LEAF GROWTH FACTORS

Thesis by

David M. Bonner

In Partial Fulfillment

of the

Requirements for the Degree of

Doctor of Philosophy

CALIFORNIA INSTITUTE OF TECHNOLOGY

Pasadena, California

1940

Foreword

For his teaching, as well as for his invaluable advice and criticism, the author would like to express his appreciation to Professor A. J. Haagen-Smit, with whom this work was carried out.

To Professor Went, who originally suggested the problem of "Leaf Growth Factors", and with whom the preliminary experiments were done, the author gives his sincere thanks. His continued interest and his many helpful suggestions were greatly appreciated.

The author is greatly indebted to Professor H. E. Hayward at whose instigation the histological examination was undertaken, and whose aid and advice made possible its carrying out.

The author wishes to thank Professor J. van Overbeek for many helpful suggestions and Professor E. G. Anderson for permission to use the facilities of the farm at Arcadia, California.

For capable technical assistance the author is greatly indebted to Messrs. L. Castle and J. Greene.

Lastly the author would like to take this opportunity of expressing his appreciation to his brother James, with whom the green plant tests were carried out, for his continued encouragement and unfailing supply of good ideas.

Table of Contents

*****

* *

Introduction

That plants require hormones, or correlation carriers, was postulated years ago by Julius Sachs. However, it was not until 1928 that the first plant hormone was clearly shown by Went (109) to be a definite chemical substance. To avoid possible confusion with animal mechanisms, the plant hormones were later termed phytohormones. The realization that organisms in other than the animal kingdom require vitamins for growth and development came even more slowly. In 1901 Wildiers (115) clearly recognized that yeast needs special growth factors which he collectively termed "bios". Linossier in 1919 (61) pointed out that molds need vitamin like substances. Bottomley (20,21) demonstrated in 1914 that many plants appeared to need accessory growth factors which he termed "auximones" and which he further showed were organic in nature. The beginning of the twentieth century was however strongly mechanistic, and these suggestions seemed too vitalistic to be accepted by the workers of the time, who spent considerable energy in attempts to show that bios and auximone effects were due to inorganic substances. With the isolation of the animal vitamins, and of the first phytohormone (Kögl and Haagen-Smit, 1931)(49) impetus was given to the study not only of growth factor requirements for microorganisms but also of the individual organs of higher plants, which has led within the past five years to the realization that the auximones of Bottomley rested upon a sounder basis than was originally thought. From this study of the hormones and vitamins of bacteria, fungi

and higher plants there has developed a gradual recognition that the chemical factor or factors limiting the growth not only of different organisms, but of different organs within a single organism vary considerably. It is possible to recite at the present time numerous chemical substances known to be the chemical factor limiting the growth of one or more organisms. It, however, has also become increasingly clear that this multitude of requirements does not indicate variation in the chemical factors controlling basic cellular processes, but rather variation in the ability of the different organisms to synthesize these factors. There is little evidence indicating differences in the basic cellular processes between the various organs of a higher plant. Differences in growth factor requirements between the various organs, may therefore be construed as owing to differences in synthetic ability of the different organs.

Julius Sachs (90) nearly sixty years ago, brought forward the first complete as well as modern theory of morphogenesis and correlation based in essence upon differences in growth factor requirements of different organs. Sach's starting point was the fact that morphological differences between plant organs must be due to differences in their chemical composition. These differences he reasoned must be present at the time of the organ's initiation, even though the chemical methods available in the days of Sachs were too crude to detect differences of such small magnitude. He clearly distinguished these morphogenetic substances from ordinary foodstuffs, i.e. those substances present in much greater abundance and out of which the bulk of the plant is made. He also realized that these substances must be present at very great dilutions, and their potency thus out of all proportion to

their concentration. The identification of these substances postulated by Sachs has, however, proceeded but slowly.

The first of Sachs' substances to be recognized was the factor concerned in stem elongation. The evidence for this substance came mainly through work on phototropism, since as shown by Charles Darwin (30) in 1880, the effects of light and gravity are perceived by the tip of the plant, and the effects of this perception are then transmitted to the lower regions which in turn react. Boysen-Jensen (22-24) in the years 1910-1913 showed that the effect of unilateral light illumination could be transmitted across a wound gap, which finding was soon confirmed by Paál (77). Paál was further able to demonstrate that the tip must be the primary seat of a growth regulating center, postulating that growth of the coleoptile is controlled by the tip through the agency of a diffusible substance (78). The demonstration of this diffusible substance as a definite chemical entity that could be freed of the plant had, however, to wait nearly ten years after the work of Paál (77,78). Went (108,109) in 1928 was the first to successfully obtain the active substance free of the plant, and show that this extract has the same growth regulating properties as the original coleoptile tip. Went through suitable application of the bending induced in Avena plants by unsymmetrical application of this active substance to the decapitated Avena coleoptile also devised a practicable quantitative bio-assay for the substance in question, the Avena test. With a quantitative assay for this substance, and with the knowledge that it could be obtained free from the plant, its isolation

was possible, the pure crystalline form being first obtained in 1931 by Kögl and Haagen-Smit (49), who suggested the name "auxin" for this crystalline substance active in promoting stem elongation. Following its isolation Kögl, Haagen-Smit and Erxleben (50-52) soon elucidated its chemical structure. Recent work of Went (110) has indicated that auxin is not the sole limiting factor for stem growth. His experiments indicate that a second factor produced in the roots, which acts in conjunction with auxin must be present for stem elongation. The chemical nature of this root factor is at present unknown, and Went (110) has tentatively termed it "caulocaline". Biotin is known to occur in pea seeds (55), and it has been shown to have a very marked promotive effect upon stem growth in excised pea embryos (55). Biotin is also known to occur(55) in roots, and has recently been shown to be synthesized in isolated roots (10). That biotin and caulocaline are probably not identical, however, may be seen in the fact that biotin is much more abundant in the leaves than in the roots (55,10). Estrone is known to have a marked promotive effect upon the growth of excised pea embryos (55), and upon green plants (91,8). That this promotive effect of estrone is primarily upon stem growth is indicated in that estrone is totally inactive in promoting growth of isolated roots (13), or leaves (table 11). Stem growth factors might be summarized as follows: auxin and caulocaline appear to be the primary factors limiting stem growth, while secondary factors include biotin and estrone.

It was clearly shown by Kögl and Haagen-Smit (54) that a different group of growth factors is required for root growth. They showed that concentrations of auxin which promote stem elongation strongly inhibit root elongation, indicating that auxin it-

self was not concerned in root elongation. In order, however, that the growth factor requirements of separate organs be successfully established it is necessary as pointed out in 1902 by Haberlandt (40) that the organs be grown entirely freed from the influences of other organs. In the early 1920's Robbins (82,83) and Kotte (59,60) were able to grow a number of different isolated root meristems. These workers noted that additions of yeast extract, meat extract, or similar substances, to the medium of inorganic salts, and sugar somewhat increased the growth of these isolated roots. "Potentially unlimited" growth through many subcultures was reported in 1934 by White (114) for tomato roots upon a medium of sugar, inorganic salts, and small amount of yeast extract. Thiamin, known to be present in yeast extract, was noted by Kögl and Haagen-Smit (55) to considerably increase root growth of excised pea embryos. Bonner (9) and Robbins and Bartley (86) found that thiamin could largely replace the effect of yeast extract on the growth of isolated root meristems of pea and tomato and was in fact essential for root growth. Thiamin alone, however, could not entirely replace yeast extract over a long period of time. Addicott and Bonner (1) found nicotinic acid necessary in addition to thiamin for continued optimum growth of pea roots, and Robbins and Schmidt (87) found vitamin B_6 a necessary second factor for growth of tomato roots. Recent work of Bonner (10,13) has indicated that thiamin requirement for root growth is quite general, and that different roots if requiring secondary factors, require nicotinic acid, or vitamin B_6, or even both. Root growth factor requirements may be summarized as follows: thiamin appears to be needed by most roots, while secondary individual requirements include such

growth factors as nicotinic acid and vitamin B_6.

Many other growth factor requirements of higher plants might be cited. Flower initiation is known to be controlled through an unidentified diffusible chemical substance (92). Initiation of root primordia is controlled by auxin in conjunction with rhizocaline (98,112). There have been few investigations, however, on the growth factor requirements of that important plant organ, the leaves. That so few investigations have been carried out upon this subject may be due to the tacit assumption that leaves containing chlorophyll are able with the aid of sunlight to synthesize all the factors they may need.

Object of the present work

The object of the present work is an attempt to establish the general nature and mechanism of the growth factor requirements of isolated leaves, with the relationship of such requirements to the growth factor requirements of stems and roots.

Chapter I

***

The study of leaf growth leading to the evidence for a hormonal control.

Prior to 1935 few direct studies of the growth factor requirements of isolated leaves were made. Of the earlier work, only that of Vyvyan (102), and of Gregory (37) bears upon this problem. Vyvyan using a photographic method with which he could measure leaf growth directly without detaching the leaf from the plant showed that the presence of the cotyledons affected the growth of leaves of seedling Phaseolus vulgaris plants, and that the removal of one of the two first foliage leaves considerably increased the growth rate of the remaining leaf. He unfortunately worked with plants grown in soil and in sunlight, thereby obscuring the importance of the cotyledons for leaf development in seedling plants, and was led to ascribe his effects as due to availability of "food", making no distinction between carbohydrates, and accessory "food", i.e. growth factors. Gregory using a photographic method similar to that of Vyvyan studied the increase in leaf area of Cucumis sativus under varying conditions. Measurement of the Q_{10} of leaf growth led him to suggest that there was formed in older leaves under the influence of light a special factor or factors necessary for the growth of younger leaves. He states, "the action of light is not inconsistent with a hypothesis of a master photochemical reaction independent of carbon assimilation leading to the formation of a substance directly involved in leaf expansion." He thus clearly differentiated between the effect of carbohydrates on leaf growth, and of other special substances formed in the light. A further interesting observation of Gregory is of a close relationship existing between root and leaf growth in barley. He

found the growth rate of the two organs closely similar, which he ascribed as due to the roots deriving their carbohydrates from carbohydrate synthesized in the leaves, and the leaves in turn deriving their nitrogen supply from the roots.

Impetus was given the problem in 1935 through the work of Avery (3). Avery, working with leaves of Nicotiana correlated growth of the midrib and probably of the larger lateral veins with the auxin concentration of the leaf tissue. He found the highest auxin concentration where greatest elongation occurred. By applying auxin in lanoline paste to the midrib of Nicotiana leaves, Avery found the leaf bent away from the point of application, thus exhibiting a typical auxin growth response. A similar application to the mesophyll was without apparent effect. Went and Thimann (113) corroborated these findings of Avery showing that auxin influences elongation of the cells of the midrib, and lateral veins with no increase in mesophyll area. On the basis of these experiments Went and Thimann (113) suggested that is is advantageous to distinguish between growth of the vein, which can be increased by auxin application, and growth of the mesophyll which is independent of auxin. The direct demonstration of a definite growth factor controlling mesophyll growth was obtained by Went (110). The nature of his evidence is as follows: A group of etiolated pea plants about ten centimeters in length were divided into four sets. The plants of the first group were left intact, the plants of the second group had their root cut off just below the cotyledons, the latter being left intact. In the third group the cotyledons were cut off close to the stem, thus leaving the stem and roots intact, while the fourth had both the root and cotyledons removed. Went found that removal of the roots did not affect leaf growth, the

mean leaf surface of such plants being exactly the same as that of plants whose roots had been left intact. Removal of the cotyledons, however, leaving the roots, exerted a very marked effect upon leaf growth, the leaves in such treated plants having but half the area of untreated plants. Removal of both cotyledons and roots had no greater effect than removal of the cotyledons alone, which would be expected since removal of the roots alone was without effect. Went's conclusion from these observations was, that in the cotyledons there is present a substance necessary for leaf growth of etiolated pea seedlings. For the sake of convenience, Went termed this substance, present in the cotyledons affecting mesophyll growth, "phyllocaline" intending that this term be used only until chemical names could be used to describe the substance or substances in question.

Although removal of the roots did not affect mesophyll growth (i.e. mean leaf surface) Went (110) found that it did affect leaf length (i.e. vein growth) as well as petiole growth. This effect was very similar to the effect upon stem growth produced by removal of the roots, thus substantiating the earlier conclusion that the growth factor requirements of vein and petiole growth are closely similar to those for stem growth. In this connection it is of interest to note Stauffert's (97) conclusion that vein growth and mesophyll growth are phenomena which are differentially affected by genes. For the laciniate form of Chelidonium majus it was concluded that this character apparently does not reflect a morphogenetic gene but results merely in a decreased mesophyll growth.

Further evidence for the existence of phyllocaline was obtained by Went through the use of transplantation experiments in which the etiolated tops of various pea varieties were grafted upon a variety of different root stocks (111). He found that the results of such experiments are obscured to some extent by the fact that not only are the responses dependent upon the cotyledon bearing root stock, but also upon the specific reactivity of the top. The pea varieties used as root stock affected leaf growth, as well as petiole, stipule and stem growth; the growth of these various organs being affected differentially. Differences in the dry weight of the seeds of different varieties were found to be small and since the bulk of the dry weight is due to stored carbohydrate, these differences, Went (111) concluded, must likely be due to differences in stored amounts of specific growth factors, as for example in amounts of stored phyllocaline.

