformation of sterols, the formation of both epimers may readily be envisaged.

Among sterols which like ergosterol (Fig. 1, Formula I, R=G) and stigmasterol (Fig. 1, Formula II, R=I) possess a double bond between C-22 and C-23 in the side chain, consideration must be given to the possible natural occurrence of sterols *cis* or *trans* oriented around the double bond. It has recently been shown that such configurations can be determined by means of the x-ray diffraction patterns (90), or more conveniently by the infrared spectra (199) of sterols. Both methods have established the *trans* configuration in sterols derived from ergosterol. It is probable that most if not all of the other known Δ^{22}-sterols also possess the *trans* configuration.

BIOSYNTHESIS OF STEROLS

In recent years considerable attention has been devoted to studies on the biosynthesis of sterols. Most of the work deals with the biosynthesis of cholesterol in vertebrate animals. In the case of primitive plants, it has been shown convincingly that acetate is the major, if not the sole carbon source of the sterol molecule. Ottke, Tatum & Simmonds (310) discovered a mutant strain of *Neurospera crassa* which requires acetate for growth, being incapable of metabolizing glucose to the acetate stage. When this mutant is grown on a medium containing normal glucose and acetate labeled by C^{13} in the carboxyl and by C^{14} in the methyl group, it produced ergosterol whose isotope concentrations were virtually the same as those of the labeled acetate (311). The acetate therefore must have been practically the exclusive carbon source of ergosterol.

The formation of sterols in growing plants has been investigated by MacLachlan (253), who observed that the germination of soybeans is accompanied by a marked reduction of the total fat, but a continuous synthesis of sterols. This synthesis was somewhat greater in the dark, and during the rapid mobilization and utilization of the fats esterification of the sterols showed a marked increase.

METHODS FOR SEPARATION AND ANALYSIS

No significant advances in the separation of sterol mixtures have been made in recent years. As has been stated above, the components of many of such mixtures differ only in the degree of their unsaturation and in the shape of their side chains at C-24. When compared with the structures of the entire molecules, such differences are rather small, and it is therefore not surprising that many sterols show such similarities in their solubilities as to make difficult their separation by conventional methods. Chromato-

graphic techniques which have been used with such conspicuous success in the separation of other steroids, which like the sex hormones and related products differ in the position of functional groups, have met only with moderate success in the separation of sterols. As a rule those sterols are most readily separated by adsorption methods which show the greatest differences in the shape of their molecules. Thus the structural differences between cholestanol and coprostanol, which are caused by the *trans* and *cis* junctures of rings A and B, are sufficient to permit a facile separation of the two sterols on an alumina column (84). Ergosterol and sterols of analogous structure are more strongly adsorbed on alumina (478) than sterols devoid of conjugated bonds in ring B, and they may therefore be separated chromatographically from mixtures of diverse origin (60, 475).

Since all sterols are colorless compounds, the possibility has been explored of making visible the progress of separation on a column. The separation of ergosterol and cholesterol may be followed by the intense fluorescence shown by the former under ultraviolet light (204). The method, however, will remain of limited value because most sterols do not fluoresce under such conditions. The majority of the sterol mixtures from higher plants are composed of compounds of very great structural similarity. It is the separation of such mixtures which presents the greatest difficulties, and one which has not yet satisfactorily been accomplished by chromatographic methods. In their studies on the chromatographic separation of the colored steryl esters of azobenzene carboxylic acid, Ladenburg *et al.* (237) observed that it was possible to separate small quantities of esters of sterols differing in the degree of unsaturation, such as ergosterol, stigmasterol, and cholesterol. The esters of homologous sterols, however, such as cholesterol and the sitosterols, formed a continuous chromatogram, and no separation of practical value was achieved. In contrast, isomeric sterols differing only in the position of a cyclic double bond are separable by means of this method. Thus Idler & Baumann (187) have recently succeeded in separating with comparative ease the azoylesters of cholesterol and its isomer, Δ^7-cholestenol (Fig. 1, III, R=A) on a silica column treated with silicone oil to prevent surface spreading. This method should prove useful in the separation of plant sterol mixtures containing Δ^5- and Δ^7-sterols.

In 1906, Windaus & Hauth (472a) accomplished the first partial separation of a plant sterol mixture through the bromination of the steryl acetates in ether-acetic acid. The authors obtained a difficulty soluble acetate tetrabromide which upon debromination with zinc in acetic acid afforded a new sterol, stigmasterol. With but minor variations this method has since been used with conspicuous success in the separation of other di-unsaturated sterols, such as brassicasterol (Fig. 1, Formula II, R=G), poriferasterol (Fig. 1, Formula II, R=H), chalinasterol (Fig. 1, Formula II, R=F), and fucosterol (Fig. 1, Formula II, R=J). This method has proved to be of practical value particularly in the isolation from mixtures of Δ^5-sterols which carry an additional double bond in the side chain, and particularly

in the Δ^{22}-position. In cases where the original mixture contains several sterols so constituted, such as C-24-methyl and ethyl epimers, mixtures of tetrabromides are obtained, and a facile isolation of a pure product cannot be expected. The bromination method is only of limited value in the separation of mixtures containing significant quantities of Δ^7-unsaturated sterols (I and III). These sterols are readily decomposed by bromine with the appearance of a green color (Tortelli-Jaffe reaction). In such cases it appears advisable to carry out a preliminary chromatographic separation of the Δ^5- and Δ^7-sterols by the method mentioned above.

With but one exception, the steryl acetate 5,6-dibromides are considerably more soluble than the steryl acetate 5,6,22,23-tetrabromides, and hence their preferential precipitation offers no serious difficulties. The exception is cholesteryl acetate dibromide which is also precipitated under the conditions normally employed, and in the case of high concentrations a similar behaviour may be shown by certain "sitosteryl acetate" 5,6-dibromides. For this reason it is advisable to triturate the precipitated bromides with ether in which the 5,6-dibromides are very soluble, and to recrystallize the remaining tetrabromides from a large volume of ether. The comparative insolubility of the tetrabromides appears to be mainly due to the presence of vicinal bromine atoms in the side chain, for the 22,23-dibromides of stigmasterylacetate (122) and neospongosteryl acetate (41) are nearly as insoluble as the 5,6,22,23-tetrabromo acetates. The isolation of difficulty soluble bromides therefore does not necessarily indicate that they consist entirely of tetrabromides.

In those, unfortunately rather common cases, where the sterol mixture consists principally of homologous and C-24-epimeric sterols, a long and tedious series of fractional recrystallizations still remains the only method offering the promise of some success. The method, however, is a wasteful one, and should be expected to meet with success only if the mixture is available in comparatively large amounts, such as a typical sitosterol mixture, or if one or another of the sterols is the predominant component.

The separation by fractional crystallizations of free sterols is less efficient in polar than in nonpolar solvents. The 3β-hydroxyl group, common to all natural sterols, is the most polar group of the molecule, and the one which diminishes the effects of structural differences. The influence of the latter may be enhanced and that of the polar group correspondingly reduced, by converting the sterols into esters, such as acetates or benzoates. Since the benzoates are the least polar, their separation as a rule offers the better promise of success. It will be found of advantage to change the solvents several times during a long sequence of recrystallizations, for it has often been found that the melting point of a fraction which had reached a constant value after numerous recrystallizations from the same solvent, is raised significantly by a single recrystallization from a different solvent. The change to a new solvent is often as effective as the change to a new derivative.

Several methods have recently been described which appear to be well

suited for the quantitative estimation of sterols in plant material. Wall & Kelley (453) make use of colorimetric and gravimetric methods in their micro- and macrodeterminations of sterols in leaf meals. The method developed by Waghorne & Ball (448) consists in the quantitative estimation of the total sterols by precipitation with digitonine followed by a dichromate oxidation of the digitonides and titration with ferrous ammonium sulfate. The precision of this method is reported to be better than 3 per cent. It is felt that this method is better than those based on color tests like the Liebermann-Burchard reaction which is negative in the case of saturated sterols. A method adapted to the determination of sterols in sea weeds has been described by Black & Cornhill (59). Differences in the absorption spectra of the colors produced by the Salkowski reaction have been described by Dhéré & Laszt (98) for sitosterol, stigmasterol, ergosterol, and zymosterol, but the practical significance of these observations remains to be evaluated.

It is because of the great difficulties encountered in the separation of sterol mixtures that many such mixtures have been and are occasionally still being described as new compounds. In addition, investigators unfamiliar with the properties of sterols are prone to report as sterols either complex mixtures of unsaponifiable materials containing sterols, hydrocarbons, batylalcohol etc., or compounds which show a superficial resemblance to sterols but are actually of quite different structure. There exists a definite pattern of relationships between the physical properties and the structure of sterols, so that compounds, described as sterols, but showing physical properties at variance with this pattern, may *a priori* be recognized as different from sterols. Thus, to cite one of several recent examples, the compound obtained from *Zyzyphus zylopora* (5) cannot have been a sterol as stated, because its melting point of 208° is substantially higher than those of the usual sterols, and that of the acetate, 62°, far lower than those of sterylacetates. It appears more likely that the material was a mixture of a high melting triterpene and a low melting hydrocarbon or alcohol. It is the purpose of the following chapters to establish a simple system of classification of natural sterols, which is based on readily recognizeable physical properties. This system should serve the uninitiated as a useful guide through the maze of information on plant sterols and assist him in the correct interpretation of his own observations in this field.

CRITERIA FOR CLASSIFYING NATURAL STEROLS

Reference has already been made to the old division of sterols on the basis of their origin into three groups: the zoosterols of the animals, the phytosterols of the plants, and the mycosterols of cryptogams, such as fungi. Cholesterol, sitosterol, and erogosterol were regarded as the most typical representatives of the three respective groups. The justification for this division appeared to be enhanced when it was found that these three typical sterols differ in the number of their carbon atoms, and that they are of the

orders C_{27}, C_{28} and, C_{29} respectively. More recent studies, however, have shown that this classification is of little value, if not actually misleading. It still remains true that cholesterol is the typical sterol of higher animals and that its occurrence in plants has not yet been demonstrated. In lower animals, however, its place is often filled by other sterols. most of which are also encountered in plants. In addition, zymosterol, a C_{27}-sterol, structurally related to cholesterol, has been found in yeast, and C_{28}-sterols, such as brassicasterol, have been shown to occur in higher plants and animals.

In 1941, Schwab (381) listed naturally occurring sterols on the basis of their empirical formulae, and arranged them in the order of the number of their carbon atoms. Past experience has shown, however, that correct empirical formulae for sterols are at times difficult to establish. As a rule, free sterols contain solvent of crystallization whose complete removal is not easily accomplished. The C- and H-values obtained by combustion of such sterols are therefore misleading. Analyses of sterylacetate and benzoates give more reliable values, but they are rarely of help in differentiating between C_{27}, C_{28} and C_{29} sterols. The differences between the theoretical C- and H-values of such derivatives of homologous sterols are so small as to fall within the limits of experimental error of the standard combustion analyses. Of greater significance are the saponification values of the acetates (364), or the combustion analyses of bromides, 3,5-dinitrobenzoates or some other derivatives, in which the differences in composition are enhanced and experimentally detectable (477). In the final analysis, however, it is best to regard only such empirical formulae as convincingly established, which are supported by chemical evidence, such as for example a conversion of the new sterol to a well known steroid. It is not surprising, therefore, that most of the older and some of the new empirical formulae for compounds believed to be sterols are incorrect. A listing of true and doubtful sterols on the basis of such questionable data appears to be of no significant value.