The work that has been reviewed leads to the following conclusions: Vein growth and mesophyll growth are controlled by different sets of growth factors, vein growth being auxin dependent, and mesophyll growth being auxin independent. In the cotyledons of pea there is present a substance or group of substances capable of increasing the growth rate of leaves in etiolated pea seedlings. Under the influence of light older leaves are able to synthesize a substance distinct from carbohydrates which is essential for the growth of younger leaves.

Chapter II

***

Preliminary experiments.

Since it was known that in etiolated pea seedlings, leaf growth depends upon growth factors stored in the cotyledons, pea cotyledons were chosen as a source of leaf growth factors. The usual plant extract has the serious disadvantage that hydrolysis of the active principal may occur, and many toxic substances be released. In view of this fact a "diffusate" similar to that used by Kögl and Haagen-Smit (55) in the preparation of biotin from pea seeds was used in preparing a crude extract of leaf growth factors. Since growth of the mesophyll under suitable conditions will be reflected mainly in increase in surface area, experiments on growth in leaf area may be used to analyze growth factors of the mesophyll. It was found that a diffusate of pea seeds, as described above, caused appreciable growth in detached growing leaf sections, and that this growth could be simulated, in part at least, by the use of sugars and amino acids as the culture solutions.

Early experiments and observations were carried out with leaves of pea seedlings, and with the leaves of Carica papaya. To determine whether the factors diffusing from the cotyledons were in any manner correlated with the effect of these same cotyledons on leaf growth in the intact plant, diffusates of Daisy and Alaska pea varieties were compared. Equal weights of peas were diffused, and the diffusates concentrated to equal volumes. They were then tested for effect on leaf growth of Little Marvel peas, which had been found to show the greatest differences in leaf size when grafted on various varieties in transplantation experiments (111). Diffusate from Daisy was found far more effective than

that from Alaska. This is in agreement with the observations that leaf growth is greater in shoots grafted on Daisy than in shoots grafted on Alaska peas (111).

When leaf strips, cut from the leaves of Carica papaya are floated on the solutions, it was observed that growth along the edges of these strips was greater than that in the middle portion of the strip. A more uniform response was obtainable by perforating the leaves with a fine steel brush. Many cells are killed and wounded by such treatment, but this allows easier penetration of the solution to the intact cells in all parts of the section, resulting in uniform growth instead of growth merely along the cut edges of the section. This effect, however, is dependent upon the kind of leaf, presumably upon the permeability of the lower epidermis, and will be discussed later in chapter IV.

Another observation was made in connection with the effect of the solutions on the parenchymatous regions of the leaf. A differential growth rate between parenchymatous and vascular tissue would be expected to result in a characteristic bulging of the tissue. When sections containing a large unstretchable vein are floated on a pea diffusate medium they show marked bulging of the intercostal regions, since the parenchyma is growing more rapidly than the veins, yet the size of the section is determined by the length of the vein. This is just the reverse of the effect obtained by auxin treatment, when the veins bulge out between tautly stretched mesophyll. If sections are used which have small veins, however, these will be stretched by the growing mesophyll, and since the size of the section is determined by the growth of the parenchyma the leaf section will be plane.

These observations may be summarized as follows:

(1) A crude source of leaf growth factors may be obtained by collecting the water in which pea seeds have been submerged.

(2) Leaf sections having small stretchable veins can be used satisfactorily in analyzing for mesophyll growth factors.

(3) There is a correlation between the factors diffusing from the cotyledons with the effect of these same cotyledons on leaf growth in the intact plant.

Chapter III

***

The leaf test.

In order that an object, a leaf in this case, be satisfactory for use in a quantitative bio-assay, (1) it must be reactive to the substance in question, and (2) the average of several leaves must be uniform from test to test. The following list gives the rather wide variety of leaves satisfying the first requirement, these being young rapidly growing leaves.

Brassica oleracea L.
Carica papaya L.
Lactuca sativa L., var. capitata L.
Lactuca sativa L., var. longifolia L.
Ludwigia L.
Nicotiana sylvestris Spegaz & Comes.
Nicotiana tabacum L.
Raphanus sativus L. (French Breakfast)
Phaseolus vulgaris L. (Kentucky wonder)

That rapidly enlarging tissue is more sensitive than slowly growing tissue was further illustrated by determining the sensitivity of different portions of a young leaf of Nicotiana sylvestris. As shown in table 1, the proximal portion is the most sensitive, and Avery (3) has shown it to be the most rapidly enlarging portion. Older leaves (6 cm. long) are found to be either only slightly sensitive, or as a general rule totally insensitive.

In order to satisfy the second requirement, it must be possible to collect the leaves in such a way that the growth rate of the leaves used for different tests, and therefore of leaves collected at different times, is limited approximately to the same extent by the leaf growth factors of some standard crude extract containing leaf growth factors, as for example pea diffusate. It was early noticed that by collecting young leaves from certain pea varieties and from Nicotiana plants, some leaves would grow almost as well in sugar as in the pea diffusate, showing that their growth

rate was limited primarily by sugar. On the other hand, some leaves of the same plant grew little in sugar but a great deal in pea diffusate, showing that their growth rate was limited by some additional factor. Such leaves obviously do not meet the second requirement just mentioned. The first foliage leaves from seedling plants, however, might be expected to be much more uniform in their sensitivity since they are dependent for their growth factors upon themselves and the cotyledons, and do not receive similar factors from older leaves. This was found to be the case in Raphanus and Nicotiana seedlings. The first foliage leaves of seedling radish and tobacco plants have been the only such leaves worked with extensively, but from the number of plants (listed above) that respond to the pea diffusate, it might be concluded that the effect of the leaf growth substances of the pea diffusate is not species specific.

It has been found that in order to obtain leaves that may be worked with satisfactorily, the plants must be grown in the light. Leaves from etiolated pea seedlings proved too small and curled to work with, and in the case of radish there is no epicotyledonary development in plants grown in the dark. An attempt was made to cause leaves to become more deficient in leaf growth factors by allowing the plants to remain in the dark a short period before collecting the leaves, and thereby increase their sensitivity. However, no further increase in sensitivity could be obtained by allowing plants to remain in the dark for 24 to 48 hours before collection. The length of the photoperiod under which the plants were grown too was found quite without effect upon the sensitivity of the leaves to crude pea diffusate.

Raphanus plants were finally selected for the leaf test, since large quantities of the seed were readily obtainable, and it is possible to grow large numbers of plants in a short time. Nicotiana can be used in the leaf test though less conveniently, as has been shown in numerous experiments. The radish variety "French Breakfast", obtained through the Ferry Morse Seed Company of San Francisco, California, was used. The plants are grown in the greenhouse under standard conditions of planting and nutrient. When the first foliage leaves are about 50 sq. mm. in surface area they are cut from the plant, the time after germination being about 16-18 days under the conditions at this laboratory. It was found necessary to adhere rigidly to these conditions, since with older leaves, and with second foliage leaves the uniformity of reaction is greatly diminished. Circular disks of approximately 19.5 sq. mm. in area are cut from the leaves by punching them out with a sharp cork borer. The variation in size from disk to disk is negligible. The disks are carefully washed in distilled water, and shaken for five minutes on an electric shaker to ensure thorough mixing of all of the sections. Fifteen to twenty sections per dish are then placed in Syracuse dishes each containing 2 cc. per dish of medium. This number of sections was found to be sufficient to reduce the variation in sensitivity from dish to dish so that a reproducibility within a single test of 2-3 per cent can generally be obtained. The dishes are placed in an incubator at 25° $\pm$ 0.5 C. for 30 hours. This length of time was found to give a maximum difference in area of about thirty per cent between control sections and sections grown in standard pea diffusate medium. Allowing the test to run for longer than thirty hours was found undesirable,

since the difference in area was enhanced little and the medium becomes badly contaminated.

Several types of direct measurements were attempted for measurement of leaf growth. Leaf strips were used, their width being measured under a binocular microscope with an eyepiece micrometer. These measurements were abandoned, however, since owing to the resistance to stretching of the larger veins the growth of the sections was rather irregular. In order to gain sufficient reproducibility for satisfactory quantitative work it was found necessary to use a considerable number of sections, making direct measurements of area increase very laborious.

Two equally reliable methods for measuring the increased growth were therefore worked out and used: (1) determination of the total wet weight of the sections from a single dish and (2) measurement of the total surface area of the sections from a single dish. The method of wet weight determination is the faster, and was therefore used in routine testing, while the alternative method may be used as a check. The method of obtaining the wet weight is as follows: all the sections from a single dish are placed upon a piece of blotting paper, taking care that the sections are separated from one another. They are dried on this paper for about one minute, and a definite number then transferred to a small beaker of known weight, their weight then being determined to a tenth of a milligram. These procedures are done in a uniform manner, and the length of time for each weighing made constant so that the error from varying degrees of dryness has been shown to be negligible. For measuring the total surface area a photoelectric cell is used. The cell is arranged in such a manner that a film may be passed between it and a suitable light source directly above the cell.

By such an arrangement, only light transmitted by clear areas in the film is caught by the cell. The galvanometer deflection is then a measure of the amount of clear area on the film. Application of this principle for measurement of leaf section areas is as follows: the sections are first dried as before and arranged on a piece of thin glass plate in sets of four, with lines of demarcation between the sections from different dishes. A shadow photograph of the sections is then made on a strip of 32 mm. positive film, using a very weak exposure and an alkaline hydroquinone developer to insure maximum contrast. Such a strip of film is shown in figure 1. An entire experiment of 20-30 dishes may be photographed on two strips of film, so that error from varying film density is minimized. In addition two or three standard areas (disks cut from a piece of steel rod) are photographed on each strip of film, so that they may be used to calibrate the measurements to the same zero point on the galvanometer for each individual film. The film is then passed over the photo electric cell with a direct light source above it. The cell catches only the light transmitted through the clear spaces on the film, these clear areas corresponding to the surface area of the original leaf sections. The galvanometer deflection is noted, and the difference between the readings is a measure of the difference in surface area of the original leaf sections. By this method the measure of activity is indicated by the increase in total surface area of twenty leaf sections grown in the crude extract over that of suitable controls. The correspondence between areas as measured in this manner, and the determination of areas by measuring the photograph under a microscope with an eyepiece micrometer is very good as is shown in table 2. The agreement between the method of determination of the wet

weight of the sections and of determination of their surface area is shown in table 3.

The test done in this manner is independent of pH from a pH of 4.0 to a pH of 7.0, table 4. Below pH 4.0 the medium becomes toxic, owing to the external pH changing the internal pH sufficiently to cause damage to the tissue. The test is light independent, but is dependent on temperature, the sensitivity being less at lower temperatures.

This chapter may be summarized as follows: A quantitative bio-assay for factors controlling the growth of leaves is as follows: circular disks ca. 19.5 mm^2 in area are cut from young first foliage leaves of Raphanus. Twenty such discs are floated on the solutions to be tested, and allowed to grow on these solutions for 30 hours at 25° C. The total wet weight of all 20 sections from a single solution is then determined by direct weighing in a standard manner. The total surface area may be determined by suitable application of a photoelectric cell.

The test is pH and light independent, but is temperature dependent.

Table 1
***

The sensitivity of different areas from a single leaf of Nicotiana Sylvestris

Section cut from	Growth in width above identical sections grown in 1% sucrose solution.
Apex of leaf	0.8 mm.
Base of leaf	1.4 mm.
Edge of leaf	0.4 mm.
Center of leaf, including mid-vein	0.5 mm.
A very young leaf	1.8 mm.

Table 2
***

A comparison of activities measured with a photoelectric cell, and of direct measurement of the film under a microscope with an eyepiece micrometer.

Sections from *Nicotiana* leaves in	Method	
	Photo	Direct
Water	100	100
1% Sucrose	111	110
1% Sucrose + pea diffusate mgs. dry wt./cc.		
1	153	150
0.1	143	142

Table 3
***

Comparison of activities determined by wet weight determination, and measuring surface area with photoelectric cell.

Sections from Raphanus leaves in	Method			
	Wet weight detn.		Area detn.	
	mgs./16 sections	Relative to water controls	Galvanometer deflection in arbitrary units	Relative to water controls
Water	42.8	100	20.3	100
1% Sucrose	50.3	117	23.4	115
1% Sucrose + pea diffusate mgs. dry wt./cc				
1	63.5	149	30.8	151
0.5	62.2	145	30.4	149
0.2	61.5	144	29.6	146
0.02	59.2	139	29.0	143

Table 4
***

Effect of pH on the leaf test

Sections in	pH of medium	Growth relative to sugar controls
1% Sucrose	Unbuffered	100
"	4.0	100
1% Sucrose + pea diffusate 10 mg. dry wt./cc.	Unbuffered	110
"	7.0	110
"	6.0	111
"	5.0	110
"	4.0	109

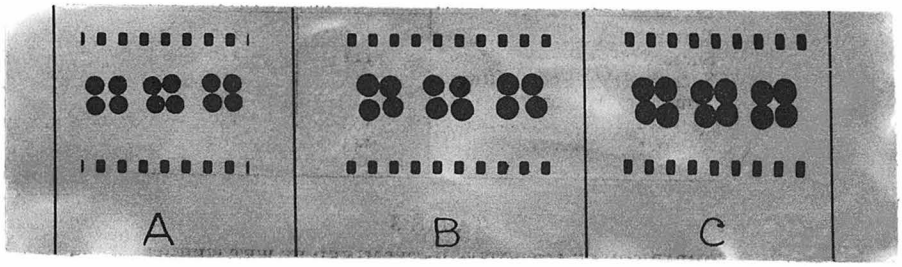


Figure 1

Film arrangement for determination of leaf section surface area: a) sections of radish leaves grown 24 hours in water; b) grown in 1% sucrose; c) grown in pea diffusate medium plus 1% sucrose.

Chapter IV
***

Additional methods of assay for leaf growth factors

As a check against the leaf test it seemed desirable to use some additional mode of assay for the growth factor requirements of leaves. The leaf test while convenient in that several extracts may be simultaneously and rapidly tested, has certain disadvantages. Partially mature, green leaf tissue is employed, and such tissue might be expected to contain some of the factors under investigation. The growth factor response and requirements of young etiolated leaf tissue might, therefore, be different. Tests of two general types were used to some extent in an investigation of this point, (1) leaf growth in excised embryos, and (2) growth of excised immature leaves.

Aseptic embryo cultures have been employed for many years. Brown and Morris (25) in 1890 described the use of excised embryos in the study of the seed-embryo relationship in barley, and other early work includes that of Hannig (43) and Dietrich (31). The usefulness of this technique in biochemical investigations has been often pointed out (81) or as has been shown by Tukey (99) it can be very successfully employed in the circumvention of embryo abortion. The observation that in general the growth of excised embryos upon medium containing the essential inorganic elements and sugar was far less than in the intact seedling, led Brunner (26) to suggest that developing plants just as developing animals require "accessory growth factors". That excised embryos do require growth factors can be seen in the investigation of Kögl and Haagen-

Smit (55), who found that additions of biotin, thiamin or estrone to the medium markedly increased the growth of embryos, biotin affecting primarily stem growth and thiamin primarily root growth. Similar observations were made by Bonner and Axtman (12), who further observed pantothenic acid and ascorbic acid (14) to exert a marked growth promotive effect upon excised embryos. Leaf development, however, as noted by Bonner and Axtman (12) was markedly less than that of the intact etiolated seedling, and was not greatly influenced by any of the added accessory factors.

The technique used in the present work is the same as that used by other workers (12,14). Seed of the variety "Perfection"* was used in all experiments. The seeds were sterilized by a brief wash of two minutes in 95% alcohol, followed by a twenty minute soaking in 0.1% $HgCl_2$. The seeds following this treatment were allowed to soak in sterile water for six hours, which sufficiently softens the seed to permit easy excision of the embryo. The embryos following excision were transferred to 50 cc. Erlenmeyer flasks containing 15 cc. of medium made up in 1% agar.

The composition of the basic medium used is shown in table 5. This medium is the same as the basic medium used for isolated root culture (11,13) which was found highly satisfactory for the growth of embryos (12). The desired amount of extract to be tested for leaf growth activity is added directly to this basic medium.

Four embryos per flask were found satisfactory. They were allowed to grow in the dark at 25° C. for three weeks. After this time the embryos were removed from the flasks, and the total wet

*Obtained thru the courtesy of Ferry Morse Seed Co., San Francisco, California.

weight of the roots, stems, and entire leaves (mesophyll plus veins) determined for all of the embryos (as a rule 20) grown in flasks of similar medium. Table 6 shows the marked effect upon leaf growth of additions of pea diffusate to the medium. As leaf growth appears to be a function of the pea diffusate concentration, it is possible that this method may be used quantitatively, though this was not carried to completion in the present work. As will be discussed in a later section, however, the requirements for leaf growth in embryos appear to be similar to the requirements found in the leaf test.

The following technique was employed in the culture of isolated immature leaves. Pea seeds of the variety "Perfection" were sterilized as described for embryo culture. Intact seeds were germinated and grown in test tubes upon sterile agar. Twelve days after germination the apical leaves were cut off using sterile technique, these etiolated leaves being then floated upon liquid medium contained in 50 cc. Erlenmeyer flasks. The same inorganic medium described in table 5 was used with the exception that a solution 1% in sucrose was used rather than the 4% shown in table 5. The desired extract was added directly to the medium. Figure 2 shows a shadow photograph of such leaves after growing one month in the dark at 25° C.

Similar results, table 7, have been obtained using young radish leaves, from radish plants grown in a manner similar to that just described for pea leaves, but left in the light, as no epicotyledonary development takes place in the dark.

The usefulness of this latter method as a quantitative bio-assay has not been fully investigated. It should be pointed

out, however, that the response of isolated immature leaves to crude leaf growth factor preparations and to crystalline active substances is similar to the response of the leaf test, (see chapter X).

This chapter may be summarized as follows:

(1) Leaf growth (mesophyll and veins) of excised pea embryos is increased by the addition of pea diffusate to the medium.

(2) Excised immature leaves of pea and radish cultured under aseptic conditions respond with markedly increased growth to the addition of pea diffusate to the culture medium.

Table 5
***

Composition of nutrient medium used in culture of excised pea embryos and isolated immature leaves.

Substance	Concentration mg./liter
$Ca(NO_3)_2 \cdot 4H_2O$	242
$MgSO_4 \cdot 7H_2O$	42
KNO_3	85
KCl	61
KH_2PO_4	20
$Fe_2(C_4H_4O_6)_3 \cdot H_2O$	1.5
Sucrose	40 grams

Table 6
***

Effect of pea diffusate upon leaf growth of excised pea embryos.

Treatment	Mgs. wet weight per leaf	Relative to controls
Control (nutrient only)	19.0	100
Nutrient + pea diffusate mg. dry wt./l		
25.0	31.5	165
2.5	25.8	136
.25	19.4	102

Table 7
***

Growth of immature leaves excised from radish seedlings grown sterilely in the light, cultured 4 weeks.

Treatment	Wet weight of 20 leaves (in grams)
Control (water only)	0.210
Inorganic salt medium + 1% sucrose	0.835
Inorganic salt + 1% sucrose medium plus pea diffusate, mg. dry wt./l	
40	Toxic
20	1.085

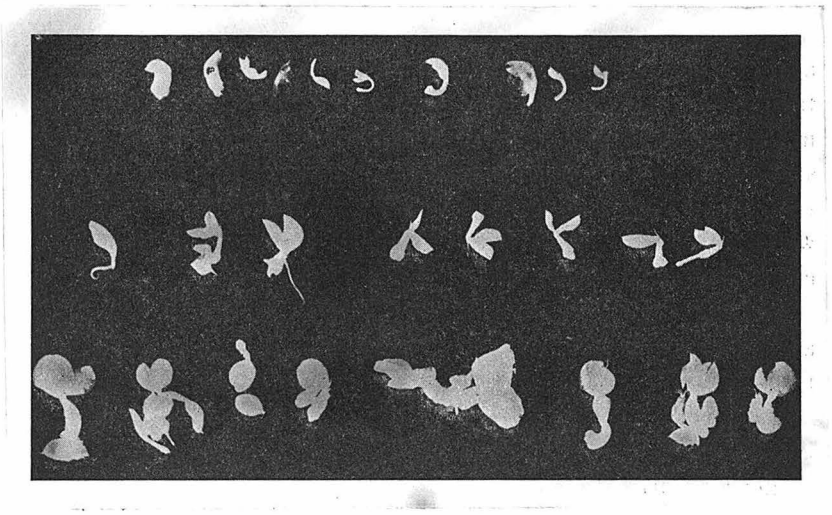


Figure 2

Growth of leaves excised from etiolated pea seedlings, cultured one month. Top row: in water; middle row: in inorganic salt medium plus 1% sucrose; bottom row: in inorganic salt and 1% sucrose medium, plus 1% standard pea diffusate solution.

Chapter V

***

Nature of the growth response.

Histological studies under the guidance of Dr. H. E. Hayward have been started on both Nicotiana tabacum and Raphanus sativa leaves. Disks were grown in a manner similar to those grown in regular leaf test, but were notched with regard to a known orientation of the midvein. The sections were killed with Navashin's solution under a slight vacuum to allow quick penetration of the fixative. They were then dehydrated in alcohol and tertiary butyl alcohol, and finally imbedded in a beeswax paraffin mixture. The sections were cut transversely and longitudinally at a thickness of 15 μ and were stained with a modified Flemming's triple stain.

Preliminary studies of both Nicotiana and Raphanus indicate that a gross enlargement in area and in thickness of the sections has taken place. Before killing the sections, measurements of area were made under a microscope with an eyepiece micrometer. They showed a 20% increase in area of sections grown in pea diffusate over sections grown in sucrose. Measurements of thickness were made after mounting the sections. The thickness was uniformly greater (measured from epidermis to epidermis) in leaves grown in pea diffusate than in sucrose. It is of interest also that in radish and tobacco the increase in thickness was rather uniform throughout the length of the sections. This would indicate that the growth factors penetrated uniformly through the epidermis, and that in these two cases lack of penetration was not a limiting factor, as mentioned earlier for Carica Papaya.

The structure and development of the leaf of Nicotiana tabacum has been excellently studied by Avery (2). The general structure of the mature lamina of the leaf of Nicotiana tabacum is shown in figure 3. Avery found that cessation of both cell division and cell elongation of the various tissues shown in figure 3 occurs independently and at different times. Cell divisions cease in the epidermis first, and in the palisade last. Moreover, cell division in the epidermal cells is generally complete by the time the leaf is one fifth to one sixth its mature size. Increase in surface area is then brought about entirely by cell elongation. While cell divisions cease first in the epidermal cells, elongation continues in them until elongation has ceased in the other tissues, elongation continuing after enlargement of the middle and lower mesophyll has ceased. Since the two tissues ceased cell division at nearly the same time, cell enlargement of the epidermis for a period longer than that of the spongy parenchyma results in a strain of the spongy parenchyma cells. This strain produced upon the cells of the spongy parenchyma eventually becomes sufficiently great to pull the cells apart with the formation of intercellular airspaces.

The palisade which is the last tissue to cease dividing is loosened, or may be pulled apart by continued enlargement of the epidermis. The points of particular interest in connection with the present work are that cell divisions are normally complete when the leaf is approximately but one fifth of its mature size, and that cessation of both cell division and cell enlargement occur at different times for the epidermal, palisade, and spongy parenchyma cells. This differential growth of the various tissues of the lamina is of particular interest in that it might indicate

differences in growth factor requirements for these three tissues.

Histological studies indicate a general trend towards a greater degree of cell enlargement in sections of both Nicotiana, and Raphanus grown in the pea diffusate than in sucrose, figure 4. In many sections examined this enlargement was very pronounced in the cells of the epidermis. No evidence of cell divisions was obtained which, in view of the findings of Avery discussed earlier, is not surprising. The leaves selected for the test had probably completed or nearly completed their cell divisions, and would be induced to further divisions only under abnormal conditions as for example high light intensity (32), or high salt concentration (44). Good evidence for a marked increase of intercellular air spaces in the spongy parenchyma is found in sections grown in crude pea diffusate. In the sections examined, however, evidence for a loosening of the cells in the palisade was inconclusive.

The formation of intercellular air spaces in the spongy parenchyma, as described earlier, is controlled by cell enlargement continuing for a longer period of time in the epidermal cells than in cells of the spongy parenchyma. Finding a marked intercellular air space formation in sections grown in pea diffusate is, therefore, suggestive that cell enlargement of the epidermis and probably of the palisade is more strongly affected than of the spongy parenchyma. Strictly speaking, therefore, the factors present in pea diffusate are not complete leaf growth factors. They do not affect vein growth, nor do they apparently markedly influence laminal spongy parenchyma. They do, however, increase cell enlargement of the laminal epidermal cells, and probably of the palisade cells. It must be emphasized that the present evidence is not conclusive, and that only tentative conclusions as to the tissue specificity of the factors present in

pea diffusate can be drawn at present.

This section may be summarized as follows:

(1) Leaf sections from leaves of Nicotiana tabacum and Raphanus sativa grown in pea diffusate show a gross enlargement in area and thickness over sections grown in medium containing sugar alone.

(2) The increase in thickness of sections grown in pea diffusate is uniform throughout the length of the section indicating that lack of growth factor penetration is not a limiting factor.

(3) Histological studies indicate a general trend towards a greater degree of cell enlargement with the formation of intercellular air spaces in leaf sections grown in pea diffusate than in the control sections.

(4) The formation of intercellular air spaces in leaf sections grown in pea diffusate might indicate that the effect is primairly upon cell enlargement of the epidermis and palisade, rather than upon cell enlargement in the spongy parenchyma.