From a practical point of view it is best to list and classify the sterols on the basis of their optical rotations and those of their esters. The directions and magnitudes of the rotations are dependent mainly upon the degree of unsaturation of a sterol and the location of the double bonds in the molecule. Thus a strongly negative specific rotation of more than $-90°$ is indicative of a structure similar to that of ergosterol (I). It is intimately associated with the presence in ring B of the system of conjugated double bonds. A more moderate *levo*-rotation of -30 to $-45°$ is typical of sterols with a single double bond at 5, 6, (II) and rotations of -50 to $-70°$ point to the presence of a double bond at 5,6 and an additional one at 22,23, as for example in stigmasterol. Specific rotations of $0°$ to $-25°$ suggest a double bond in the 7,8-position (III), the more negative rotations indicating an additional double bond at 22,23. The absence of unsaturation in the ring system (IV) is revealed by positive specific rotations ranging from $+10$ to $+30°$, and more pronounced dextro-rotations of $+40$ to $+50°$ point to the presence of a double bond in the 8,9-position (V). Specific rotations in excess of $+50°$

usually indicate that the compound in question is not a sterol, but rather one of the triterpenoids, frequently encountered in plant material. In addition, the differences between the molecular rotations,

$$[M]_D = [\alpha]_D \times \frac{\text{molecular weight}}{100}$$

of the sterols, their acetates and benzoates furnish significant clues to the uniformity of the material assumed to be a sterol. The classification of the sterols on the basis of their rotatory power is therefore practically synonymous with a classification based on definite structural features. In the following survey, therefore, the sterols from plants have been classified on the basis of the magnitude of their optical rotatory power.

CLASSIFICATION OF STEROLS ON BASIS OF OPTICAL ROTATION, AND THEIR DISTRIBUTION IN PLANTS

Group I (Structure I)

$\Delta^{5,7}$-Sterols with Negative Optical Rotations in Excess of $[\alpha]_D - 90°$

Sterols belonging to this group possess readily recognizable physical and chemical properties which set them apart from sterols belonging to other groups. The most characteristic structural feature of these sterols, and the one which is responsible for their peculiar properties, is the system of conjugated double bonds in ring B. It accounts for the high negative rotation and also for the very characteristic ultraviolet absorption spectrum of $\Delta^{5,7}$-sterols [Fieser & Fieser (125)]. Since sterols devoid of this structural feature do not absorb ultraviolet light in the regions customarily considered, it is possible to detect and determine quantitatively the presence of considerably less than 1 per cent of sterols belonging to group I in mixtures of sterols or other lipids. It should be kept in mind, however, that the spectrographic evidence only indicates the presence of $\Delta^{5,7}$-unsaturation and does not detect other structural features, such as the nature of the side chain. The older literature contains many references to the presence of ergosterol in plant sterol mixtures which are based entirely on spectrographic evidence. In the absence of supporting data such evidence should be interpreted only as showing the presence of some $\Delta^{5,7}$-sterol rather than a specific compound.

The presence of the conjugated system in ring B confers exceptional chemical reactivity upon the sterols, and it is for this reason that $\Delta^{5,7}$-steroids are particularly attractive to investigators interested in converting readily available steroids into cortical hormones. It is also well known that the irradiation of such sterols brings about a series of rearrangements leading, among other products, to the vitamins D. Since the chemical reactions of $\Delta^{5,7}$-sterols have been most adequately covered in Fieser & Fieser's monograph (125), the present review restricts its comments only to those features which are of use in the determination and isolation of sterols of group I. It has already been mentioned that $\Delta^{5,7}$-sterols may be separated chromato-

graphically from other sterols. The comparative ease with which this is accomplished is probably due to the conjugated system which significantly alters the shape of the molecule. As has been shown by Fieser & Campbell (124), $\Delta^{5,7}$-sterols, like other conjugated substances, show a coupling reaction with *o*-nitrobenzene diazoniumchloride which is accompanied by the appearance of a yellow color. This color reaction and the recently described colorimetric determination by means of zinc chloride (320) appear of value only in such places where ultraviolet spectrophotometers are not available.

When irradiated in an alcoholic solution and in the presence of equimolecular amounts of eosin and in the absence of oxygen, $\Delta^{5,7}$-sterols are dehydrogenated to *bis*-compounds which because of their great insolubility readily precipitate from solution (195, 468). This method has been used to determine the presence of small amounts of ergosterol in the sterol mixtures from *Dactylis glomerata* (322) and *Scopolia carniocola* (467). When irradiated in an alcoholic solution with small amounts of eosin and in the presence of oxygen, $\Delta^{5,7}$-sterols readily absorb oxygen and form nicely crystalline and rather stable transannular peroxides (VII) (45, 470). Ergosterol-peroxide, (VII, R=G) has been isolated at times from the sterol mixtures of fungi (446), and it has been inferred that it is an artefact formed by air oxidation of ergosterol during the isolation procedure. It is quite probable, however, that the peroxide does indeed occur in the fungus where it may play an important role as an intermediate in the biochemical transformation of ergosterol. The natural occurrence of such transannular peroxides in plants would not be unique for it has long ago been shown that ascaridole, the principal constituent of chenopodium oil, is an analogous peroxide of the terpenoid series.

Ergosterol (I, R=G), $C_{28}H_{44}O$, m.p. 166°; $[\alpha]_D$ −132°; ac., m.p. 181°; $[\alpha]_D$ −90°, is the most widely distributed of the $\Delta^{5,7}$-sterols and the one most readily available. Since its discovery by Tanret in 1889 (417a) in the ergot of rye, *Claviceps purpurea*, it has been shown to be the principal sterol of the fungi, lichens, and of at least one green alga. Until now commercial ergosterol has been obtained exclusively from yeast which may contain sterols well in excess of 2 per cent and which can synthesize ergosterol from glucose and other carbohydrates as the only carbon sources. Large potential sources of ergosterol may be found in the mycelia of organisms raised in great amounts in the manufacture of citric acid, various antibiotica and other products.

In many of the fungi, ergosterol is not present exclusively in the free state but also as esters, particularly ergosterylpalmitate. Crude ergosterol is often contaminated by other sterols such as dihydrogosterol, zymosterol, which can be completely removed only by repeated fractional crystallization or chromatographic methods.

Mycosterol, $C_{30}H_{48}O_2$ (?), m.p. 150°; $(\alpha]_D$ −139°; ac., m. p. 169°, has been isolated from various fungi (188). It appears to be an impure sample of ergosterol.

Neosterol, m.p. 164°; $[\alpha]_D$ −105°, one of the minor yeast sterols (465), has recently been shown to be a mixture of ergosterol and 5,6-dihydroergosterol (28a).

22,23-Dihydroergosterol (I, R=C), $C_{28}H_{46}O$, m.p. 153°; $[\alpha]_D$ −109°; ac., m.p. 158°; $[\alpha]_D$ −75°, has not yet been isolated from plants, but it is probably a minor component of many fungi sterol mixtures. It has been prepared by the preferential hydrogenation of ergosterol (473).

14-Dehydroergosterol (VI, R=G), $C_{28}H_{42}O$, m.p. 198–201°; $[\alpha]_D$ −396° (CCl_4); ac., m.p. 164–167°; $[\alpha]_D$ −332° (CCl_4), is the latest addition to the list of natural sterols. Barton & Bruun (27) isolated it from the sterol mixture of a strain of *Aspergillus niger* and beyond doubt proved the structure of this remarkable compound. Because of its exceptionally high negative rotation, this sterol should be readily detectable in mixtures.

The following sterols of group I have not yet been isolated from plant material but there is valid reason to believe that some of them may prove to be components of sterol mixtures showing absorption of ultraviolet light.

7-Dehydrocampesterol (I, R=B), $C_{28}H_{46}O$, m.p. 165°; $[\alpha]_D$ −109°, the 24-epimer of 22,23-dihydroergosterol, has been prepared from campesterol (352).

7-Dehydro-β-sitosterol (I, R=E), $C_{29}H_{48}O$, m.p. 145°; $[\alpha]_D$ −116°; ac., m.p. 152°; $[\alpha]_D$ −71°, has been prepared from a β-sitosterol sample of doubtful uniformity (480).

7-Dehydroclionasterol (7-dehydro-γ-sitosterol) (I,R=D), $C_{29}H_{48}O$, m.p. 138°; $[\alpha]_D$ −98°; ac., m.p. 140°; $[\alpha]_D$ −72°, is believed to be the 24-epimer of 7-dehydro-β-sitosterol. It has been prepared from clionasterol which is common in many sponges (44).

7-Dehydrostigmasterol (I, R=I), $C_{29}H_{46}O$, m.p. 154°; $[\alpha]_D$ −113°, ac., m.p. 172°, has been prepared from stigmasterol (126, 157, 246).

Organisms in which ergosterol has been shown to be one of the principal sterols.

FUNGI. PHYCOMYCETES: *Phycomyces blakesleeanus* (49); *Rhizopodus japonicus* (245).

ASCOMYCETES: *Aspergillus fischeri* [Thom. 5041 (340)]; *A. fumigatus* mut. helvola Yuill, also ergosteryl palmitate and ergosterolperoxide (466); *A. niger* (51, 441); *A. sydour* (408); *Claviceps purpurea*, recent papers (154, 155b, 170b, 176, 350a); *Helvella esculenta* (226); *Neurospora crassa* (309); *Penicillium brevi-compactum*, also ergosteryl palmitate (312); *P. carmino-violaceum* (178); *P. chrysogenum* (79); *P. citrinum*, also ergosteryl palmitate (419); *P. glaucum* (136); *P. luteum* (15); *P. luteum purpurogenum* (15); *P. notatum* (15, 79, 370, 491); *P. notatum B2.1* (1); *P. notatum* var. chrysogenum strain Q176 (418); *P. puberulum* (56); *Saccharomyces cerevisiae* and related species, numerous publications.

BASIDIOMYCETES: *Agaricus campestris* (181); *Amanita muscaria* (486a,e); *Armillaria edodes* (188); *A. matsutake* (290); *A. mellea* (486h); *Boletus cavipes* (132a); *B. edulis* (345); *B. granulatus* (265); *B. luridus* (303); *Cantharellus cibarius* (181); *Calocera viscosa* (132a), *Cortinellus shiitake*, also neoergosterol (?) (188, 412b); *Exidia auricula judae* (486j); *Exobasidium vaccinii* (486g), *Ganoderma lucidum* (250); *Geaster fimbriatus* (354); *Hydnum ferrugineum* (486i), *H. imbricatum* (250); *Hypholoma fasciculare* (486f); *Lactuarius piperatus; rufus, L. scrobiculatus* (486h,k,i); *Lentinus lepideus*, synthesized from glucose and ethanol (378); *L. squamosus* (132b); *Lenzites sepiaria* (486j); *Lycoperdon bovista* (22); *L. gemmatum* (188); *Marasmius scorodonius* (132a), *Panus stypticus* (486j); *Pholiota squarrosa* (486h); *Polyporus applanatus* (486i); *P. confluens* (132b); *P. hispidus* (486k); *P. ignarius* (486d); *P. nigricans* (112b, 486h); *P. pinicola* (156); *P. sulfureus* (487); *Polysticus velubinus* (354); *Psalliota campestris* (486b); *Trametes suaveolens* (486c);

HYPHOMYCETES: *Fusarium lycopersici* (128); *F. solani* D_2 (300); *Helminthosporium velutinum* (342).

LICHENES: *Evernia vulpina* (38); *Gyrophora Dillenii, Parmelia furfuracea* (486v); *P. physodes* (486u); *Peltigera canina* (486t).

ALGAE. CHLOROPHYCEAE: *Chlorella pyrenoidosa* (223).

GROUP II (STRUCTURE II)

Δ^5-STEROLS WITH SPECIFIC ROTATIONS FROM $[\alpha]_D$ $-30°$ TO $-70°$

This group includes the sterols most commonly encountered in higher plants. Their most characteristic structural feature is the 5,6-double bond, whose presence is indicated by the *levo*-rotation and which may be definitely ascertained by comparing the molecular rotations of the sterol with those of its acetate and benzoate. According to the principles of rotational shift first studied by Hudson and by Freudenberg in the carbohydrate series the differences between the molecular rotations of the acetates, benzoates and sterols must be of the same sign and the same order of magnitude if the respective compounds possess the same structural features in the ring system. That such is indeed the case has been shown in recent years particularly by Barton (26). By analyzing a great volume of data and by averaging the rotational values reported for well-established sterols, Barton found that the conversion of a Δ^5-sterol to its acetate is accompanied by an increase of the molecular rotation of -35, and that the molecular rotation of the benzoate is more positive by $+81$ than that of the sterol. The limits of experimental error in these cases are about ± 10. If a newly found sterol undergoes such rotational changes upon acetylation and benzoylation it may safely be regarded as a Δ^5-sterol, the double bond being the only one in the ring system. If, on the other hand, a compound suspected of being a Δ^5-sterol affords acetates and benzoates whose molecular rotation differences are significantly at variance with the known values, it may safely be as-

sumed that this material either is lacking in uniformity or of an entirely different structure. Several of the Δ^5-sterols are additionally unsaturated at 22, 23 in the side chain. The presence of this double bond increases the negativity of the molecular rotation by about -60 ± 20 to 30. It exerts, however, no noticeable influence on the differences between the molecular rotations of the sterols, their acetates and benzoates.

The Δ^5-sterols and their derivatives, unsaturated in the side chain, which are known at present to occur in plants, fall into two groups, the C-24-methyl sterols of the order C_{28} and the C-24-ethyl sterols of the order C_{29}. The C_{28}-sterols do not appear to be as widely distributed as the C_{29}-sterols, and as a consequence comparatively little is known about their presence in many of the typical, complex plant sterol mixtures. As has been pointed out earlier in this review, both C-24 epimers of these sterols are found in nature. In the case of the Δ^5-sterols which may be regarded as C-24-methyl derivatives of cholesterol, those with an α-oriented methyl group (R=C) are distinctly more levorotatory than the β-epimers (R=B), the differences between the molecular rotations being about -55 ± 10. Among the corresponding $\Delta^{5,22}$-sterols these differences are not as clearly pronounced. (43, 407)

Campesterol (II, R=B), $C_{28}H_{48}O$, m.p. 158°; $(\alpha)_D$ −33°; ac., m.p. 138°; $[\alpha]_D$ −35°, has been isolated from rape seed oil (*Brassica campestris*), soybean oil and wheat-germ oil (119). It probably also occurs in many other plant sterol mixtures. Its structure has been firmly established (121c).

Brassicasterol (II, R=G), $C_{28}H_{46}O$, m.p. 146–148°; $[\alpha]_D$ −61 to −64°; ac., m.p. 152°; $[\alpha]_D$ −65°, was first isolated from rape seed oil (*Brassica rapa*) (476) by way of its sparingly soluble acetate tetrabromide. The structure of brassicasterol has been established by ozonization and hydrogenation (122), and through its conversion to ergosterol (38). The last transformation shows that the side chain double bond has the same *trans*-orientation as that of ergosterol.

It is of interest to note that campesterol and brassicasterol which have been isolated from closely related source materials differ in their configuration at C-24. There appears little doubt that the C-24 epimers of the two respective sterols will also eventually be found in plant sterol mixtures. It is in anticipation of their isolation that the properties of these sterols are described below.

22,23-Dihydrobrassicasterol (II, R=C), $C_{28}H_{48}O$, m.p. 158°; $[\alpha]_D$ −46°; ac., m.p. 145°; $[\alpha]_D$ −46°, has been prepared by the selective hydrogenation of brassicasterol (121b).

Chalinasterol (II, R=F), $C_{28}H_{46}O$, m.p. 144°; $[\alpha]_D$ −42°; ac., m.p. 136°; $[\alpha]_D$ −46°, has been isolated from various marine invertebrates (43) in which it is at times accompanied by brassicasterol. Its structure has been established through ozonolysis, and selective hydrogenation to campesterol (47).

β-Sitosterol (II, R=E), $C_{29}H_{50}O$, m.p. 137°; $[\alpha]_D$ −37°; ac., m.p. 127°;

$[\alpha]_D$ −42°, is probably the most widely distributed of all plant sterols. It is the principal sterol of cotton-seed oil (454), tall oil (363), and sugar cane wax (284, 444). The last two industrial products represent the largest potential sources of plant sterols. In addition, β-sitosterol appears to be one of the major components of the many poorly characterized plant sterol mixtures which have been mentioned in the literature during the past 60 years, and a survey of which forms part of this chapter. Notwithstanding its great relative abundance, β-sitosterol does not yet seem to have been isolated in a high state of purity from any natural source. Jones *et al.* (198) and Barton & Jones (29) have shown that specimens of β-sitosterol obtained from tall oil and wheat-germ oil with constants in good agreement with those reported in the literature, were actually far from homogeneous. They tested the homogeneity by first oxidizing the sterol with Oppenauer's reagent and then chromatographing the resulting ketone. They obtained a mixture of unsaturated ketones, sitostanone, an unidentified ketone and hydrocarbons. The principal impurities to expect in β-sitosterol samples are higher aliphatic alcohols and hydrocarbons, γ-sitosterol, dihydrositosterols, C_{28}-sterols such as campesterol and also some stigmasterol which may have escaped separation during the bromination of the acetates.

The purest samples of β-sitosterol known at present is probably the one which has been made by selective sidechain hydrogenation of stigmasterol (50, 121b). The physical data of the 22,23-dihydrostigmasterol thus obtained are sufficiently close to those of the best samples of natural β-sitosterol to dispel doubts concerning the identity of the two compounds.

It has been generally recognized that higher animals excrete the plant sterols of their diet unchanged. This observation has been verified by Rosenheim & Webster (350b) who administered pure β-sitosterol to rats on a sterol-free diet. If, however, cholesterol-free brain powder or Thudichum's phrenosin was administered simultaneously, the β-sitosterol was hydrogenated and excreted as copro-sitostanol, identical with the 24-ethylcoprostanol prepared by Marker & Wittle (262). Stigmasterol and ergosterol remained unchanged under such conditions.

γ-Sitosterol (clionasterol?) (II, R=D), $C_{29}H_{50}O$, m.p. 148°; $[\alpha]_D$ −43°; ac., m.p. 144°; $[\alpha]_D$ −45°, is also widely distributed in plants. It is the principal sterol of soybean oil (36, 63), and a minor constituent of the sterols from wheat-germ oil (14), rye-germ oil and probably also from many other plant fats. It is believed that γ-sitosterol is the 24-epimer of β-sitosterol because upon oxidation γ-sitosterol yields the *levo*-form of 6-methyl-5-ethylheptanone-2 and β-sitosterol the dextro form (103). As yet, however, γ-sitosterol has not been degraded to an acid such as Δ^5-3-hydroxynorcholenic acid which would prove beyond doubt that the configuration of all other carbon atoms are the same as those of β-sitosterol. The possibility, however unlikely it may be, has therefore not completely been excluded that γ-sitosterol may differ from β-sitosterol in the configuration of yet another carbon atom, such as C-20.

On the other hand, Bergmann & Low (43) have presented evidence

indicating that γ-sitosterol is identical with clionasterol, one of the commonest of the sterols of lower marine invertebrates. Clionasterol has also been prepared by the selective reduction of the side chain of poriferasterol (see below) which in turn has been shown to be the 24-epimer of stigmasterol. Thus, since there is good reason to believe that clionasterol and γ-sitosterol are identical, it follows that γ-sitosterol is 24β-ethylcholesterol and that β-sitosterol is 24α-ethylcholesterol.

Stigmasterol (II, R=I), $C_{29}H_{48}O$, m.p. 170°; $[\alpha]_D$ −51°; ac., m.p. 144°; $[\alpha]_D$ −56°, is the best characterized of all plant sterols. It was first isolated by way of its acetate tetrabromide from the sterol mixture of the Calabar bean (*Physostigma venenosum*) (472a), and is at present isolated by an analogous method from soybean sterols (63, 364, 405). It is a component of many plant sterol mixtures and available in potential quantities in sugar cane wax (201). The presence of stigmasterol in moulds such as *Penicillium notatum* (353) remains to be confirmed. The structure of stigmasterol has been established through selective ozonization of the sterol to ethylisopropylacetaldehyde (149) and Δ^5-3β-hydroxybisnorcholenic acid (118). The comparative ease with which the sidechain can be partially removed has lead to extensive investigations of practical conversions of stigmasterol to steroid hormones (Fieser & Fieser (125)].

Poriferasterol (II, R=H), $C_{29}H_{48}O$, m.p. 156°; $[\alpha]_D$ −49°; ac., m.p. 147°; $[\alpha]_D$ −53°, has been shown to be the 24-epimer of stigmasterol (43, 251). So far it has only been isolated by way of its acetate tetrabromide from the sterol mixtures of marine invertebrates, but its isolation from plant material is to be anticipated.

Fucosterol (II, R=J), $C_{29}H_{48}O$, m.p. 124°; $[\alpha]_D$ −38°, ac., m.p. 118°; $[\alpha]_D$ −45°, has already been mentioned as the typical sterol of the brown algae, *Phaeophyceae*. It is also present in minor amounts in a few algae of other classes. Its structure, first suggested by McPhillamy (255) has recently been firmly established by two independant groups of investigators (42, 169). Reviews on the occurrence of fucosterol in algae have been published by Carter *et al.* (77), Black & Cornhill (59) and Shirahama (388c).

Black & Cornhill (59) have recently carried out quantitative studies on the fucosterol content of sea weeds and have observed that it varies on a dry basis between 0.09 and 0.28 per cent. They also found that a correlation exists between the fucosterol content and the total crude fats; a high fat content is associated with a high sterol content. The amount of fat and hence of fucosterol varies with the season. In *Fucus vesiculosus* it increases from 1.84 per cent in February to 3.75 per cent in October. The percentage of total fats decreases with the depth of immersion. Thus, species growing in shallow water, like *Pelvetia caniculata* contain as much as 8.1 per cent of fat, and the *Laminariaceae*, growing in deeper waters, less than 1 per cent. The best yields of fucosterol may therefore be expected from brown algae growing in shallow water and harvested in autumn.

Pelvesterol, which had been described by Japanese investigators as occurring

in various marine algae, has been shown to be identical with fucosterol (388).

The following survey lists in order of the plant families those sterol mixtures in which Δ^5-sterols have been shown or may be assumed to be the principal components. Plants marked with an asterisk have been found to contain phytosterolins which will be discussed in the last chapter.

MYXOMYCETES: *Aethalium septicum*, m.p. 134°; $[\alpha]_D$ −29° (paracholesterol) (136, 346).

CHLOROPHYCEAE: *Cladophera sauteri*, m.p. 136°; ac., m.p. 135°; $[\alpha]_D$ −45° (77, 165). *Enteromorpha compressa*, m.p. 137° ;ac., m.p. 135° (77, 412a). *Nitella opaca*, sitosterol (163). *Pleurococcus Naegelii*, m.p. 127° (77). *Vaucheria harmata* m.p. 130° (77). *Zygnema pectinatum*, m.p. 136°, ac. m.p. 128° (77).

XANTHOPHYCEAE: *Botrydium granulatum*, m.p. 135° (77).

RHODOPHYCEAE: *Ahnfeltia plicata*, m.p. 147°; ac., m.p. 121° (77). *Ceramium rubrum, Chondrus crispus*, m.p. 134°; ac., m.p. 134° (77). *Corallina officinalis*, m.p. 147°; ac., m.p. 121° (77). *Gigartina stellata, Lemania mamillosa, Phyllophora membranifolia, Placamium coccineum, Polysiphonia fastigiata*, m.p. 132–134°; ac., m.p. 134° (77).

LYCOPODIACEAE: *Lycopodium clavatum*, sitosterol (412a).

FILICALES: *Nephrodium filix mas, N. eu-spinulosum, N. austriacum, N. cristatum*, phytosterols (257). *Osmunda regalis*, sitosterol (412a).

GINGKOACEAE: *Gingko biloba**, m.p. 139°; ac., m.p. 128° (134, 401c).

PINACEAE: *Juniperus occidentalis*, m.p. 136°; $[\alpha]_D$ −21°; ac., m.p. 128° (α-sitosterol) (233). *Picea excelsa*, sitosterol, dihydrositosterol (365). *P. mariana*, m.p. 136°; ac., m.p. 119°; $[\alpha]_D$ −36° (479). *Pinus caribaea** (153), *P. echinata, P. palustris* (232a), *P. pinea* (274), *P. sabiniana* (140e), *P. silvestris* (131b), m.p. 135–138°; $[\alpha]_D$ −23°; ac., m.p. 120–127°; $[\alpha]_D$ −30°. *P. Thunbergii*, m.p. 131°; $[\alpha]_D$ −23°; ac., m.p. 117°; $[\alpha]_D$ −21° (matsusterol) (358). *P. sp.*, tall oil, β-sitosterol, dihydrositosterol, stigmasterol (158, 249), sterol esters (113); pinebark oil, β-sitosterol, sitostanol (313), pine wood, β-sitosterol, sitostanol, no stigmasterol (215), pettu oil, m.p. 139°; α_D −32° (313). *Pseudotuga taxifolia*, m.p. 135°; ac., m.p. 126° (147, 232b).