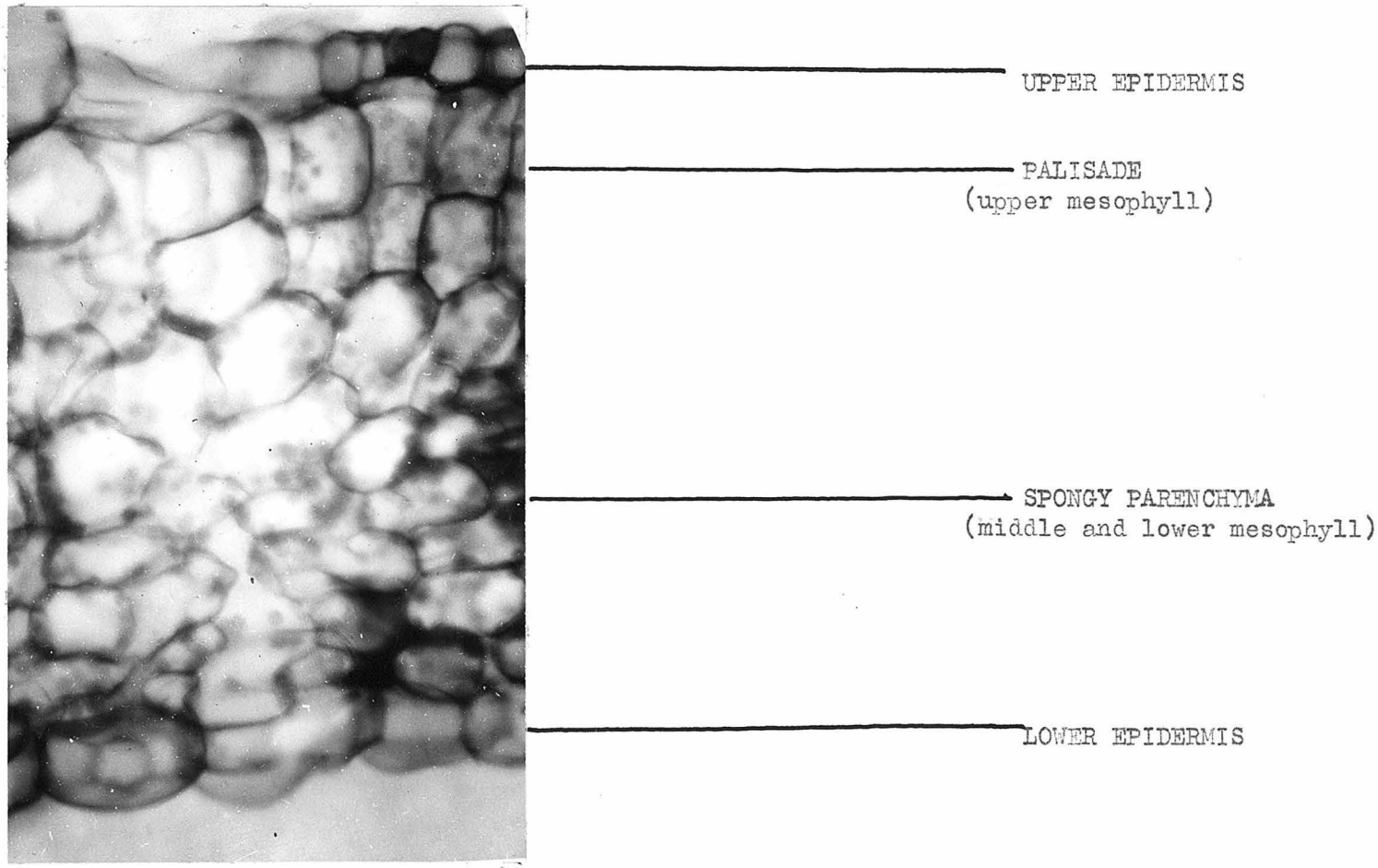


Figure 3

Structure of the Lamina of a Nicotiana tabacum Leaf
Photomicrograph of a portion of the lamina of a tobacco leaf, X 200

Figure 4

Photomicrographs of the lamina of *Nicotiana tabacum* leaf sections cultured for 30 hours in different solutions

a- water
b-1% sucrose
c-1% sucrose plus pea diffusate

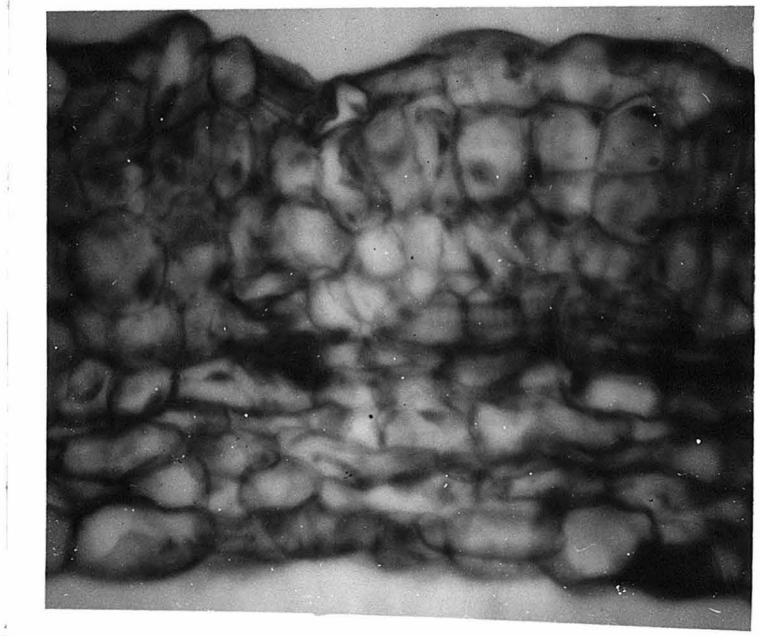


Figure 4-a

Transverse section of a tobacco leaf
section cultured 30 hours in water
X 200

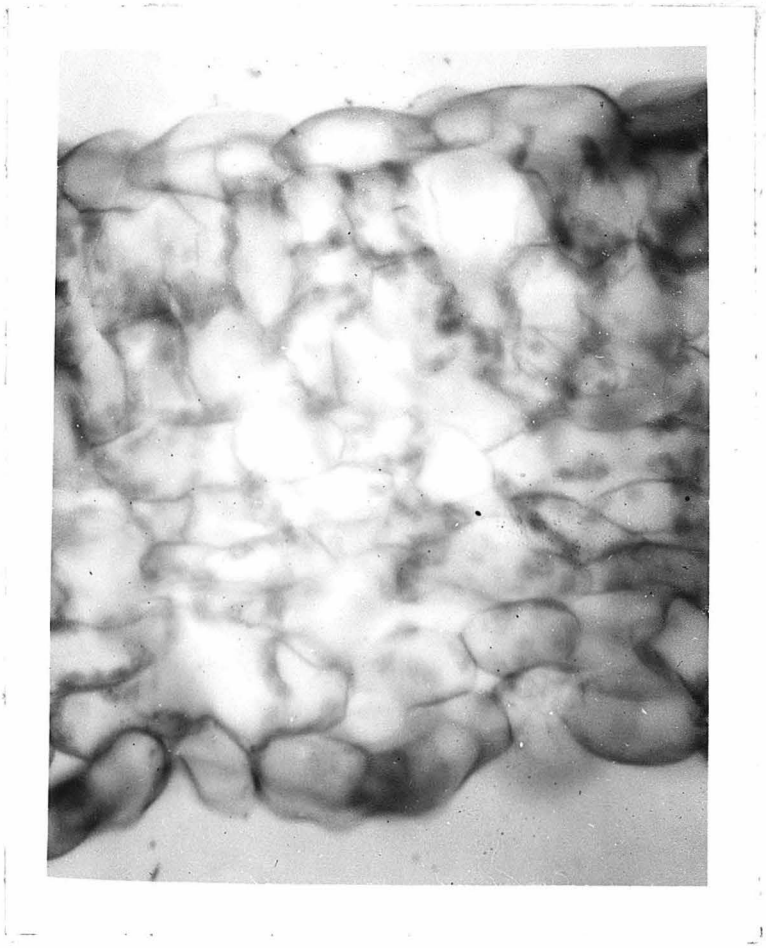


Figure 4-b

Transverse section of a tobacco leaf section cultured 30 hours in 1% sucrose
X 200

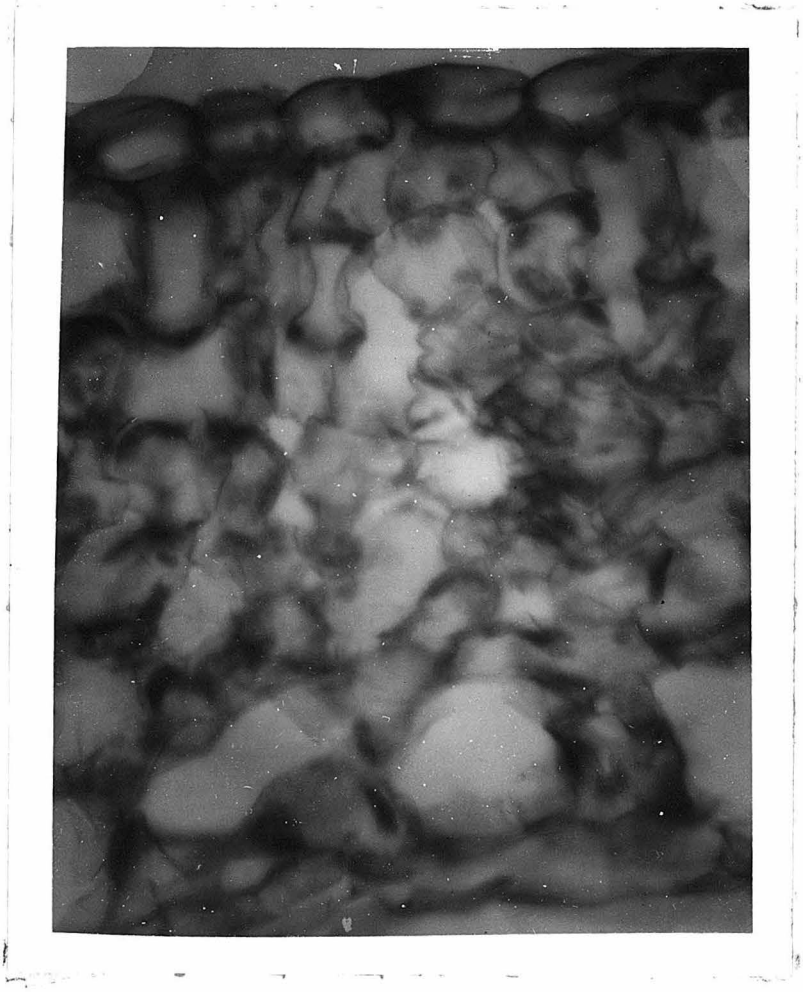


Figure 4-c

Transverse section of a tobacco leaf section
cultured 30 hours in 1% sucrose plus
pea diffusate
X 200

Chapter VI

***

Units and Standards.

In order that different extracts or pure substances be compared as to activity in promoting leaf growth, it was necessary to devise an arbitrary measure of activity. Sugars, as mentioned earlier, proved to be active in the leaf test. Figure 5 shows the activity concentration curve obtained for sucrose. The particular type of sugar used plays an important role. Both sucrose and glucose have the effect shown in figure 5. Mannose and galactose on the other hand give no appreciable effect, which indicates that the effect of sugars is not merely an osmotic one. The diffusate by itself has a high activity, as shown in figure 6. To remove the possibility of a sugar being the limiting factor in the growth reaction, a medium containing sugar was added to all extracts tested and the growth compared with that of sections grown on sugar alone. The sugar selected was sucrose, it giving a good reaction, figure 5, and as seen from table 9 pea diffusate contains 40% sucrose calculated as percentage dry weight. Two per cent by weight of sucrose gives optimum growth when sucrose is used by itself. By trying several dilutions it was found that in the presence of pea diffusate optimum growth was obtained with a solution one per cent by weight in sucrose, figure 7. All extracts were tested in a medium consisting of 1% sucrose, and activity was measured in terms of the added growth beyond that of sections grown in the basic medium. In order that activities be compared from day to day it is necessary to have a solution of "standard activity". It was found convenient to define such a solution as one containing 10 mg. dry weight of pea diffusate per cubic centimeter of one per cent sucrose solution. This solution is tested in a standard dilution series, consisting

of four dilutions from 50 to 2 per cent, figure 8. It has been found that if the diffusate is concentrated to a syrup and stored in the cold, standard solution preparations from this concentrated diffusate give a relatively constant activity over a period of 2-3 months. The standard solution itself, however, must be made up fresh every other day from the concentrated diffusate, since after that time its activity becomes variable. That such a solution may be used as a standard is due to the fact that standard solution preparations made from concentrated diffusates which were prepared at widely different times but from the same variety of pea have nearly the same activity.

In calculating activities of extracts it is necessary to select an arbitrary unit. One leaf unit (L.U.) was defined as the activity of a solution containing one tenth of a cubic centimeter of the standard solution; that is, one milligram dry weight of pea diffusate per cubic centimeter of one per cent sucrose solution. The leaf unit concentration of an extract is determined by plotting the percentage increased growth (growth above that of the sugar controls) against concentration in mg./cc. The concentration of the extract which shows the same activity as the standard solution at a concentration of one mg. dry weight of pea diffusate per cc. of 1% sucrose solution is determined graphically. This concentration then gives the milligrams dry weight of extract per leaf unit.

This chapter may be summarized as follows:

(1) The basic medium used in the leaf test is a solution 1% in sucrose.

(2) A solution of "standard activity" is defined as one containing 10 mgs. dry weight of pea diffusate per cubic centimeter of one per cent sucrose solution.