TYPHACEAE: *Typha angustata*, m.p. 138°; $[\alpha]_D$ −33.5°; ac., m.p. 128°, sitosterol (α-typhasterol) (159, 217, 234).

GRAMINEAE: *Agrostis sp.*, m.p. 138° (422), *Andropogon sorghum*, m.p. 141°; ac., m.p. 137° (437). *Coix lacrima-jobi*, m.p. 138°; $[\alpha]_D$ −20°; ac., m.p. 215°; $[\alpha]_D$ −37° (236c). *Cryptostegia grandiflora*, m.p. 139°, ac., m.p. 126° (391b). *Dactylis glomerata*, m.p. 139°; $[\alpha]_D$ −36° (322). *Echinochloa crusgalli**, m.p. 137°; $[\alpha]_D$ −22°; ac., m.p. 216°; $[\alpha]_D$ −26° (193b). *Hordeum sativum*, sitosterol, sitostanol, sitosterylpalmitate (420). *Oryza sativa**, β- and γ-sitosterol, sitostanol, stigmasterol (16, 141, 216, 292, 415, 436). *Panicum milliaceum*, m.p. 136°; $[\alpha]_D$ −31°; ac., m.p. 128°; $[\alpha]_D$ −38° (193a). *Poa sp.*, m.p. 138° (422). *Saccharum officinarum**, β-sitosterol, stigmasterol (201, 283, 444), brassicasterol (?) (446). *Secale cereale*, sitosterol, dihydrositosterol (11, 91, 213, 421). *Setaria italica*, m.p. 139°; $[\alpha]_D$ −22°; ac., m.p. 122°; $[\alpha]_D$ −25° (193c).

*Triticum sativum**, β- and γ-sitosterol, sitostanol (10b, 14, 106, 293), no stigmasterol (411), campesterol (119), sitosterylpalmitate (95), other esters (206, 403). *Zea mays*, β- and γ-sitosterol, sitostanols, stigmasterol, sitosterylpalmitate (8a, b, 13, 139a, 183, 493).

CYPERACEAE: *Cyperus esculentus*, m.p. 135°; ac., m.p. 123° (33).

PALMAE: *Areca catechu*, m.p. 137°; $[\alpha]_D$ −29°; ac., m.p. 218° (236a). *Cocos nucifera*, m.p. 135–140°; ac., m.p. 126–128° (7, 61, 267). *Elaeis guineensis*, m.p. 136°; $[\alpha]_D$ −34°; ac., m.p. 130°; $[\alpha]_D$ −37° (7, 439).

ARACEAE: *Arisaema triphyllum*, m.p. 135° (arisaesterol) (260a). *Pinellia tuberifera*, m.p. 136°; $[\alpha]_D$ −31°; ac., m.p. 121° (295).

BROMELIACEAE: *Hechtia rosea*, *H. scariosa*, sitosterol (261).

LILIACEAE: *Colchicum autumnale*, m.p. 133°; $[\alpha]_D$ −33° (315). *Convallaria majalis* sitosterol (261). *Fritillaria roylei*, m.p. 137°; $[\alpha]_D$ −30°; ac., m.p. 132° (85). *Gagea lutea*, m.p. 136°; $[\alpha]_D$ −34°; ac., m.p. 126° (343). *Gloriosa superba**, stigmasterol sitosterol (86). *Nolina bigelovi*, *N. macrocarpo*, *N. paraviflora*, *N. texana*, *sitosterol* (261). *Scilla maritima*, m.p. 134°; ac., m.p. 126°; m.p. 164°; ac., m.p. 134° (scillisterol) (72) *Smilax off.*, m.p. 148° (303). *S. pseudo-china*, phytosterol (248). *S. sp.**, stigmasterol, β-sitosterol (332e, 394, 440). *Trillium sessile californicum*, sitosterol (261). *Yucca arizonica*, *Y. thompsonia*, sitosterol (261). *Zygadenus intermedius*, m.p. 135°; $[\alpha]_D$ −29°; ac., m.p. 123° (174).

AMARYLLIDACEAE: *Agave melliflua*, *A. palmaris*, *Amaryllis sp.*, sitosterol (261). *Buphane disticha**, m.p. 132° (429a). *Mescal azul* and others, magueys sitosterol (261).

IRIDACEAE: *Iris versicolor**, m.p. 148°; $[\alpha]_D$ −36° (*α-sterol*) (335b).

SALICACEAE: *Salix purpurea*, m.p. 135°; ac., m.p. 119° (486s).

JUGLANDACEAE: *Juglans nigra*, m.p. 137°; ac., m.p. 125°. *J. regia*, *var. sinensis*, m.p. 138°; $[\alpha]_D$ −34°; ac., m.p. 121°; $[\alpha]_D$ −40° (274, 281, 435, 486m). *J. sieboldiana* m.p. 133° (435).

BETULACEAE: *Carpinus betulus*, m.p. 136°; ac., m.p. 122° (65) *Corylus avellana* m.p. 135°; ac., m.p. 122° (66).

FAGACEAE: *Castanea sativa*, m.p. 135° (96). *Fagus silvatica*, m.p. 134° $[\alpha]_D$ −31°; ac., m.p. 121°; $[\alpha]_D$ −32° no stigmasterol (87). *Quercus suber*, m.p. 135°; ac., m.p. 120° (488).

ULMACEAE: *Ulmus campestris*, m.p. 134°; ac., m.p. 118°, sito- and stigmasterol (372, 373, 386m).

MORACEAE: *Cannabis sativa*, m.p. 138° (61). *Ficus carica*, sitosterol, stigmasterol (221, 372). *Humulus lupulus**, m.p. 135°; $[\alpha]_D$ −31; ac., m.p. 122° (337). *Maclura aurantiaca*, m.p. 148° (221). *Morus nigra*, m.p. 132° (133).

URTICACEAE: *Urtica urens*, m.p. 137°; $[\alpha]_D$ −34°; ac., m.p. 127°, no stigmasterol (433, 452, 484a).

OLACACEAE: *Coula edulis*, m.p. 135°; ac., m.p. 129° (449b).

SANTALACEAE: *Santalum album*, m.p. 131°; ac., m.p. 118° (256).

ARISTOLOCHIACEAE: *Aristolochia indica**, m.p. 138°, 146°; ac., m.p. 121° (229). *A. serpentaria**, β-sitosterol (218).

HYDNORACEAE: *Prosopanche burmeister*, m.p. 133° (486 *l*).
POLYGONACEAE: *Radix Bardanae*, m.p. 137°; ac., m.p. 127° (372). *Rheum emodi*, m.p. 136°; ac., m.p. 121° (285). *R. off., ac.*, 118° (verosterol) (430b). *Rumex crispus*, (34), *R. ecklonianus** (430a), m.p. 134°; ac., m.p. 122°.
CHENOPODIACEAE: *Beta vulgaris var. rapa*, m.p. 135°; $[\alpha]_D$ −34 (247).
NYCTAGINACEAE: *Hermidium alipes*, m.p. 136°; $[\alpha]_D$ −19°; ac., m.p. 120° (67).
RANUNCULACEAE: *Adonis vernalis**, m.p. 138°; $[\alpha]_D$ −36°; ac., m.p. 128° (173). *Clematis vitalba*, sito- and stigmasterol (430e). *Hydrastis canadensis*, m.p. 133° (375).
BERBERIDACEAE: *Epimedium macranthum*, m.p. 132°, ac., m.p. 125° (82), *Caulophyllum thalictroides**, m.p. 153° (332c).
MENISPERMACEAE: *Coscinium fenestratum**, m.p. 136°; ac., m.p. 122° (210).
CALYCANTHACEAE: *Calycanthus floridus*, β-sitosterol (89).
ANONACEAE: *Anona muricata**, m.p. 133° (73).
MYRISTICACEAE: *Myristica fragrans**, m.p. 135° (335a).
LAURACEAE: *Actinodaphne hookeri*, m.p. 131°; ac., m.p. 119° (341). *Laurus nobilis*, m.p. 133°; ac., m.p. 125° (275).
PAPAVERACEAE: *Adlumina fungosa**, m.p. 136°; m.p. 152°; ac., m.p. 137° (adluminasterol) (260b). *Argemone hispida**, m.p. 136° (399). *Papaver rhoeas*, sitosterol (20). *P. somniferum*, m.p. 134–137°; ac., m.p. 122–126° (papaveristerol) (61, 62, 69).
CRUCIFERAE: *Brassica campestris*, m.p. 139°, ac., m.p. 136°, sitostanol (410), campesterol (119). *B. juncea*, m.p. 142° (410). *B. napus*, m.p. 142° (61). *B. oleracea capitata alba*, brassicasterol (?) (112a) *B. rapa*, brassicasterol (476), sitosterol (374). *Cheiranthus cheiri*, m.p. 136°; $[\alpha]_D$ −32°; ac., m.p. 129°; $[\alpha]_D$ −21° (268). *Eruca sativa*, m.p. 137–140°, ac., m.p. 126° (211, 410). *Raphanus raphanistrum*, m.p. 136°; $[\alpha]_D$ −32°; ac., m.p. 125°, dihydrosterol, m.p. 155° (?) (raphanisterol) (70). *R. sativus*, m.p. 135° (396). *Sisymbrium irium*, sitosterol (3b).
MORINGACEAE: *Moringa pterygosperma*, m.p. 135° (192).
SAXIFRAGACEAE: *Ribes rubrum*, m.p. 133° (230).
HAMAMELIDACEAE: *Hamamelis virginica*, m.p. 137°, sterylesters (148).
PLATANACEAE: *Platanus orientalis*, m.p. 134° (486m).
ROSACEAE: *Alchimilla vulgaris*, m.p. 132°; ac., m.p. 122° (289). *Gillenia stipulata*, m.p. 135°; ac., m.p. 121° (259). *Parinarium macrophyllum*, m.p. 153°; $[\alpha]_D$ −29°; ac., m.p. 126° (parinariumsterol A); m.p. 147°; $[\alpha]_D$ −24°; ac., m.p. 131° (parinariumsterol B) (401a). *Pirus malus*, ac., m.p. 122° (338). *Prunus armeniaca*, m.p. 134°; ac., m.p. 120° (68, 432). *P. emarginata*, m.p. 137°; ac., m.p. 121° (127). *P. virginiana**, m.p. 136°; $[\alpha]_D$ −34°; ac., m.p. 119°; $[\alpha]_D$ −33° (330a). *P. mume*, sitosterol (414). *Rosa canina*, m.p. 135°; $[\alpha]_D$ −28°; ac., m.p. 127° (443). *R. polyantha*, sitosterol (302).
LEGUMINOSAE: *Acacia confusa*, m.p. 137° (200). *Adenanthera pavonina*, m.p. 135° (288). *Arachis hypogaea*, m.p. 138°; ac., m.p. 125° (7, 61). *Butea frondosa*, m.p. 134°; ac., m.p. 126° (207). *Caesalpina bonducella**, m.p. 133°; ac.,

m.p. 120° (209). *Cassia absus*, m.p. 136°; ac., m.p. 130° (4). *C. angustifolia**, m.p. 143°; $[\alpha]_D$ −38°; ac., m.p. 128° (429b). *C. tora*, m.p. 134°; ac., m.p. 120° (409). *Ceratonia siliqua*, m.p. 135° (486c). *Cicer arietinum*, m.p. 137°; ac., m.p. 128° (slanutosterol) (490), sitosterol (52). *Daviesa latifolia*, m.p. 136° (332f). *Erythrina hypaphorus subumbrans*, m.p. 136°; $[\alpha]_D$ −34°; ac., m.p. 130° (88b). *Erythrophleum guineense*, m.p. 133° (335c). *Glycine soja**, γ-sitosterol, stigmasterol (63, 119, 196, 423), campesterol (299). *Lupinus luteus*, m.p. 136°; $[\alpha]_D$ −36°; ac., m.p. 124°; $[\alpha]_D$ −25°, (caulosterol) (346, 379). *Medicago sativa*, m.p. 133°; ac., m.p. 121° (medicagosterol I) (93). *Pentaclethra macrophylla*, m.p. 141°, ac., m.p. 132° (450). *Phaseolus radiatus*, sito- and stigmasterol (194). *Ph. vulgaris*, β-sitosterol, stigmasterol (paraphytosterol), (193d, 244, 307). *Physostigma venenosum**, sito- and stigmasterol (162, 472a,b, 332b). *Piscidia erythrina*, m.p. 145°; $[\alpha]_D$ −51°; ac., m.p. 124° (piscidiasterol) (94). *Pisum sativum*, m.p. 130°; ac., m.p. 120° (244). *Psorolea corylifolia*, m.p. 128° (384). *Robinia pseudacacia*, sito- and stigmasterol (486s). *Trifolium incarnatum**, *T. pratense**, m.p. 136°; $[\alpha]_D$ −34 to −42°; ac., m.p. 126°; $[\alpha]_D$ −34° (332b, 348a). *Ulex europaeus*, sitosterol (376). *Vicia sativa*, m.p. 135°; ac., m.p. 120° (346), *V. faba* (viciosterol) (54).

LINACEAE: *Linum usitatissimum*, m.p. 133° (182).

ERYTHROXYLACEAE: *Erythroxylon hypericifolium*, m.p. 136°; $[\alpha]_D$ −35° (18).

RUTACEAE: *Casimiroa edulis**, m.p. 135°; $[\alpha]_D$ −20°; ac., m.p. 127° (326). *Citrus aurantiacum*, m.p. 137° (hydrocarotin) (356, 406). *C. grandis*, m.p., 138–140°, m.p. 150°; $[\alpha]_D$ −0.37(?); ac., m.p. 114° (263, 266a). *Dictamnus albus*, m.p. 142° (203). *D. sp.*, m.p. 143°; $[\alpha]_D$ −35°; ac., m.p. 138°; $[\alpha]_D$ −40° (235). *Limonia warnecki*, m.p. 124°; ac., m.p. 138° (449b). *Phellodendron amurense* (291). *Ph. molle*, m.p. 142°; $[\alpha]_D$ −29°; ac., m.p. 137° (202). *Xanthoxylum caroliniarum*, m.p. 140° (101).

SIMARUBACEAE: *Ailanthus glandulosa*, m.p. 135° (486s). *Brucea antidysenterica*, m.p. 136°, 147° (334a). *B. sumatrana*, m.p. 133°; $[\alpha]_D$ −38° (329, 360).

BURSERACEAE: *Canarium polyphyllum*, m.p. 137°; ac., m.p. 123° (449a).

EUPHORBIACEAE: *Aleurites fordii*, m.p. 138°; $[\alpha]_D$ −33°; ac., m.p. 123°; $[\alpha]_D$ −33° (382). *Cluytia similis**, m.p. 159°; $[\alpha]_D$ −45°; ac., m.p. 139° (cluytiasterol) (430c). *Croton gubouga**, ac., m.p. 117° (145). *Euphorbia cyparissis*, stigmasterol (?) (185). *Hevea brasiliensis**, β-sitosterol steryl esters (164, 458), *Jatropha curcas*, *J. glandulifera*, m.p. 135°; ac., m.p. 122° (105, 6). *Joannesia princeps*, m.p., 131° (114). *Putranjiva roxburghii*, m.p. 145°; ac., m.p. 130 (228), sitosterol (135a).

ANACARDIACEAE: *Anacardium occidentale*, sitosterol (317). *Rhus glabra*, m.p. 138°; ac., m.p. 118° (252). *Rh. succedanea*, m.p. 135°; $[\alpha]_D$ −32° (269).

AQUIFOLIACEAE: *Ilex paraguarensis*, ac., m.p. 135° (344), steryl esters (361).

CELASTRACEAE: *Celastrus paniculatus*, m.p. 136°; ac., m.p. 119° (231); m.p. 149° (385). *Evonymus atropurpureus**, m.p. 138°; $[\alpha]_D$ −28°; ac., m.p. 118° (evonysterol), m.p. 134°; ac., m.p. 130° (homoevonysterol) (348b). *E. europaeus*, m.p. 130°; ac., m.p. 119° (486s.)

ACERACEAE: *Acer campestre*, m.p. 128°; $[\alpha]_D$ −25° (117). *A. pseudoplatanus*, m.p. 132°; ac., m.p. 118° (486q).

HIPPOCASTANACEAE: *Aesculus pavia*, m.p. 134°; ac., m.p. 118°, no stigmasterol (486q).

SAPINDACEAE: *Dodonea viscosa*, sitosterol (224). *Xanthoceras sorbifolium*, m.p. 158° (321).

BALSAMINACEAE: *Impatiens noli tangere*, m.p. 166°, stigmasterol (?) (485c).

RHAMNACEAE: *Cascara sagrada*, m.p. 133° (rhamnol) (197). *Chailletia toxicaria*, m.p. 135°; $[\alpha]_D$ −48° (336d). *Rhamnus frangula*, β-sitosterol (rhamnol) (471).

VITACEAE: *Vitis vinifera*, sitosterol (ampelosterol) (17, 367, 368, 369).

TILIACEAE: *Corchorus capsularis*, m.p. 128° (383). *Tilia europaea*, m.p. 126°; $[\alpha]_D$ −30°; ac., m.p. 119°; $[\alpha]_D$ −40° (222a).

MALVACEAE: *Althaea off.*, m.p. 135° (131). *Gossypium sp.**, β-sitosterol, stigmasterol (9, 327, 424, 454). *Hibiscus esculentus*, sitosterol (395).

BOMBACACEAE: *Ceiba pentandra*, m.p. 136°; ac., m.p. 126° (271).

STERCULIACEAE: *Theobroma cacao*, sito- and stigmasterol (273, 279, 339, 353a); [theobromsterols, α-theosterol (166)].

THEACEAE: Teaplant oil, β- and γ-sitosterol, stigmasterol (142).

GUTTIFERAE: *Hypericum perforatum*, m.p. 131° (485b). *Pentadesma kerstingii*, m.p. 154°; $[\alpha]_D$ −55°; ac., m.p. 137°; $[\alpha]_D$ −38° (451). *Vateria indica*, m.p. 134°; ac., m.p. 119° (341).

FLACOURTACEAE: *Gynocardia odorata*, m.p. 132° (328). *Hydnocarpus anthelmintica*, m.p. 133° (324). *Taractogenos kurzii*, m.p. 132° (328).

OLINIACEAE: *Ongoka klaineana*, sito- and stigmasterol (78).

LECYTHIDACEAE: *Bertholletia excelsa*, m.p. 130° (68).

THYMELACEAE: *Daphne genkwa*, m.p. 137°; $[\alpha]_D$ −49°; ac., m.p. 124° (294). *Lasiosophon meissnerianus*, m.p. 133°; $[\alpha]_D$ −31°; ac., m.p. 110° (α-sterol) (348c).

MYRTACEAE: *Eugenia jambolana**, m.p. 135°; $[\alpha]_D$ −42°; ac., m.p. 120° (155a).

ONAGRACEAE: *Oenothera biennis*, m.p. 133°; ac., m.p. 117° (220, 434).

UMBELLIFERAE: *Aethusa cynapium*, m.p. 141°; $[\alpha]_D$ −36° (336c). *Angelica archangelica*, m.p. 135°; $[\alpha]_D$ −37° (402). *A. grosserata*, m.p. 143°; ac., 135° (81). *Daucus carota**, m.p. 138°; $[\alpha]_D$ −36° (115, 484b). *Echinophora spinosa*, m.p. 148°; ac., m.p. 125° (413). *Ferula sumbul**, m.p. 135°; $[\alpha]_D$ −30°; ac., m.p. 122° (172b). *Heracleum spondylium*, m.p. 142°; ac., m.p. 118° (130). *Oenanthe crocata*, m.p. 135° (429c). *Petroselinum sativum*, sito- and stigmasterol (270).

CORNACEAE: *Cornus florida*, m.p. 124°; ac., m.p. 111° (362). *C. sanguinea*, m.p. 132° (486n).

ERICACEAE: *Arbutus unedo*, m.p. 129°; $[\alpha]_D$ −15°; ac., m.p. 110° (arbusterol) (366). *Kalmia polyfolia*, m.p. 128° (116).

OLEACEAE: *Fraxinus excelsior*, sito- and stigmasterol (57). *Ligustrum vulgare*, m.p. 135°; ac., m.p. 117° (486p). *Olea europaea**, m.p. 135–136°; ac., m.p. 121–123° (7, 61, 139b, 336h).

LOGANIACEAE: *Gelseminium sempervirens*, m.p. 136°; $[\alpha]_D$ −40°; ac., m.p. 127° (286b). *Strychnos nux vomica*, m.p. 158° (162).

GENTIANACEAE: *Gentiana lutea*, m.p. 143°; $[\alpha]_D$ −29° (gentiosterol) (55) *Sweertia chirrata*, m.p. 130°; ac., m.p. 129° (258b).
APOCYNACEAE: *Aspidosperma quebracho blanco*, m.p. 125°; $[\alpha]_D$ −29°; ac., m.p. 115° (quebrachol) (168). *Holarrhena febrifuga*, sitosterol (389), *Nerium oleander*, m.p. 137° (276). *Strophanthus hispidus*, m.p. 140°; ac., m.p. 130° (162a, 272). *Tabernaemontana coronaria*, sitosterol (200b). *Thevetia neriifolia**, m.p. 137°; ac., m.p. 130° (53). *Writia tinctoria*, sitosterol (314).
ASCLEPIADACEAE: *Calotropis gigantea*, sito- and stigmasterol (278). *Hoya carnosa*, m.p. 137°; ac., m.p. 128° (214). *Sarcostemma acidum*, m.p. 142° (191).
CONVOLVULACEAE: *Argyreia speciosa*, sitosterol (58). *Convolvulus scammonia**, m.p. 136°; $[\alpha]_D$ −30°; ac., m.p. 123° (331c). *Cuscuta reflexa*, m.p. 135°; $[\alpha]_D$ −31°; ac., m.p. 125° (2). *Ipomoea batatas** (266b), *I. orizabensis** (verosterol) (331b), *I. purga** (333d), *I. purpurea** (331d, 336e), m.p. 133–137°; $[\alpha]_D$ −32 to −34°; ac., m.p. 119–129°.
HYDROPHYLLACEAE: *Eriodyction glutinosum*, m.p. 137° (336f).
BORAGINACEAE: *Pulmonaria off.*, m.p. 133° (485b).
VERBENACEAE: *Clerodendron infortunatum*, m.p. 148°; $[\alpha]_D$ −26°; ac., m.p. 128° (23). *Lippia scabberina**, m.p. 134° (336b).
LABIATAE: *Mentha aquatica* (146), *M. piperita** (64), m.p. 134–135°. *Micromeria chamissonis*, m.p. 135° (333a). *Monarda fistulosa*, m.p. 131°; ac., m.p. 118° (140d). *Orthosiphon stamineus*, m.p. 139°; ac., m.p. 124° (212). *Perilla nankinensis*, m.p. 130°; ac., m.p. 118° (80).
SOLANACEAE: *Atropa belladonna*, m.p. 134° (375). *Datura stramonium* m.p. 138°; ac., 132° (140a). *Fabiana imbricata*, m.p. 134°; ac., m.p. 117° (111). *Mandragora autumnalis*, m.p. 136°; ac., m.p. 123° (404). *Nicotiana tabacum*, m.p. 137°, 141°, ac., m.p. 127° (347, 380, 445). *Physalis peruviana*, m.p. 131°; $[\alpha]_D$ −34° (151). *Scopolia carniocola*, β-sitosterol (scopoliasterol) (375, 469b). *Solanum angustifolium**, m.p. 134°; ac., m.p. 121° (430d). *S. nigrum*, m.p. 129°; $[\alpha]_D$ −30°; ac., m.p. 120° (318). *Withania somnifera**, m.p. 136°; ac., m.p. 128° (258a, 332g).
SCROPHULARIACEAE: *Digitalis purpurea*, sitosterol (140c). *Leptandra virginica*, m.p. 136°; ac., m.p. 120° (verosterol) (331a). *Linaria vulgaris*, m.p. 136–138°; ac., m.p. 117–138°; $[\alpha]_D$ −39° (222c, 472b).
BIGNONIACEAE: *Catalpa ovata*, m.p. 137°; ac., m.p. 114° (180).
PEDALIACEAE: *Sesamum indicum*, m.p. 137°; $[\alpha]_D$ −34°; ac., m.p. 131° (7, 61, 62).
GLOBULARIACEAE: *Globularia nudicaulis*, m.p. 132° (485d).
RUBIACEAE: *Cinchona sp.* β- and γ-sitosterol (cupreol, cholestol, cinchol, quebrachol) (102, 168, 243). *Coffea arabica*, sito- and stigmasterol (31, 35, 97), m.p. 149°; ac., m.p. 149° (coffeasterol) (31). *Galium verum*, phytosterol (296). *Mitragyna inermis*, β-sitosterol (21). *Morinda longiflora*, m.p. 130° (25). *Psychotria ipecacuanha*, m.p. 162°; $[\alpha]_D$ −54°; ac., m.p. 140° (75).
CAPRIFOLICAEAE: *Sambucus nigra* (486p), *S. racemosa* (274), m.p. 134–136°; ac., m.p. 118–126°. *Viburnum opulus**, m.p. 138°; ac., m.p. 123°; $[\alpha]_D$ −38° (170c).