(3) One leaf unit (L.U.) is defined as the activity of a solution containing 0.1 cc. of the standard solution.

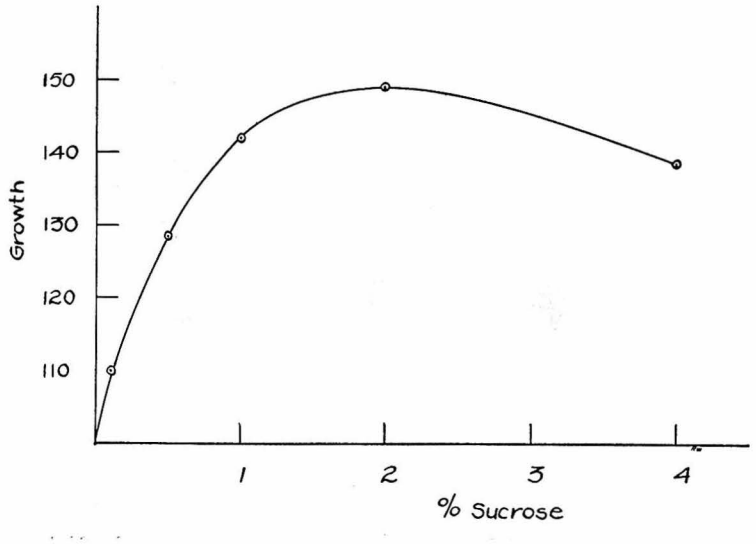


Figure 5

Effect of sucrose on leaf growth expressed as growth above growth of sections in water.

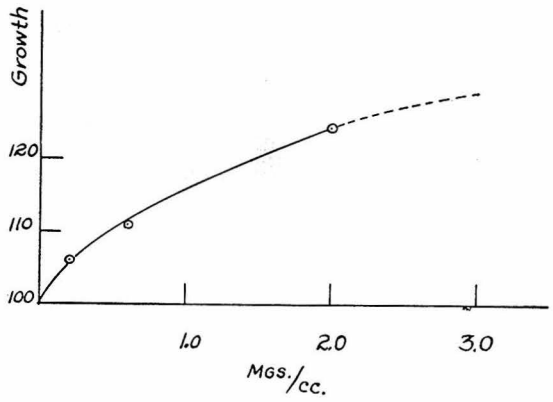


Figure 6

Effect of pea diffusate on leaf growth. Concentration of pea diffusate in terms of mg. dry weight of pea diffusate per cc. of water. Growth in water alone = 100.

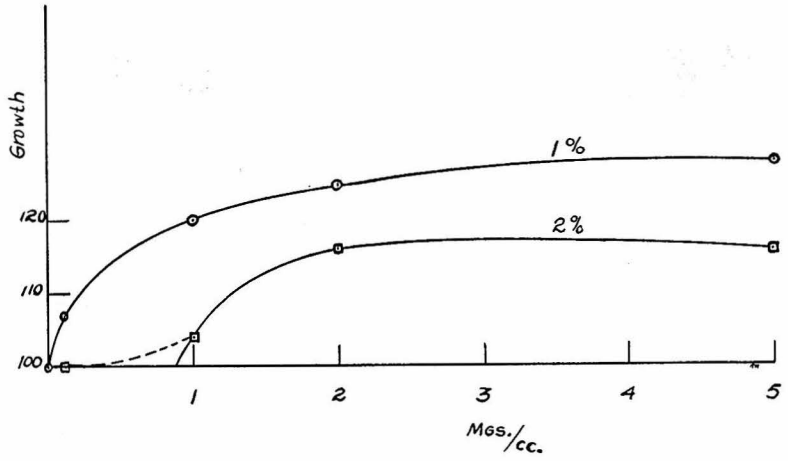


Figure 7.

Growth of leaves in pea diffusate with different concentrations of sucrose. Growth expressed as growth above growth of sections grown in appropriate sucrose concentration.

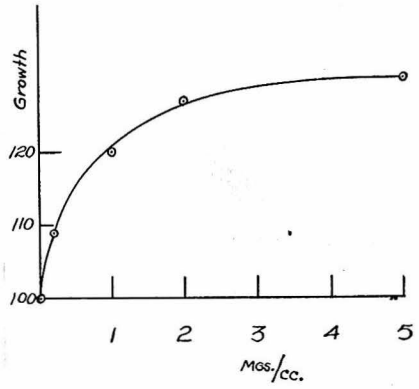


Figure 8.

Standard dilution series. Growth expressed as growth above growth of sections grown in 1% sucrose solution.

Chapter VII

***

The activity of various extracts and natural products in the leaf test.

A wide variety of different extracts and natural products have been tested for activity in promoting leaf growth, as measured by the leaf test. The extracts and natural products may be classified as follows: (1) seed diffusates, (2) urines, (3) leaf extracts, and (4) miscellaneous.

The method used in obtaining a seed diffusate is as follows:

a) Sterilization of the seed:

1. Wash the dried seeds 10-12 times in tap water.
2. Wash 6-8 times in distilled water.
3. Place in a container from which the diffusate is to be collected (a large separatory funnel has been found excellent for this purpose).
4. Soak 2 minutes in 95% alcohol.
5. Rinse 5-6 times with sterile (boiled) distilled water.
6. Soak 30 minutes in 0.1% mercuric chloride.
7. Rinse 6 times with sterile distilled water.

b) Obtaining the diffusate:

After the above treatment the seeds are covered with sterile water and left 12 hours. The water is then drained off and replaced by fresh sterile water. The water that has been drained off is concentrated in vacuo at 30° C.

Pea seeds treated in this manner remain sterile indefinitely, and the leaf growth hormones (as biotin 55) continue to be given

off at a constant rate for a very long time. Even after a month of diffusing leaf growth hormones are given off at nearly the same rate as when the diffusion was first started. Diffusates active in the leaf test have been obtained in a fashion similar to that just described from corn, radish, and pea seeds. A comparison of their activities in leaf units (L.U.) per milligram dry weight is shown in table 8. Of these three diffusates, pea diffusate was the most active. This might be expected from the fact that the seed coat of corn allows little diffusion, and radish probably stores very little leaf growth factor, the first foliage leaves appearing only in the light. It should be noted, however, that the diffusates from these three widely different plants are all active in causing an increased growth rate in the radish leaf. This lends additional support to the view that the active principle, or principles in each of the three diffusates is probably the same, and that common growth factors govern leaf growth in a wide variety of plants.

Cow, mare, and human urine have all been tested for activity in the leaf test. Their activities in comparison to pea diffusate are shown in table 8. Cow urine was found to be highly active in leaf growth, as measured by the leaf test, being approximately one hundred times more active than the standard pea diffusate preparation. Human urine, while less active than cow urine still possesses considerably greater activity than pea diffusate. Mare urine was the least active of any of the urines tested, though still ten times more active than pea diffusate.

No active extract has been obtained by water extraction of fresh ground-up leaf tissue. However, by placing the leaves intact in sterile water and allowing them to remain in it at a low temperature (to keep down infection) it is possible to obtain a leaf

diffusate that is active in the leaf test. An extract of greater activity can generally be obtained by allowing the leaves first to soak in ether for 1-2 days, thereby inactivating destructive enzymes. A water extract of these ether killed leaves is then made. From young palm leaves (Washingtonia filifera) an exceedingly potent extract may be prepared by allowing the etiolated immature leaves to soak in sterile water for a week, draining the water off, and concentrating this diffusate in vacuo. This diffusate was the only highly active leaf extract obtained. Many other leaf extracts have been prepared, viz. radish, tobacco, bean, cocklebur, etc. table 8. While they all possessed activity, their activities were considerably lower than the activity of pea diffusate.

Yeast extract and "Acme" beer were both found active in the leaf test, and both had activity greater than pea diffusate, table 8. A water extract of egg yolk was inactive, while a similar extract of egg white had low but definite activity in the leaf test. Fruit juices as a rule were found to have either very low activity, or to be totally inactive.

Pea diffusate was selected from among these possible crude sources for the fractionation and isolation of constituents active in leaf growth. While it is true that pea diffusate is considerably less active than cow urine, pea diffusate is of particular interest due to the fact that it is prepared from pea cotyledons, known to be rich in the naturally occurring leaf growth factors.

This chapter may be summarized as follows:

a) Seed diffusates obtained from pea, radish, and corn were found active in the leaf test.

b) Cow, mare, and human urine were found highly active in the leaf test.

c) The water extract of young etiolated palm leaves was found highly active in the leaf test. Other leaf extracts, though active, possessed low activity.

d) Yeast extract, "Acme" beer, and egg white were found active in the leaf test.

Table 8

***

Activity of various extracts and natural products in the leaf test, expressed as leaf units (L.U.) per milligram dry weight of substance.

Substance tested	L.U./mg. dry weight
Pea diffusate	1.0
Corn diffusate	0.2
Radish diffusate	0.5
***	
Cow urine	100.0
Human urine	20.0
Mare urine	10.0
***	
Washingtonia filifera leaves	80.0
Radish) "	
Nicotiana) "	
Xanthium) "	0.1
Phaseolus) "	
Daucus) "	
Lactuca) "	
***	
Yeast extract	40.0
Beer (Acme)	15.0
Egg white	0.1

Chapter VIII

***

Chemical fractionation of pea diffusate.

A fractionation of pea diffusate has been carried out which has led to the recognition of several distinct classes of substances contributing to the activity of the crude material. All of the active constituents have not as yet been recognized however, due in part to the lack of specificity exhibited by the leaf test, and in part to the multiple nature of leaf growth factor requirements.

Table 9 shows a general analysis of the pea diffusate. The bulk of the diffusate is accounted for by carbohydrates and ash, both of which are active in leaf growth. The activity of the carbohydrate fraction can be duplicated and its effect obviated through the use of sucrose in the medium. The activity of the ash on the other hand contributes to the activity of the crude material. The magnitude of this contribution will be discussed later in this chapter.

Several different methods of fractionation have been used. It would be of little value to discuss those yielding inconclusive information concerning the nature of the growth factors. Rather the two yielding the most fruitful results will be described, using a typical fractionation as example. The fractionation schemes are shown diagrammatically in figures 9 and 10.

I. Benzene and petrol ether extraction:

95 grams of pea diffusate concentrated to a syrup was extracted in a continuous extractor for 24 hours with benzene, followed by 24 hours extraction with petrol ether (boiling point 30-40). Initial analysis showed 9,000 L.U. present in the total sample. One

gram of material proved soluble, with the entire activity remaining in the water fraction.

Total dry weight of water soluble fraction - 95 grams

Total number of leaf units - - - - - - - - -9,000

L.U./mg. 0.1

II. Alkaline ether extraction:

The extract was made alkaline with barium hydroxide and extracted with peroxide free ether on a continuous extractor for 48 hours. 200 milligrams of inactive ether soluble material was obtained. The barium was removed through precipitation as barium sulfate by adding an equivalent amount of sulfuric acid. This precipitation carried with it 10 grams of inactive material, and gave an increase in the total number of leaf units present.

Total dry weight of water soluble fraction -- 86 grams

Leaf units - - - - - - - - - - - - - - - - 30,000

Enrichment - 2.8 X.

III. Acid ether extraction.

The active water extract was made acid with sulfuric acid, and extracted with peroxide free ether in a continuous extractor. After extraction was complete the sulfuric acid was removed as barium sulfate by addition of barium acetate. The acetic acid formed in this treatment was then removed by continued extraction with ether.

Water soluble	Ether soluble
Total dry weight - - - - - 85 grams	Total dry weight - - - 1 gram.
Leaf units - - - - - - 50,000	Leaf units - - - - 6,600
Enrichment 6X	

IV. Mercuric acetate precipitation

The water soluble fraction from the preceeding step was fractionated with mercuric acetate according to the method of Klein (47). The extract was maintained slightly alkaline at all times during the precipitation by adding a 10% sodium carbonate solution. Mercuric acetate solution (25%) was added giving rise to a fine white precipitate. It was added until precipitation was complete as indicated by the formation of a bright red mercury complex. The precipitate was filtered off, suspended in water and decomposed with hydrogen sulfide gas. The mercuric sulfide formed was filtered off, and the extract concentrated in vacuo. The excess mercuric acetate present in the mother liquor was removed in a similar fashion, and the acetic acid formed during the original precipitation was removed by ether extraction.

Precipitate	Mother liquor
Total dry weight - - - - - - 26.5 grams.	Inactive.
Leaf units - - - - - - - 30,000	
Enrichment - 11X	

The water extract obtained from the above precipitate was allowed to remain in the ice box for several days. A fine white precipitate formed which was filtered off, and was recrystallized from alcohol-water as the potassium salt. This substance was found to have a low activity in the leaf test, giving a maximum increase in wet weight of 10%. Elementary analysis gave: *

*Microanalysis by G. A. Swinehart.

	%C	%H	%N	%O(by difference)
Found	55.19	5.36	7.28	32.17
Calculated for $C_9H_{10}O_4N$	55.20	5.12	7.15	32.13

The free acid showed a decomposition point of 250° C., and corresponds in many respects with 3,4 dioxy phenylalanine (decomposition point 279° C.). The definite identity of the two has not yet been further established, however, since the substance has low activity (5 L.U./mg.), and accounts for a negligible fraction of the total activity of the pea diffusate.