CUCURBITACEAE: *Citrullus colocynthis**, m.p. 125°; ac., m.p. 110° (6).
CAMPANULACEAE: *Platycodon grandiflorum*, m.p. 148°, m.p. 157°; $[\alpha]_D$ −34° (428b).
COMPOSITAE: *Achillea millefolium*, sito- and stigmasterol (140b, f, 254). *Anacyclus pyrethrum*, m.p. 137° (308). *Antennaria dioica*, m.p. 130°; $[\alpha]_D$ −29° (222d). *Anthemis nobilis**, m.p. 139° (325b). *Arctium majus*, m.p. 132°; $[\alpha]_D$ −36°; ac., m.p. 119°; $[\alpha]_D$ −43° (485a). *A. minus*, m.p. 129°; ac., m.p. 111–115° (gobosterol) (386). *Brauneria angustifolia**, sito- and stigmasterol (140b, 172a). *Carthamus oxycantha*, m.p. 135° (396). *Cichorium intibus*, m.p. 133° (283). *Dicoma anomala** m.p. 159°; ac., m.p. 133° (431). *Helianthus annus**, m.p. 136°; ac., m.p. 127° (298, 489). *Lactuca scariola*, m.p. 135°; ac., m.p. 127° (135b). *Matricaria chamomilla**, m.p. 134°; ac., m.p. 123° (222c, 325c). *Parthenium argentatum*, m.p. 128° (221). *Petasites off.*, m.p. 132° (485d). *Tanacetum vulgare*, m.p. 135° (277). *Tussilago farfara*, sito- and stigmasterol (371, 485a), (*l*-sterol 222b).

GROUP III (STRUCTURE III)

Δ^7-STEROLS WITH SPECIFIC ROTATIONS FROM $[\alpha]_D$ −20° to +10°

As a rule Δ^7-sterols, also called γ-stenols, are readily recognizable. Their optical rotations are close to zero degrees if the Δ^7-bond is the only point of unsaturation in the molecule, but more negative if an additional double bond is situated in the sidechain, as for example, at 22:23. The differences between the molecular rotations of the sterols and their acetates are $=15 \pm 15$, and of the sterols and their benzoates $+20 \pm 14$ (26). Of considerable practical importance in recognizing sterols belonging to this group is the fact that the Δ^7-sterols melt significantly lower than their acetates. With but very few exceptions the reverse is true of sterols, monounsaturated in the ring system, which belong to other groups. It has recently been shown by Idler & Baumann (187) that Δ^7-sterols can be separated from mixtures with comparative ease by the chromatography of the colored azoylesters on a silicagel column.

Several methods have recently been introduced which are of considerable use in detecting and estimating small quantities of Δ^7-sterols in mixtures. Wall & Kelley (453) have found that there is a distinct difference between the transmittance curves of color produced by the Liebermann-Burchard reaction [Fieser & Fieser (125)] of Δ^5- and Δ^7-sterol digitonides. The former show a broad band from 620–680 mμ and the latter a sharper band at 680 mμ. Of greater practical importance are the colorimetric determinations of Δ^7-sterols recommended by Moore & Baumann (287) and by Fieser (123). The first method makes use of modified Schoenheimer-Sperry reagents (377), and the other of the reduction of the colorless selenious acid to the colored selenium.

Interest in naturally occurring Δ^7-sterols has recently increased because of the possible use of such compounds in the preparation of cortisone. When treated with mercuric acetate, Δ^7- and also $\Delta^{5,7}$-sterols are dehydro-

genated and converted into compounds with an additional double bond at 9:11. (VIII) The presence of the new double bond facilitates the introduction of oxygen at C-11, one of the most important features in the partial synthesis of cortisone.

Fungisterol, Δ^7-ergostenol (III, R=C), $C_{28}H_{48}O$, m.p. 146°; $[\alpha]_D$ 0°; ac., m.p. 160°; $[\alpha]_D$ −5°, first isolated by Tanret (417b) as a companion of ergosterol in ergot has been shown to be a minor component of the ergosterol mixtures of many of the fungi listed in the survey of group I. More recently fungisterol has been shown to be identical with Δ^7-ergostenol (γ-ergostenol) which is obtainable by partial hydrogenation of ergosterol (459). Many of the older samples of fungisterol including Tanret's original specimen were probably contaminated with ergosterol and 5,6-dihydroergosterol (460).

5,6-Dihydroergosterol, α-dihydroergosterol (III, R=G), $C_{28}H_{46}O$, m.p. 176°; $[\alpha]_D$ −20°; ac., m.p. 180°; $[\alpha]_D$ −20°, is a minor component of yeast sterol mixtures and probably also present in small amonts in the ergosterol samples obtained from other fungi. It is best prepared by the reduction of ergosterol with Raney nickel (240). The sterol has also been reported to be present in tea-seed oil (427), but the evidence merely shows the compound to be a Δ^7-sterol, possibly identical with α-spinasterol (see below).

Episterol (III, R=?), $C_{28}H_{46}O$, m.p. 151°; $[\alpha]_D$ −5°; ac., m.p. 160–162°; $[\alpha]_D$ −3.5°, is a minor yeast sterol of somewhat doubtful uniformity (461, 464). The position of the cyclic double bond has been deduced from molecular rotation data (26). A second double bond must be situated in a terminal position of the side chain, for upon ozonolysis episterol affords formaldehyde in a yield of 45 per cent of theory (464).

α-Spinasterol (III, R=I), $C_{29}H_{48}O$, m.p. 168°; $[\alpha]_D$ −3°; ac., m.p. 187°; $[\alpha]$ −5°, is one of the principal components of the sterol mixtures of spinach (155), alfalfa (120, 219), and of senega root (394). It is probably also the principal component of some of the poorly identified Δ^7-sterol mixtures mentioned in the survey at the end of this chapter. The structure of α-spinasterol has been adequately established by conversion of the sterol to stigmastanol (239), ozonolysis to ethylisopropylacetaldehyde (121), and through its preparation from 7-dehydrostigmasterol (126).

β-Spinasterol, m.p. 148–150°; $[\alpha]_D$ +6°; ac., m.p. 153–155°; $[\alpha]_D$ +5°; γ-Spinasterol, m.p. 160°; $[\alpha]_{5461}$ 0°; ac., m.p. 140°(?); $[\alpha]_{5461}$ −14°; and δ-Spinasterol, m.p. 144°; $[\alpha]_D$ +6°; ac., m.p. 133° (?); $[\alpha]_D$ +1°, (175, 219), are minor components of the sterol mixtures from spinach and alfalfa. The sterols whose uniformity is somewhat uncertain are all structurally related for they afford the same Δ^8,(14)-stigmastenol when hydrogenated under conditions favoring isomerization. On the basis of rotational data they must be assumed to be Δ^7-sterols. If uniform componuds, their differences must be located in the side chain (26).

Chondrillasterol (III, R=H), $C_{29}H_{48}O$, m.p. 169°; $[\alpha]_D$ −2°; ac., m.p. 175°; $[\alpha]_D$ −1°, first isolated from a sponge (46) has since been shown to be the principal sterol of the alga, *Scenedesmus obliquus* (39). It has been shown to be the 24-epimer of α-spinasterol through its hydrogenation to poriferastanol and ozonolysis to ethylisopropylacetaldehyde (46).

Bessisterol, first isolated from the roots of *Momordica cochinchinensis* (CUCURBITACEAE) has been shown to be identical with α-spinasterol (236b). Medicagosterol II, m.p. 164°; $[\alpha]_D$ −2°; ac., m.p. 173°, which has been isolated from alfalfa (93) appears to have been an impure sample of α-spinasterol (120, 219).

Verbasterol, m.p. 142–144°; $[\alpha]_D$ −3.3°; ac., m.p. 108–109°; $[\alpha]_D$ −2.6°, which has been isolated from *Verbascum thapsus* (SCROPHULARIACEAE) (222c) might have been a rather impure sample of a Δ^7-sterol. The possibility, however, is not excluded that the rotations were incorrectly reported, and that the compound is actually a mixture of Δ^5-sterols.

Anasterol, m.p. 157–159°; $[\alpha]_D$ −8°, and Hyposterol, m.p. 100–102°; $[\alpha]_D$ +12°, are compounds of questionable uniformity which have been obtained from yeast sterol mixtures (461). At present nothing definite is known about their structures.

The following survey lists in order of the plant families those sterol mixtures in which Δ^7-sterols have either been shown or are assumed by the reviewer to be some of the principal components. It is of interest to note the presence of Δ^7-sterols appears to be quite typical for the CUCURBITACEAE and to some extent also for the SAPOTACEAE.

SCHIZOMYCETES: *Azotobacter chroococcum*, m.p. 158°; $[\alpha]_D$ −16° (392).

BACILLAROPHYCEAE: *Nitzschea closterium*, ac., m.p. 149–161° (77).

CHLOROPHYCEAE: *Scenedesmus obliquus*, chondrillasterol (42).

CHENOPODIACEAE: *Beta vulgaris*, var. crassa, m.p. 155°; $[\alpha]_D$ ±0° (297). *Spinacia oleracea*, spinasterols [see above, (175, 177, 280)].

TROCHODENDRACEAE: *Trochodendron araloides*, m.p. 145–155°; ac., m.p. 155–158°, 190°, (481).

LEGUMINOSAE: *Acacia confusa*, m.p. 158–160° (200a). *Gleditschia triacanthos*, m.p. 153°; $[\alpha]_D$ ±0°; ac., m.p. 164° (19). *Gymnocladus dioica canadensis*, m.p. 166° (24), *Medicago sativa*, spinasterols (120, 219) (Medicagosterol II) (93). *Mex europaens*, m.p. 153°; $[\alpha]_D$0°; ac., m.p., 146° (376).

THEACEAE: *Thea sinensis*, m.p. 167–168°; ac., m.p. 181°, mistaken for dihydroergosterol? (427). Teaseed oil, ac., m.p. 157–176° (338).

SAPOTACEAE: *Bassia latifolia*, m.p. 156°; ac., m.p. 175° (37). *Butyrospermum parkii*, m.p. 158°; ac., 170° (karitesterol A) (32). *Mimusops hexandra*, m.p. 158°; ac., 176° (mistaken for ergosterol) (316).

CUCURBITACEAE: *Bryonia dioica**, m.p. 137°; $[\alpha]_D$ ±0°; ac., m.p. 157° (330d, 332d), m.p. 159° (485c). *Ecballium elaterium*, m.p. 148°; $[\alpha]_D$ +3.2°; ac., m.p. 157° (330b). *Citrullus colocynthus**, m.p. 162°; ac., m.p. 177°; also,

m.p. 160°; $[\alpha]_D$ +8°; ac., m.p. 170° (330c). *Cucurbita citrullus**, m.p. 164° (333c). *C. melo*, m.p. 160°; ac., m.p. 173° (129), *C. pepo*, m.p. 162–163°; ac., m.p. 175° (cucurbitasterol) (241, 333b). *Trichosanthes dioica*, m.p. 160° (301).

Group IV (Structure IV)

Sterols with Specific Rotations from $[\alpha]_D$ +15 to +30°

All sterols belonging to this group are saturated in the ring system and, with but one exception, also saturated in the side chain. The exception is neospongosterol which, however, has so far only been isolated from a sponge (41), and has not yet been detected in any plant material. Apart from their rotations, the sterols of group IV can therefore readily be distinguished from other natural sterols by their failure to add bromine or to react with perbenzoic acid. When quite pure these sterols do not give any of the typical color reactions of unsaturated sterols. Saturated sterols can easily be freed of unsaturated impurities by subjecting them to a treatment with acetic anhydride and concentrated sulphuric acid in a carbon tetrachloride solution. In this reaction only the unsaturated sterols form colored products, which because of their solubility in alkali can readily be separated from the unreacted saturated sterols (10c, 474). In carrying out this reaction, which has become known as the Anderson-Nabenhauer method, it is best to follow the directions given in Organic Syntheses (304). Saturated sterols occur in small amounts in practically all plant sterol mixtures, but only in a few cases have they been isolated and characterized.

Stigmastanol, dihydro-β-sitosterol (IV, R=E), $C_{29}H_{40}O$, m.p. 145°; $[\alpha]_D$ +25°; ac., m.p. 138°; $[\alpha]_D$ +15°, is a minor constituent of the sterol mitxures of corn oil (10a), wheat-germ oil (10b), cottonseed oil (454), tall oil (363), and of many other plant fats (11, 13, 14). Pure samples of this sterol are best obtained by the hydrogenation of stigmasterol (469a).

γ-Sitostanol, (poriferastanol?) (IV, R=D), $C_{29}H_{50}O$, m.p. 144°; $[\alpha]_D$ +19°; ac., m.p. 144°; $[\alpha]_D$ +10°, is also probably a constituent of many plant sterol mixtures (13, 14). It has been isolated from soybean sterols (63) and prepared by the hydrogenation of γ-sitosterol (14, 63). Since the latter, as has been mentioned earlier, is not readily obtained in a pure state, its hydrogenation products may also be of doubtful uniformity. It has been suggested on the basis of optical evidence that γ-sitostanol and poriferastanol are identical and that γ-sitostanol is therefore the 24-epimer of stigmastanol (43).

Group V (Structure V)

$\Delta^{8(9)}$-Sterols with Specific Rotations from $[\alpha]_D$ =+30° to +50°

Sterols belonging to this group have so far been found only as minor components of yeast sterol mixtures. They are generally obtained by extensive fractional crystallization of the residues left after removal of most of the ergosterol. Their most characteristic feature, unique among natural

sterols, is the location of the cyclic bond. The $\Delta^{8(9)}$-sterols, also called δ-sterols, show some resemblance in their color reactions to the Δ^{7}-sterols. They may, however, readily be differentiated from sterols of other groups by their pronounced dextro-rotations which are the highest of all natural sterols now known. The molecular rotation differences between the sterols and their acetates are -46.5 ± 1, and between the sterols and their benzoates $+11.5 \pm 3.5$ (26).

Zymosterol (V, R=K), $C_{27}H_{44}O$, m.p. 110°; $[\alpha]_D$ +49°; ac., m.p. 108°; $[\alpha]_D$ +35°, is one of the more abundant and best characterized of the minor yeast sterols. It is also the only C_{27}-sterol which has so far been isolated from plants. Since its discovery by Smedley-Maclean in 1928 (398) it has been studied by several groups of investigators and its structure has now been firmly established through the hydrogenation of zymosterol to the known $\Delta^{8}(9)$-cholestenol and cholestanol and through the formation of acetone upon ozonolysis (28b, 161, 463, 464). The isolation of zymosterol from commercial ergosterol residues is difficult to accomplish. It requires numerous crystallizations of the benzoate, chromatographic separations, and purification over the somewhat unstable dibromides (161, 463).

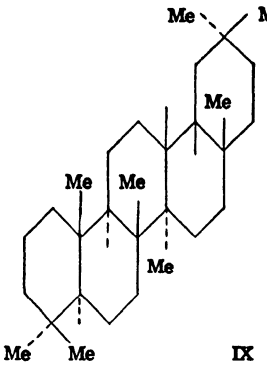
Ascosterol (V, R=?), $C_{28}H_{46}O$, m.p. 142°; $[\alpha]_D$ +45°; benzoate, m.p. 128°–130°; $[\alpha]_D$ +38°, is one of the minor yeast sterols (463, 465). It possesses two double bonds, one of which has been inferred to be in the $\Delta^{8(9)}$-position on the basis of optical evidence (28b). The second double bond is located in the side chain (125).

Faecosterol (V, R=?), $C_{28}H_{46}O$, m.p. 162°; $[\alpha]_D$ +42°; ac., m.p. 159–161°; $[\alpha]_D$ +20°, is another of the minor yeast sterols (461) and closely related to ascosterol (465). The location of a cyclic double bond has been deduced from rotational data (28b). Another double bond must be located in a terminal position of the side chain for upon ozonolysis faecosterol affords formaldehyde in a yield of 30 per cent of theory (465).

Pseudosterols and Other Compounds to which the Term "Sterol" has been Incorrectly or Prematurely Applied

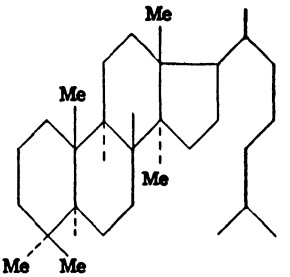
Prior to the recognition of the fundamental structure of sterols many alcohols from plants have been and are still being mistaken for sterols because of their appearance in the unsaponifiable fraction and their resemblance to sitosterol. Since then it has been shown that such resemblances are superficial, and that many of such alcohols are not sterols but triterpenoids of the order C_{30} and that they are derivatives of ring systems such as IX and X.

The nonsterol nature of many of the pseudosterols may often be recognized by their physical properties. Many of the pseudosterols are dextrorotatory in excess of $[\alpha]_D$ +50°, which is the highest specific rotation observed for any true natural sterol (zymosterol). In addition, several pseudosterols and their acetates melt significantly higher than the known sterols and their acetates. Thus, the triterpenoid alcohol, taraxasterol, is readily recognized as a pseudosterol by its high melting point, 221°, and its high



IX

Oleanane



X

Lanostane

dextrorotation, $[\alpha]_D$ +96°. In the absence of such extremes in physical properties, pseudosterols may be recognized by a comparison of their molecular rotations with those of their acetates (26). Among natural sterols devoid of conjugated double bonds the acetates are always more *levo*-rotatory than the corresponding sterols. The opposite is true for most, if not all of the pseudosterols whose acetates are more dextro-rotatory. The only exceptions to this rule are sterols of group I which are more *levo*-rotatory than their acetates. Such sterols, however, may readily be recognized by their characteristic ultraviolet absorbtion spectra.

The following survey lists the mono- and polyvalent alcohols which are certainly pseudosterols. Those which have been definitely identified as triterpenoids have been marked with an asterisk. It is hoped that in future publications the suffix -stol will replace that of -sterol in the trivial names of pseudosterols. Such a change has already been adopted in the case of cafesterol, a pseudosterol now referred to as cafestol (456). In order to point out the types of plants in which pseudosterols are most likely to be encountered, the various named and unnamed pseudosterols have been arranged in order of their source material. It may be noted that triterpenoids are particularly common among the Euphorbiaceae, Asclepiadaceae and Compositae.

ASCOMYCETES: *Elaphomyces hirtus*, mikosterol, $C_{30}H_{54}O_3$, m.p. 265° (190). *Saccharomyces cerevisiae*, cryptosterol=lanosterol*, $C_{30}H_{50}O$, m.p. 139; $[\alpha]_D$ +61°; ac., m.p. 131°; $[\alpha]_D$ +65° (355); cerevisterol, $C_{28}H_{46}O_3$(?), m.p. 254°; $[\alpha]_D$ −40° (184, 460).

BASIDIOMYCETES: *Scleroderma aurantiacum*, m.p. 178°; m.p. 197 (190).

GRAMINEAE: *Oryza sativa*, α-orysterol*, $C_{30}H_{50}O$, m.p. 121°; $[\alpha]_D$ +49; β-orysterol*, $C_{30}H_{50}O$, m.p. 113°; $[\alpha]_D$ +51°; γ-orysterol*, $C_{30}H_{50}O$, m.p. 119°; $[\alpha]_D$ +52° (425, 462); α_3-sitosterol, see below (141). *Triticum sativum*, α-tritisterol*, $C_{30}H_{50}O$, m.p. 114°; $[\alpha]_D$ +54° ac., m.p. 107°; $[\alpha]_D$ +70°; β-tritisterol*, $C_{30}H_{50}O$, m.p. 97°; $[\alpha]_D$ +49° (106, 205 425, 462,); cpd. m.p.

163°; $[\alpha]_D$ +98° (186); α-sitosterol*, $C_{30}H_{50}O$, m.p. 163°; $[\alpha]_D$ −2°; ac., m.p. 137°; $[\alpha]_D$ +29°; α_2-sitosterol*, $C_{30}H_{50}O$, m.p. 156°; $[\alpha]_D$ +4°; ac., m.p. 124°; $[\alpha]_D$ +17°; α_3-sitosterol, $C_{30}H_{50}O$, m.p. 142 ; $[\alpha]_D$ +5; ac., m.p. 52°; $[\alpha]_D$ +6° (50, 141); cpd., m.p. 106°; $[\alpha]_D$ +58°; ac., m.p. 100°; $[\alpha]_D$ +66° (107).

PHYTOLACCACEAE: *Phytolacca americana*, phytolaccasterol, m.p. 170°; $[\alpha]_D$ +35; ac., m.p. 183° (144).

LEGUMINOSAE: *Mimosa pudica*, m.p. 209–211° (3).

RUTACEAE: *Fagara xanthoxyloides*, m.p. 214°; $[\alpha]_D$ +20°; ac., m.p. 118° (306). *Xanthoxylum budrunga*, xanthosterol, m.p. 213–214° (99).

EUPHORBIACEAE: *Euphorbia electa*, cryptosterol (see above) (351). *E. latyris*, euphorbiasterols, $C_{28}H_{36}O_7$, m.p. 200°; $C_{29}H_{36}O_7$, m.p. 247° (108). *E. pilulifera**, $C_{25}H_{40}O$(?), euphosterol, m.p. 275°; ac., m.p. 297°; $[\alpha]_D$ +8°. *E. tirucalli*, taraxasterol (see below) (152). *E. sp.* m.p. 110°; $[\alpha]_D$ +9°; ac., m.p. 117–122°; $[\alpha]_D$ +25° (88). *Phyllanthes acidus*, m.p. 229°; $[\alpha]_D$ +40°, ac., m.p. 266° (438).

ANACARDIACEAE: *Toxicodendron capense*, m.p. 265°; $[\alpha]_D$ −22°; ac., m.p. 244° (167).

AQUIFOLIACEAE: *Ilex paraguarensis*, matesterol, m.p. 276°; $[\alpha]_D$ +65° (pyridine) (160).

CELASTRACEAE: *Lophopetalum toxicum*, m.p. 279–281° (100).

RHAMNACEAE: *Zyzyphus vulgaris*, m.p. 259–260°; m.p. 288–290° (416).

TILIACEACEAE: *Tilia parvifolia*, m.p. 183°; ac., m.p. 161–165° (400).

MALVACEAE: *Gossypium sp.*, m.p. 172°; $[\alpha]_D$ +37° (269).

ALANGIACEAE: *Alangium lamarckii*, $C_{30}H_{48}O_3$, m.p. 302–307° (238).

ERICACEAE: *Arbutus unedo*, unedosterol, $C_{29}H_{48}O$(?), 218°; $[\alpha]_D$ +29°; ac., m.p. 220° (401b). *Kalmia polyfolia*, m.p. 250–255° (116).