V. Fractionation with silver acetate:

Saturated silver acetate solution was added to the above water extract until no further precipitate was formed after standing 48 hours in the cold room, (2° C.). The precipitate was filtered off and decomposed after suspension in water with hydrogen sulfide gas. The silver sulfide formed by this treatment was filtered off, and the resulting water solution concentrated in vacuo. The mother liquor was treated in similar manner, with the exception that the acetic acid formed from the silver acetate was removed by ether extraction.

Precipitate	Mother liquor
Total dry weight - - - - - - 4.5 grams	Total dry weight - - 22.5 grams
Leaf units - - - - - - - 20,000	Leaf units - - - - 40,000
Enrichment - 45X	Enrichment 18X

VI. Picric acid fractionation:

Excess of a saturated water solution of picric acid was added at 2° C. to the water extract obtained from the insoluble fraction of step V. A fine yellow precipitate formed which was filtered off, and recrystallized from water. The picrate was

hydrolysed with dilute sulfuric acid, and the picric acid removed by ether extraction. The sulfuric acid was removed as barium sulfate, and the resulting acetic acid removed by a second ether extraction. The final extract gave no crystalline material but had an activity of 40,000 L.U./gram, representing a 400 times enrichment.

Elementary analysis of the picrate showed no ash, and gave the following composition: *

	%C	%H	%N	%O(by difference)
Found - - - - - - - -	36.80	2.31	27.62	33.27
Calculated for $C_{11}H_7N_7O_8$ - - - - - -	36.19	1.99	26.84	35.08

The nitrogen value might indicate, that the substance in question is a purine picrate, and its elementary analysis as well as its physical properties correspond closely to those of the hypoxanthine picrate, $C_{11}H_7N_7O_8$. Adenine and hypoxanthine are the only two purines giving insoluble picrates. The elementary analysis of hypoxanthine picrate corresponds more closely to the substance in question than does adenine picrate as shown by the following comparison:

	Hypoxanthine	Found	Adenine
% carbon	36.2	36.8	36.2
% hydrogen	1.9	2.3	2.2
% nitrogen	26.8	27.6	30.8
% oxygen	35.1	33.3	30.8

Adenine and hypoxanthine picrates may be distinguished by difference in their physical properties. Adenine picrate forms only in HCl solution. It is highly insoluble in both hot and cold water, decomposes when heated to 279° C. Hypoxanthine picrate on

*Microanalysis by G. A. Swinehart

the other hand forms in either neutral or acid solution. It is quite insoluble in cold water, more soluble in hot. It discolorizes at 200° C. with decomposition. The picrate obtained from the pea diffusate is formed from neutral solution, very insoluble in cold water, very much more soluble in hot water. It showed strong discoloration at 195° C., and decomposed upon heating to higher temperatures.

One active substance obtained from the pea diffusate on these grounds is, therefore, hypoxanthine. Crystalline hypoxanthine was found active in the leaf test, table 12, with an activity equal to that found for the substance obtained by hydrolysis of the picrate (i.e. 40,000 L.U./gram). The lowest active concentration of either substance is 0.05 mg. per one cc. of 1% sucrose solution. This concentration represents a dosage of 2.5 γ per leaf section, thus having activity comparable to other known growth factors.

VII. Fractionation of the mother liquor from the silver acetate precipitation:

The factors responsible for activity in this fraction are as yet undetermined. Lead acetate precipitation, charcoal adsorption, HCl-CH_3OH precipitations, and many other methods have been employed with little enrichment of activity.

VIII. Fractionation of the organic acid fraction:

The ether soluble fraction, obtained in procedure III, was dried over anhydrous sodium sulfate. The ether was then removed and the residue taken up in fresh alcohol-free ether. The acids were esterified with diazomethane (CH_2N_2). This reagent was prepared from nitroso methyl urea in strongly alkaline water solution (73), the gas being caught in ether cooled at 5° C. This ether solution of diazomethane was then dried over KOH so that the extract and all

reagents were dry. Esterification was carried out at 5° C. by slow addition of the diazomethane to the ether extract of the organic acids. Completion of esterification was indicated by no further evolution of gas. After esterification was complete, the ether was removed, and the residue transferred to the distilling flask. The type of micro distilling flask used was similar to that used in the isolation of auxin (50). Distillation was carried out at a pressure of 20 microns, obtained by the use of a "high vacuum" oil pump. The following fractions were collected from the side arm of the distilling flask:

I-60-80° C.

II-80-120° C.

III-120-180° C.

IV-180-210° C.

These four fractions were hydrolyzed by refluxing two hours in a saturated alcoholic potassium hydroxide solution. The alcohol was removed by vacuum distillation, and the residue taken up in dilute sulfuric acid. The acids were then removed by ether extraction. These acids were tested as the potassium salts, it making no difference in activity whether tested as the sodium or potassium salts as was shown by tests carried out with succinic and glutaric acids, table 15. Fractions I and II proved to have little activity, the bulk of the activity being found in fractions III, and IV. The residue was inactive.

Fraction I	53 mgs.	1.3 L.U./mg.	69 L.U.
Fraction II	50 mgs.	2.0 L.U./mg.	100 L.U.
Fraction III	48 mgs.	10 L.U./mg.	480 L.U.
Fraction IV	50 mgs.	40 L.U./mg.	2,000 L.U.

Fractions III and IV give crystalline material from water

solution under suitable conditions. The structure of the active acid, however, has not yet been established.

The ash of pea diffusate was shown to be active in the leaf test with an activity of 6.6 L.U./mg. Qualitative analysis, using a hydrogen sulfide scheme, showed the presence of either potassium or sodium ions, as well as mercuric ion. The presence of mercuric ion is undoubtedly an artifact, being a contaminant from the use of mercuric chloride in the sterilization of the seeds. Potassium nitrate was found extremely active in promoting leaf growth, table 16. The effect of potassium nitrate was not, however, additive to that of the crude diffusate. Such an addition was found to increase the activity of the pea diffusate, but by only 50% of the activity shown by an equivalent concentration of potassium nitrate when tested alone.

The following separations were made, in an attempt to establish the contribution of the inorganic salts present in the diffusate to the total activity. Fifteen and three tenths grams of crude diffusate, shown by analysis to contain 12.2% ash, and 15,300 L.U. were taken up in methyl alcohol to a final concentration of 95%. It was then placed for 12 hours at -20° C. The precipitate that had formed was centrifuged off, the mother liquor concentrated and again taken up in methyl alcohol to a concentration of nearly 100%. It was again allowed to stand at -20° C. for 12 hours, and the precipitate which formed centrifuged off. A similar treatment for a third time gave no further precipitation. The following distribution of dry weight, ash and activity was found:

Fraction	Total dry wt. in grams	Milligrams of ash	L.U. of extract	L.U. of ash
1st pp't	5.3	960	10,650	6,400
2nd pp't	2.5	254	3,560	1,690
soluble	7.6	805	4,740	5,360

In the methyl alcohol soluble fraction, therefore, the number of leaf units of ash are greater than the total number of leaf units found in the extract. An acid ether extraction of this fraction gave 300 mgs. of extract having a total activity of 2,000 L.U. Assuming that the activity of the ash is diminished by 50% in the presence of the extract, of the 4,740 L.U. present in the extract, 2,780 are accounted for by the activity of inorganic salts, and 2,000 by the activity of the organic acids, thereby accounting for the total activity found in this fraction.

The combined precipitates give 8,000 L.U. of ash, and a total of 14,200 L.U. found in the extracts. Again assuming a 50% reduction in activity of the ash, 4,000 units would be accounted for by the activity of the inorganic salts. Acid ether extraction of these fractions gave no appreciable extract, so that 10,000 L.U. are left unexplained. The purines would be found in this fraction, they being very insoluble in alcohol. On the basis of the previous fractionation, hypoxanthine would account for at least 3,500 of the 10,000 leaf units leaving undetermined 6,500 presumably due to the fraction discussed in procedure VII. It is possible on this basis to make a rough estimate of the distribution of activity of pea diffusate, which is shown diagramatically in figure 10:

Ash	6,800	36.1%
Hypoxanthine	3,500	18.6%
Organic acids	2,000	10.8%
Undetermined	6,500	34.5%

At best this can be but a rough estimate. The activity of the ash as determined after ashing in air is largely due to chlorides and carbonates, known to have low activity in the leaf test, table 16. Pea seedlings, and cotyledons are known (19) to contain some

nitrate nitrogen which might increase the relative activity of the inorganic consituents of the pea diffusate, potassium nitrate being highly active in the leaf test. A portion, at least, of the inorganic salts exist as the salts of the organic acids, which would considerably alter their relative activities. Possible synergistic effects between the various active fractions, and effects with possible inactive cofactors would also change the relative activities, though no such effects have been found as yet. This distribution of activity must, therefore, be considered as purely tentative. Interactions between the various growth factors will be discussed more fully in the next chapter, together with a discussion of the increase in total number of L.U. during the course of fractionation.

This chapter may be summarized as follows:

(1) Two different methods employed in the fractionation of pea diffusate are described.

(2) A crystalline compound of low activity was isolated and its empiric formula established as $C_9H_{10}O_4N$. It is thought possible that this substance is identical with 3,4 dioxy phenyl alanine.

(3) A crystalline picrate was isolated. This picrate was found upon hydrolysis to give a substance with an activity of 40,000 L.U./gram. This picrate was shown to be identical with the picrate of the purine, hypoxanthine ($C_{11}H_7N_7O_8$).

(4) The organic acid fraction from pea diffusate was found to be active. Esterification and high vacuum distillation gave a fraction of high activity distilling between the temperatures of 180-210° C. at 20 microns pressure.

(5) The inorganic salts present in the pea diffusate were shown to be active.

(6) A tentative estimate of the distribution of activity in the pea diffusate is given as follows:

Ash	36.1%
Hypoxanthine	18.6%
Organic acids	10.8%
Undetermined	34.5%

Table 9*
***

General analysis of pea diffusate.

Fraction determined	Percentage dry wt.
Total nitrogen (micro-dumas)	4.36
Total nitrogen (micro-Kjeldahl)	3.2
Protein nitrogen	0.0
Non-protein nitrogen	3.2
Ammonia nitrogen	0.3
Amino nitrogen	1.9
Amide nitrogen	0.05
Polypeptide nitrogen	0.02
Reducing sugars	5.6
Sucrose	40.5
Non-carbohydrate reducing substances	10.5
Ascorbic acid	0.0
Ash	12.2

*The author is indebted to Prof. H. Borsook and Mr. W. McRary for the nitrogen fraction determinations.

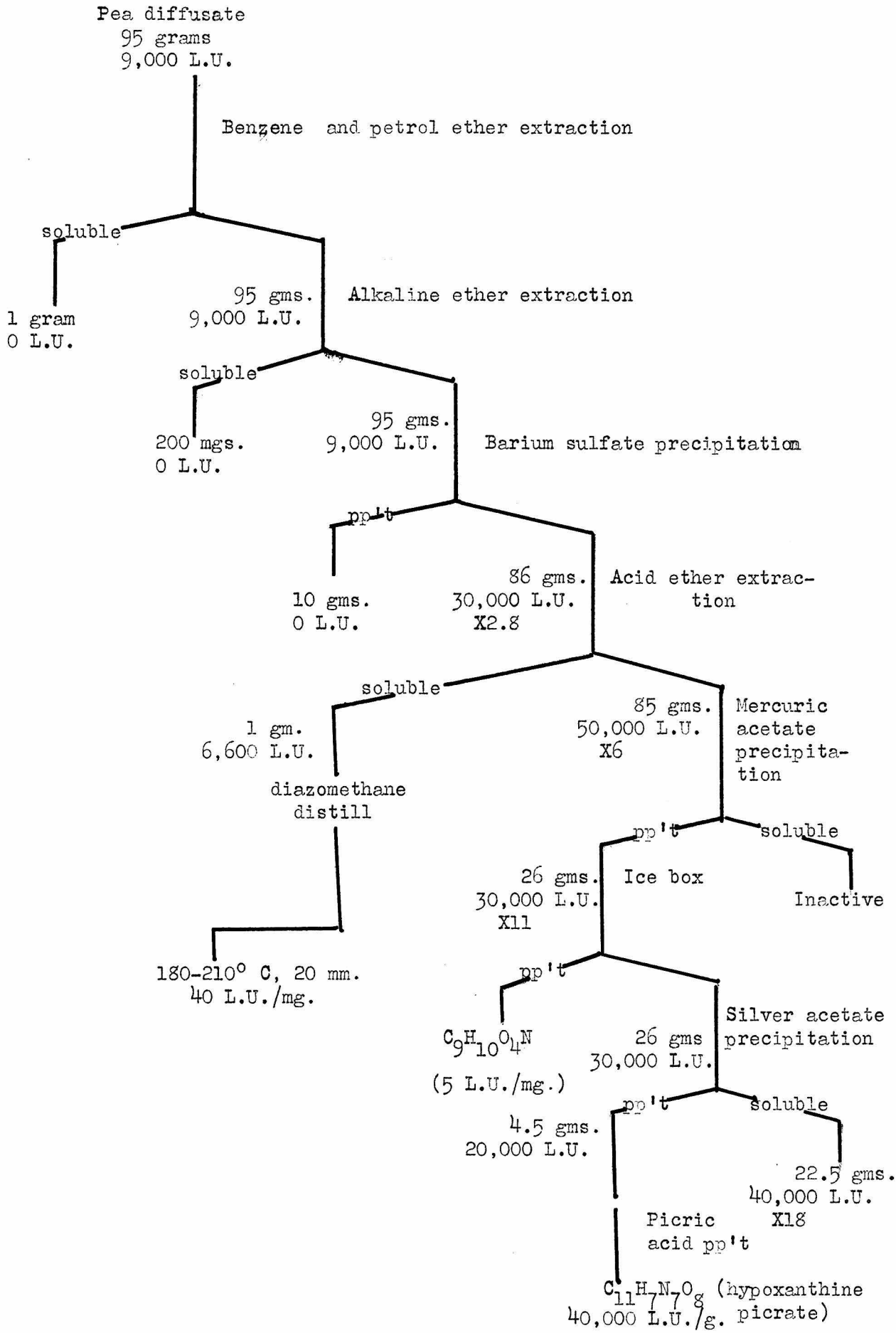


Figure 9.
Fractionation of pea diffusate.