SAPOTACEAE: *Bassia latifolia*, bassisterol, $C_{27}H_{46}O$(?), m.p. 211°; $[\alpha]_D$ +26° (428a). *Butyrospermum parkii*, triterpenoid alcohols (163). *Palaquium calophyllum*, m.p. 210°; ac., m.p. 212° (349).

OLEACEAE: *Nycanthes arbortristis*, nycosterol, $C_{27}H_{44}O_2$, m.p. 222°; $[\alpha]_D$+91° (442). *Olea europaea*, oleasterol, $C_{20}H_{34}O$(?), m.p. 174° (336g).

Apocynaceae: *Alstonia costulata*, m.p. 158°; $[\alpha]_D$+60° (179). *Apocynum androsaemifolium**, androsterol, m.p. 214°; $[\alpha]_D$+30°; homoandrosterol, m.p. 236°; $[\alpha]_D$+83° (286a) *Rauwolfia serpentina*, serposterol, $C_{30}H_{48}O_2$, m.p. 160°; $[\alpha]_D$−68.5° (390). *Tabernaemontana coronaria*, coronasterol*, $C_{30}H_{50}O$, 160°; $[\alpha]_D$+44° (200b).

ASCLEPIADACEAE: *Calotropis gigantea*, $C_{28}H_{44}O$(?), calosterol, m.p. 203°; ac., m.p. 211°; $[\alpha]_D$ +105° (30). *Daemia extensa*, sterol A, m.p. 173°; $[\alpha]_D$ +70; ac., m.p. 203°; *sterol B*, m.p. 163°; $[\alpha]_D$ +60°; ac., m.p. 194° also compounds *C* and *D* (109). *Hemidesmus indicus*, $C_{34}H_{60}O$(?), hemidosterol, m.p. 182°; $[\alpha]_D$ +83°; ac., m.p. 198° (110).

SOLANACEAE: *Solanum pseudocapsicum*, m.p. 229°; ac., 202° (391a). *S. xanthocarpum* carpesterol, $C_{36}H_{54}O_4$(?), m.p. 248°; $[\alpha]_D$ −80°; ac., m.p. 194° (357).

ACANTHACEAE: *Blepharis edulis*, $C_{27}H_{42}O_3$(?) m.p. 117°; $[\alpha]_D$ −35°;

$C_{28}H_{48}O_2$(?), m.p. 250°; $[\alpha]_D$ −61° (319). *Hygrophila spinosa*, hygrosterol, $C_{28}H_{46}O$, m.p. 194°; ac., m.p. 208°; $[\alpha]_D$ +28° (138).

RUBIACEAE: *Coffea arabica*, cafestol (cafesterol) $C_{30}H_{48}O_2$, m.p. 155°; $[\alpha]_D$ −138° (397, 456).

CAPRIFOLIACEAE: *Viburnum prunifolium**, $C_{27}H_{46}O$(?), m.p. 187°; $[\alpha]_D$ +115°; ac., m.p. 234° (171).

COMPOSITAE: *Ambrosia artemisifolia*, ambrosterol, $C_{20}H_{34}O$, m.p. 149°; ac., m.p. 113°; $[\alpha]_D$ +28° (170a). *Anthemis nobilis*, α-anthesterol*, $C_{30}H_{50}O$, m.p. 223°; $[\alpha]_D$ +54°; β-anthesterol, m.p. 164° and 195°; $[\alpha]_D$ +57°; δ-anthesterol*, m.p. 150–160°; $[\alpha]_D$ +45°; γ-anthesterylbenzoate, m.p. 240–250°; $[\alpha]_D$ +67° (222a), Probably impure taraxasterol samples (71, 489). *Calendula off.*, calendulasterol*, $C_{30}H_{50}O_2$, m.p. 210–212°; $[\alpha]_D$ +40° (483, 489). *Chrysanthemum cineriaefolium*, homocholesterol, $C_{28}H_{48}O$(?), m.p. 183°; ac., m.p. 223° (492). *Eupatorium chinense* m.p. 213; ac., m.p. 231° (242). *Helianthus annuus*, helisterol*, $C_{30}H_{50}O_2$, m.p. 245°; $[\alpha]_D$ +45° (484b, 489). *Parthenium argentatum*, m.p. 137°; ac., m.p. 196° (373). *Spilanthes acmella*, m.p. 185° (143). *Sp. oleracea*, m.p. 178°, dextrorotatory (137). *Taraxacum off.*, taraxasterol*, $C_{30}H_{50}O$, m.p. 221°; $[\alpha]_D$ +96°; ac., m.p. 251°; $[\alpha]_D$ +120°; ψ-taraxasterol*, m.p. 200°; $[\alpha]_D$ +47°; ac., m.p. 234°; $[\alpha]_D$ +53° (71, 393, 489); homotaraxasterol, m.p. 163°; $[\alpha]_D$+25°; ac., m.p. 219°; $[\alpha]_D$+28° (325, 486r). *Taraxacum platycarpum*, $C_{22}H_{36}O$, $[\alpha]_D$+66°, ac., m.p. 179° (189).

Higher aliphatic alcohols and possibly also hydrocarbons have occasionally been mistaken for sterols. Thus the components of grasses, chortosterol and hippocoprosterol, m.p. 79°; ac., m.p. 61°, have been shown to consist principally of cerylalcohol (323). It is certain that the following alleged sterols also belong to this group: compound, m.p. 74°, from *Equisetum arvense* (74), rhamnosterol, m.p. 85°, from *Rhamnus catharticus* (426), and conduransterol, m.p. 52°, from *Marsdenia condurango* (*Asclepiadaceae*) (76). The sterol from the seed oil of *Zyzyphus xylopora* (*Rhamnaceae*), m.p. 208°; ac., m.p. 62° (5) appears to be a mixture of triterpenoids and higher aliphatic alcohols. Daucosterol, m.p. 283°, from *Daucus carota* (115) has been shown to be a sterolin by its hydrolysis to D-glucose and β-sitosterol (484b). Shesterol, from *Rhamnus catharticus* was found to be quite unrelated to sterols and identical with emodinanthranol glucoside (227).

Lack of data does not yet permit a temporary classification of the "sterols" isolated from the following plants: *Endocarpon minitiatum* (LICHENES) m.p. 133° (168b); *Lilium candidum*, liliosterol (282); *Phytolacca americana*, m.p. 108°; $[\alpha]_D$ +70°, no acetate (?) (144); *Celastrus paniculatus*, m.p. 184° (455); *Garcinia mangostana* (Guttiferae), mangostansterol, m.p. 106°; ac., m.p. 113° (104); *Peudecanum decursivum* (UMBELLIFERAE), m.p. 123°; $[\alpha]_D$ −183°, ac., m.p. 125° (83); *Ipomoea hederaca* (CONVOLVULACEAE), *coprosterol*(?) (206); *Solanum xanthocarpum*, m.p. 122°; $[\alpha]_D$ +16°, and m.p. 142°; $[\alpha]_D$ −83° (150); *Grindelia robusta* (COMPOSITAE), m.p. 166° (336a). *Oryza sativa* (GRAMINEAE), *satisterol*, m.p. 156°; $[\alpha]_D$ −140°; ac., m.p. 111°; $[\alpha]_D$ −10° (216).

PHYTOSTEROLINS

During the first decade of the present century, Power and his associates observed in a number of plant oils a polyhydric alcohol, which melted with decomposition around 285–290°, and which gave an acetate of m.p. 160°. The first compound of this type was found in *Ipomoea purpurea* (CONVOLVULACEAE) and it was consequently named ipuranol (336e). Compounds either identical with ipuranol or closely related to it, such as citrullol (332c) and trifolianol (348a) were isolated from widely different species of plants. Eventually, other products with properties reminiscent of those of ipuranol were discovered and described under a variety of names such as bryonol, grindelol, anonol, ipurganol, cluytianol, calabarol, and cucurbitol (332d).

Since these compounds gave the color reactions of sterols but differed from them in their oxygen content and high melting points, Power & Salway (332d) suspected them of being the glucosides of plant sterols, such as sitosterol. When they hydrolyzed ipuranol in amylalcoholic hydrochloric acid they indeed obtained a sitosterol of m.p. 136° and glucose, and therewith proved the compound to be sitosterylglucoside. Analogous compounds upon similar treatment gave sterols and a carbohydrate. Power & Salway therefore assumed all the compounds mentioned above to be phytosterol glucosides for which they proposed the collective name of phytosterolins. At present this term is often used in its abbreviated form, sterolins. The differences between the sterolins probably parallel the differences between the sterol mixtures from various plants. Salway (359) synthesized a sterolin from sitosterol and acetobromglucose, and obtained a product, m.p. 295–300°; tetraacetate, m.p. 166–167°, which very closely resembled the natural sitosterolglucoside (ipuranol). Sterolins have been obtained from the plants marked with an asterisk in the various surveys included in this review. For references to additional plants see (208, 264, 334b). The sterolins are not restricted to higher plants but are also found in algae, such as *Nitella opaca* (165). Most of the sterolins are glucosides of Δ^5-sterols, and their rotations in pyridine are close to $[\alpha]_D$ −40° (458); those of their tetraacetates in chloroform are about $[\alpha]_D$ −20° to −25°. Glucosides of sterols from other groups are also known such as a γ-spinasterol glucoside, m.p. 275–280°; $[\alpha]_{5461}$ −33° (pyridine); tetraacetate, m.p. 178.5°; $[\alpha]_{5461}$ −13° (chloroform) (175).

Two methods have been recommended for the isolation of sterolins from plant oils. Jantzen & Gohdes (196) saponify the fat, extract the unsaponifiable material, and then acidify the soap solution. The fatty acids are then extracted with ether. If sterolins are present, they collect at the interphase as a flocculent material. It is collected and recrystallized from amylalcohol or a mixture of pyridine and ethanol. Additional small quantities of sterolins are found in the unsaponifiable and acid fractions.

In the isolation of large quantities of soybean sterolins Thornton, Kraybill & Mitchell (423) treated crude soybean oil with a synthetic silica absorbent which removes almost completely lipids such as phosphatides,

sterols, glucosides and pigments. The adsorbent was then extracted with acetone and the oil thus obtained was cooled. The precipitate was collected, washed with acetone and recrystallized from amylalcohol. Further purification of the sterolin was carried out over the tetraacetate. In this manner, using 30 kg. adsorbent, the authors obtained 235 g. of steroline from 4.450 kg. of oil.

Thornton, Kraybill & Broome (424) also recommend the use of milder conditions in the hydrolysis of sterolins which avoid decomposition of the carbohydrate. Hydrolysis is effected by refluxing the sterolin with 2 per cent ethanolic hydrochloric acid. Most of the ethanol is then removed under reduced pressure, water is added to the residue and the sterol is filtered and recrystallized. The aqueous filtrate is then refluxed for 6 hr., neutralized with silver carbonate, and the glucose is isolated as its benzimidazole derivative, m.p. 213–214°.

The authors showed that the soybean sterolin was a mixture of at least two components, for the sterols obtained upon hydrolysis could be separated by way of the acetatebromides into stigmasterol (24 per cent) and sitosterol of uncertain uniformity. The composition of this sterol mixture was approximately the same as that of the sterol mixture obtained by alkaline saponification of soybean oil. It may therefore be assumed that most of the other sterolins which have been reported are also mixtures.

It appears certain that neither of the two methods mentioned above fully recovers the sterolins present in plants. Sterolins are only slightly soluble in fats, and Kondo & Mori (225) have shown that an extraction of soybean press cake with ethanol yields additional quantities of sterolins.

Whitby (457) has described a color reaction which appears to be typical for sterolins and hence of use in differentiating such products from certain high-melting triterpenoid alcohols (pseudosterols). The reaction consists in warming a few particles of material in a small tube with concentrated sulfuric acid until dissolved. The solution is then cooled and a saturated, aqueous solution of thymol is added carefully without mixing. In the presence of sterolins the lower layer turns orange with strong fluorescence, and a violet color appears at first at the interphase and later spreads throughout the upper layer. The last effect is not shown by free sterols.

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