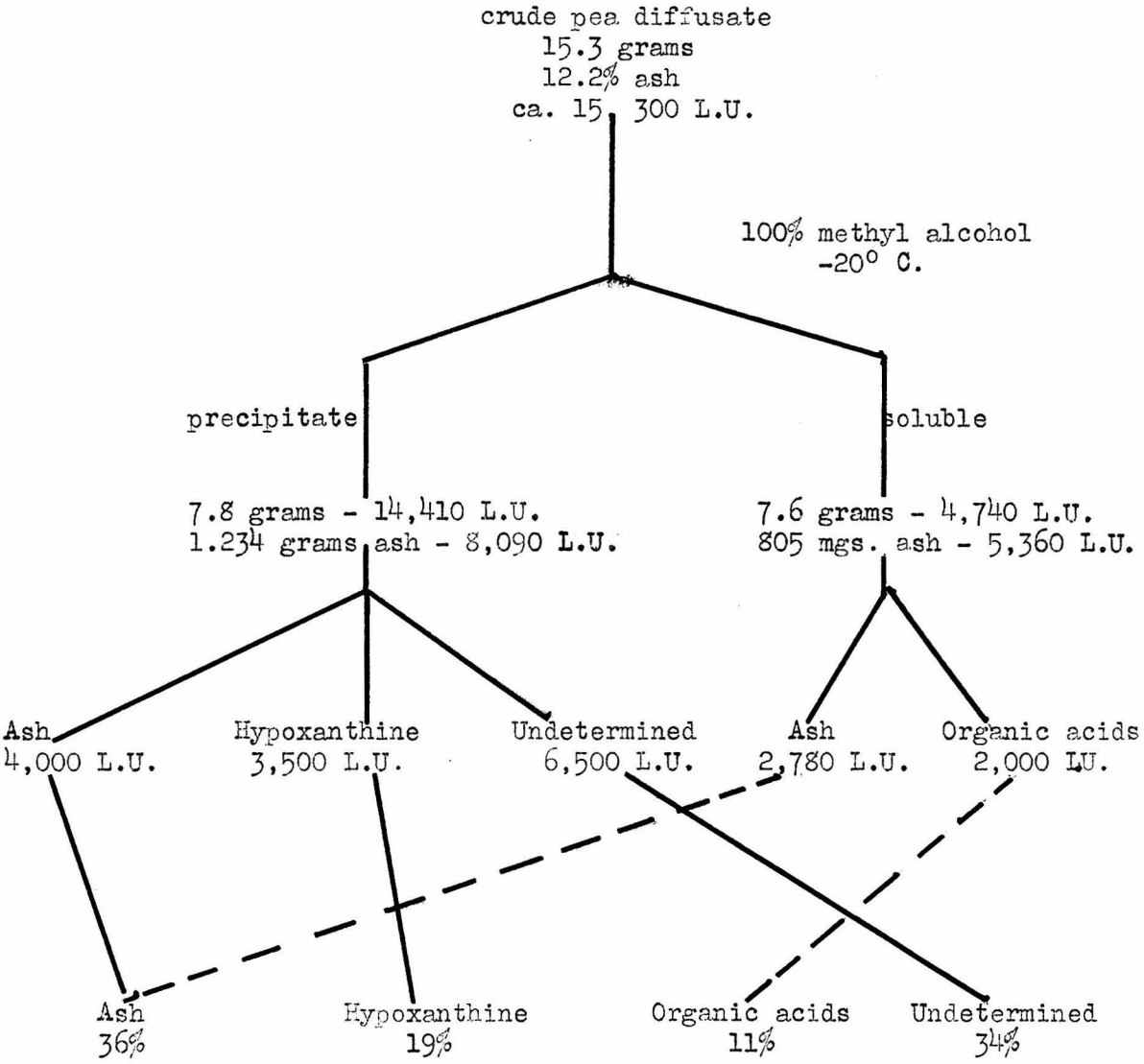


Figure 10.

Distribution of activity in pea diffusate.

Chapter IX

***

Activity of pure substances in leaf growth.

A second method for investigation of the general nature of leaf growth factors has been extensively employed. This method has been the testing of pure compounds of known chemical constitution for activity in the leaf test. The study of such compounds has shown that activity in the leaf test is found in four general classes of compounds, (1) amino acids, (2) purines, (3) dicarboxylic acids and (4) inorganic salts.

Table 10 lists a variety of substances found to be active in different biological phenomena which have been tested in the leaf test. In every instance they have been found to be totally inactive with the exceptions of nicotinic acid amide, and urea, both of which showed slight activity at rather high concentrations. The amino acids that have been tested in the leaf test are listed in table 11. When radish leaves are used as test objects asparagin and proline proved the most active. However, in the case of the amino acids it seems likely that the species of plant from which the leaf is taken determines the amino acid requirements; thus when Nicotiana sylvestris is used as the test object arginine proved to be the most active, while with Raphanus leaves arginine was totally inactive. The growth obtained by the use of amino acids is, however, never as great as that obtained for the S.S.A. (solution of standard activity), nor are the amino acids active by themselves at high dilution. Yeast nucleic acid was found to possess very definite activity. Upon testing of various crystalline purines adenine proved to be active at the highest dilution (20γ per liter) Hypoxanthine had activity at a somewhat higher concen-

tration, while xanthine, uric acid, guanine and caffeine had slight activity, or were totally inactive, table 12. That adenine, hypoxanthine, or a related purine is the only factor concerned in mesophyll growth seems unlikely not only from the chemical studies of the pea diffusate already discussed, but also from the fact that in only one or two instances was the amount of growth obtainable from leaf sections in adenine plus 1% sucrose as great as that obtainable from the S.S.A. This means as shown in table 13, that the amount of growth at the optimum concentration of adenine was not as great as that obtainable at the optimum concentration of the S.S.A.

In view of the activity of the acid ether soluble fraction, various organic acids have been tested for activity in leaf growth. The fatty acids were without activity. The dicarboxylic acids on the other hand have good activity, as shown in table 14. Of the acids tested malonic acid was the least active, the activity of succinic acid being somewhat greater. The acids from glutaric to sebacic had the highest activities, their individual activities being nearly equal. Acids longer than sebacic acid, as decane dicarboxylic acid, showed a decided decrease in activity. This optimum activity appears in the dicarboxylic acids from C_5- to C_{10}. The amount of growth obtainable from leaf sections in acids of the glutaric acid group, in contradistinction to adenine, was greater than that obtainable from sections grown in pea diffusate. It is of interest that the presence of a single double bond made no apparent change in activity. Fumaric acid proved to have the same activity as succinic and decene dicarboxylic acid to have the same activity as decane dicarboxylic acid. Due to the low pH of the

solutions, the free acids proved toxic at the higher concentrations. Their salts were, therefore, always used in testing, the potassium salts being generally employed. As shown in table 15, no apparent difference in activity could be found between acids tested as the potassium or as the sodium salts, which should be expected if the acid is active only inside the cell. The pH of the cell sap would then determine the form of acid present within the cell. In this respect, the dicarboxylic acids resemble auxin, which has been shown to be equally active in the Avena test when tested as the free acid, the potassium salt or the sodium salt (D. Bonner 7).

The most surprising group of compounds found active in the leaf test is the inorganic salts. Table 16 gives the activities of those tested. Few inorganic salts, of those tested, have marked activity, these being potassium, sodium, and calcium nitrate, and potassium and sodium chloride. Potassium nitrate is by far the most active, being non-toxic at high concentrations and retaining activity to comparatively low concentrations. The lowest active concentration is 0.1 mg. per cc. of 1% sucrose solution. This corresponds to a concentration of 50 mg. per liter which is high in comparison to the activities of hormones or accessory growth factors in general. On the other hand it corresponds to a dosage of 2.5γ per leaf section, which is within hormonal concentration. Concentrations as high as 10 mg. per cc. are without toxicity, while the other active salts tested at similar concentrations were found to be either inhibitory, or toxic. The effects produced by potassium nitrate are often considerable giving increases in area and wet weight of 100% in 40 hours, i.e. an increase far greater than has been observed for any other active

substance. What the effect is, or its importance is still not clear. That an impurity is responsible for this activity seems unlikely. Samples obtained from three different manufacturing chemical concerns showed unappreciable differences in activity, table 17. Whether the effect is produced by the potassium-ion, or nitrate ion, or both is not certain since both other potassium and other nitrate salts have activity. That it is entirely a permeability phenomenon seems unlikely in that calcium nitrate and potassium nitrate are more nearly additive in their effects than antagonistic as shown in table 18.

Sensitivity of leaf sections to potassium nitrate can be influenced by the previous treatment of the leaves from which the sections are taken. Leaves allowed to grow over night in a 1% sucrose and 1% potassium nitrate solution were found very much less sensitive to potassium nitrate as well as nearly insensitive to pea diffusate when used in the regular leaf test, than leaves that had been 24 hours in 1% sucrose alone, table 19. The sensitivity to pea diffusate as well as to potassium nitrate, of radish leaf sections, was found to be influenced by the previous nutrition of the plants from which the leaves were taken. Leaves of the same age but collected from flats that had been given a) no nutrient, b) Shive's nutrient (see chapter 11) twice a week, and c) Shive's nutrient every day from germination of the seed were tested for sensitivity to both pea diffusate and potassium nitrate, table 20. Shive's nutrient every day almost abolishes sensitivity in the leaf test. This experiment is of course open to the objection that the leaves from the flat given nutrient every day were much larger, and so more mature than the

control leaves. The response to pea diffusate might, therefore, naturally be diminished.

Possible interactions between these various groups of active substances have been investigated. No clear evidence of synergistic effects have been found using the various possible combinations at many different concentrations. The effects at low concentrations are as a rule nearly additive. At higher concentrations the interaction proved inhibitory, i.e. the sum of the individual activities are greater than the observed activity in the combination. It was further observed that the activity of the combination tends to approach that of the compound present at the highest concentration. Whether this means that each group stimulates a particular tissue of the mesophyll, or whether they are all capable of causing growth in a single tissue cannot be settled without further data. It is an interesting correlation, however, that the lamina consists of three main tissues, epidermis, palisade and spongy parenchyma, and that three main classes of active substances are found, purines, organic acids and inorganic salts. During the course of the fractionation described in chapter VIII the total number of leaf units present in the combined extracts increased from 9,000 to 66,000. During the first two extractions with benzene and petrol ether the total number of units remained constant. The total number of leaf units after the next step, however, increased. In that step inorganic reagents were employed, and possibly some of the reagents remained in the extract, thereby increasing the total activity. A more likely explanation, however, is that in this step an inhibiting fraction was removed, giving an apparent increase in total activity of the fraction. A second

factor influencing this increase, is the separation of the active fractions. As mentioned above, the activity of these substances when tested together is not additive, but rather the activity of the combination tends toward that of the factor present at the highest concentration. As these different active fractions are separated and tested independently the sum of the individual activities will give a total activity higher than that originally found.

The study of pure substances shows the same general classes of active substances as found by fractionation of the pea diffusate. Fractionation of pea diffusate showed inorganic salts, organic acids, the purine, hypoxanthine, and some as yet undetermined fraction all contributing to the activity. Four general types of compounds were found active by study of pure substances, inorganic salts, dicarboxylic acids, purines, and amino acids. Went's "phyllocaline" would appear, therefore, to be multiple in nature.

This chapter may be summarized as follows:

(1) A large number of different substances found to be active in different biological phenomena were found inactive in the leaf test.

(2) The activity of amino acids was found to be species specific. Amino acids were found to have low activities in the leaf test, if active at all.

(3) Purines were found active in the leaf test. Adenine was found most active, minimum dosage 0.004γ per leaf section. The maximum growth of leaf sections in adenine was not so great as for pea diffusate.

(4) Dicarboxylic acids were found active in the leaf test. Highest activity was found for the acids from glutaric (C_5) to sebacic (C_{10}). The maximum growth of leaf sections in these acids

was greater than for pea diffusate.

(5) Inorganic salts were found active in the leaf test. Potassium nitrate was found to be the most active inorganic salt tested. Minimum dosage 2.5γ per leaf section. The maximum growth obtained by use of potassium nitrate was far greater (ca.100X) than that obtained from pea diffusate.

(6) The interaction between these various active substances was found to not be synergistic. At low concentrations the effects were additive. At higher concentrations the interaction proved inhibitory, the activity of the combination tending to be that of the compound present at the highest concentration.

(7) The factors involved in the observed increase in total number of leaf units during the course of fractionation of pea diffusate were given as, a) removal of an inhibiting substance, and b) separation of the active fractions.

Table X
***

Substances tested between the concentrations of 0.5 - 0.005 mg. per cc. of 1% sucrose solution, and found to be inactive in the leaf test using Raphanus leaves as test objects.

Barbituric acid	Pantothenic acid
Biotin	Vitamin B_1
Estrone	Vitamin B_2
Indole (3) acetic acid	Vitamin B_6
Nicotinic acid	Vitamin E
Nicotinic acid amide	Vitamin K
2-methyl, 6-amino pyrimidine	Inositol
2-methyl,6-hydroxy pyrimidine	Uracil
6-amino pyrimidine	Thiazole of vitamin B_1
	Urea

Table 11
***

Activity of amino acids in the leaf test using Raphanus leaves as test objects. Activity on basis of growth in 1% sucrose solution is equal to 100.

Active amino acids	Concentration expressed as mg. per cc. of 1% sucrose solution			
	0.5	0.1	0.02	0.005
Proline	124	114	107	105
Asparagine	116	112	103	101
Threonine	115	110	103	100
d-Valine	112	105	103	100
Glutamic acid	Toxic	108	102	100
Alanine	110	100	100	100
Leucine	110	100	100	100

Amino acids tested between the concentrations of 0.5-0.005 mg. per one cc. of 1% sucrose solution and found to be inactive:

Histidine, Glycine, Aspartic acid Arginine, Citrulline, Serine, Phenyl-alanine, Cystine, and beta-Alanine.

Table 12
***

Activities of purines in the leaf test using Raphanus leaves as test objects. Activity on basis of growth in 1% sucrose solution equal to 100.

Purine tested	Concentration expressed as mg. per cc. of 1% sucrose solution				
	0.5	0.1	0.05	0.01	0.005
Adenine	-	-	118	117	113
Hypoxanthine	-	109	116	104	100
Xanthine	-	115	107	100	100
Caffeine	102	112	108	100	100
Uric acid	118	108	100	100	100
Guanine	100	100	100	100	100

Table 13
***

Comparison of the activities of adenine and pea diffusate in the leaf test using Raphanus leaves as test objects, with comparison on several different days.

Activity on basis of growth in 1% sucrose solution is equal to 100.

Date of test	Activity at optimum concentration of pea diffusate	Activity at optimum concentration of adenine
November 5, 1938	125	118
November 9, 1938	135	125
November 12,1938	113	100
November 14,1938	130	110
December 1, 1938	118	107
January 13, 1939	118	108

Table 14
***

Activities of dicarboxylic acids in the leaf test using Raphanus leaves as test objects. Activity on basis of growth in 1% sucrose solution equal to 100.

Acid tested	Concentration expressed as mg. per cc. of 1% sucrose solution				
	0.5	0.2	0.06	0.02	0.01
Malonic	110	105	100	-	-
Succinic: Fumaric	115	110	105	100	-
Glutaric: Adipic: Pimelic: Azulaic: Sebacic	125	122	116	109	104
Decene dicarboxylic: Decane dicarboxylic:	116	108	100	100	-

Table 15
***

Activities of the potassium and sodium salts of dicarboxylic acids in the leaf test using Raphanus leaves as test object. Activity on basis of growth in 1% sucrose solution equal to 100.

Acid tested	Salt	Concentration expressed as mg. per cc. of 1% sucrose solution		
		1.0	0.5	0.1
Succinic	K	114	113	107
	Na	115	113	107
Glutaric	K	120	119	110
	Na	120	118	112

Table 16
***

Activities of inorganic salts in the leaf test using Raphanus leaves as test object. Activity on basis of growth in 1% sucrose solution equal to 100.

Inorganic salts	Concentration expressed as mg. per cc. of 1% sucrose solution					
	5.0	2.5	1.0	0.3	0.1	0.05
KNO_3	175	160	140	125	110	100
$NaNO_3$	130	130	120	110	100	100
$Ca(NO_3)_2$	110	120	110	105	100	100
KCl	130	110	100	-	-	-
NaCl	120	105	100	-	-	-
K_2CO_3	124	110	100	-	-	-
Na_2CO_3	115	110	100	-	-	-

Inactive inorganic salts $(NH_4)_2SO_4$, K_2SO_4, $Mg(NO_3)_2$, $(NH_4)_2CO_3$, $(NH_4)_2NO_3$.

Table 17
***

Activity of three different brands of potassium nitrate in the leaf test using Raphanus leaves as test object. Activities on basis of growth in 1% sucrose solution equal 100.

Concentration mg./cc.	Brand "Merck"	"Kahlbaum"	"Baker"
5.0	150	147	149
2.5	140	141	140
1.0	130	132	128
0.3	118	120	118
0.1	106	104	107
0.05	100	100	100

Table 18
***

Interaction of potassium nitrate and calcium nitrate in the leaf test, using Raphanus leaves as test object. Activities on basis of growth in 1% sucrose solution equal to 100. Concentrations expressed in mgs. per cc. of 1% sucrose solution.

Calcium nitrate Conc.	1.0	0.3	0.1
Activity	124	110	100
Potassium nitrate Conc. / Activity	(Interaction)		
1.0 / 135	151	142	132
0.3 / 120	132	128	124
0.1 / 108	123	120	110

Table 19
***

Sensitivity of leaf sections to potassium nitrate as influenced by previous treatment of the leaves from which the sections were taken. Raphanus leaves as test object. Activity as growth above sections in 1% sucrose solution equal 100.

Concentration of potassium nitrate mg./cc. of 1% sucrose solution	Leaves 24 hours in 1% sucrose solution	Leaves 24 hours in 1% sucrose and 1% potassium nitrate solution
5.0	155	119
1.0	148	108
0.1	109	100

Table 20
***

Sensitivity in the leaf test as influenced by the previous nutrition of the plants from which the leaves were taken. Raphanus leaves as test object. Activity of sections in 1% sucrose expressed as growth above sections in water alone equal 100. Activity of sections in pea diffusate and potassium nitrate expressed as growth in 1% sucrose solution equal 100.

Treatment	Sections from leaves of plants having received Shive's nutrient solution		
	Control (no nutrient)	Twice weekly	Every day
Water	100	100	100
1% sucrose	132	134	140
	(100)	(100)	(100)
1% sucrose plus pea diffusate mg. dry weight/cc.			
5.0	111	110	102
2.0	112	114	105
0.2	109	108	100
1% sucrose plus potassium nitrate mg./cc.			
5.0	142	150	128
1.0	126	118	103

Chapter X
***

The response of excised pea embryos, and excised immature leaves to known leaf growth factors.

Additional assay methods for leaf growth factors were discussed in chapter IV. The two methods described were the culture of excised pea embryos, and young immature leaves. A few preliminary tests have been made on the response of these two tests to known leaf growth factors.

Combinations of different amino acids, either alone or in conjunction with thiamin chloride (for good root growth) were found to have little effect upon leaf development in excised pea embryos. Dicarboxylic acids are also without effect upon leaf growth in excised pea embryos (10). The potassium nitrate concentration in the medium, however, markedly influenced leaf growth as shown in table 21. Embryos grown in medium containing optimum potassium nitrate concentration showed increased leaf growth, however, when small supplements of pea diffusate were added, table 21. This effect of pea diffusate was found to be largely replaceable by additions of adenine to the medium at a concentration of 0.1 milligram per liter. Higher concentrations of adenine were found to be inhibitory, table 21. Whether adenine entirely replaces the effect of pea diffusate for pea embryos has not been definitely proved, since the experiments reported here are few, and were not carried for a period of time longer than four weeks. In certain tests, however, and for this length of time, adenine at a suitable concentration completely replaced the effect of pea diffusate. The present data suggest that the beneficial effect of pea diffusate

upon leaf growth of excised pea embryos is mainly through the effect upon leaf growth of inorganic salts and adenine.

A similar situation was found in the culture of excised etiolated pea seedling leaves. When these were grown upon inorganic salts, sucrose, and adenine (0.1 mg./l.) the growth was found to be approximately as great as that of leaves grown in a medium containing inorganic salts, sucrose, and pea diffusate. The culture of immature first foliage leaves of radish, from plants grown sterilely in the greenhouse, showed that the growth effect of adenine (in conjunction with inorganic salts and sucrose), was as great after a period of five weeks as the growth effect of pea diffusate, table 22. These experiments are again open to criticism in that they are few in number, and that they were maintained only for a short period of time (four weeks). They do, however, indicate that the promotive effect of pea diffusate upon growth of entire excised leaves, as for excised embryos, can be replaced for a period of four weeks by the use of suitable concentrations of potassium nitrate and adenine.

The results from these two tests differ from the results of the leaf test in so far as they tend to place much greater emphasis upon the importance of adenine for leaf growth, and might indicate that the factor most strongly limiting leaf growth is a purine, hypoxanthine or adenine. The importance of the purine fraction is further substantiated and strengthened by a study of adenine as a possible phytohormone discussed in the next chapter.

This section may be summarized as follows:

(1) Leaf growth in excised etiolated pea embryos is promoted through the presence in the medium of potassium nitrate and adenine.

The optimum concentration of adenine was found to be not greater than 0.1 milligram per liter.

(2) Growth of excised etiolated immature pea seedling leaves, as well as of excised immature first foliage leaves of radish seedlings, is promoted by the presence of adenine in the medium.

(3) In all of these cases the promotive effect of pea diffusate appears to be largely replaceable by adenine and inorganic salts.

Table 21
***

Leaf growth of excised pea embryos in response to leaf growth factors.

Treatment	Concentration mg./l.	Average wet weight per leaf relative to control
Inorganic medium: potassium nitrate	85 (control)	100
	324	114
	850	120
Inorganic medium: (KNO_3 conc. 850 mg./l.) plus pea diffusate mg. dry weight/l.	50	Toxic
	5.0	144
Inorganic medium: (KNO_3 conc. 850 mg./l.) plus adenine	0.5	130
	0.1	150

Table 22
***

Response of isolated immature leaves excised from radish seedlings grown sterilely in the light and cultured 4 weeks.

Treatment	Average wet weight per leaf of twenty leaves. Weight in mgs.
Water	10.5
Inorganic salt medium plus 1% sucrose	41.7
Inorganic salt and 1% sucrose medium plus pea diffusate mg. dry weight/l.	
40	Toxic
20	52.2
Inorganic salt and 1% sucrose medium plus adenine mg./l.	
0.5	46.6
0.1	52.2

Chapter XI

The influence of known leaf growth factors on the growth of green plants.

Bonner and Greene (17,18) have shown that certain plants grown in nutrient sand culture respond with markedly increased growth to small additions of thiamin in the nutrient solution. The effect of such additions was shown to be not only upon the root growth, but upon the growth of the shoots as well. Thiamin has no direct effect upon shoot growth (12,55), but as Went (110) has shown there is a factor present in the roots necessary for the growth of stems. Increased root growth would bring about an increased production of this factor, which in turn would cause more vigorous shoot growth. It may well be that a similar case exists in the growth of leaves. It is known that older leaves produce a factor required for the growth of immature leaves (37). If their requirements for this factor are not fulfilled, its addition to the nutrient solution should increase the growth rate of these younger leaves. This in turn would increase the total photosynthetic area of the plant increasing the centers of thiamin and auxin production. The end result should, therefore, be a more vigorous growth of the plant as a whole. To test this view the following experiments were done.

Plants were grown in washed quartz sand contained in 2-gallon glazed crocks supplied with drainage. The crocks were supplied on alternate days with Shive's R_2S_5 nutrient solution containing the minor elements Fe, Mn, Cu, Zn, Mo, and B. On the intervening days the crocks were flushed with water. Additions of growth substances were made directly to the nutrient solution.

In a few experiments plants were grown in an open garden soil contained in 6 or 8-inch pots. In these cases the growth factor additions were made to the water with which the plants were supplied.

Photoperiodically sensitive plants were maintained under controlled photoperiod. "Long photoperiod" below refers to a daily photoperiod of 18-20 hours, the natural day being supplemented by light from Mazda lamps. "Short photoperiod" refers to a daily photoperiod of 9 hours. In all the experiments described below Cosmos plants were maintained under conditions of long photoperiod, while mustard plants were maintained under conditions of short photoperiod.

Dry weight determinations were made at the end of each experiment, that is, unless otherwise noted, approximately 30 days after the beginning of treatment. For these determinations the plants were separated into roots and shoot, shoot in this case signifying the entire aerial portion of the plant. In a few cases thiamin assays were made upon the dry material (8) by the Phycomyces method.

That adenine can increase leaf growth of Cosmos is easily seen in figure 11. This figure shows a comparison between control plants and plants treated with adenine at a concentration of 0.1 mg./l. five weeks after germination. In one experiment which is outlined in table 23, seeds of a late flowering variety of Cosmos sulphureus were planted in 20 crocks. When the seedlings were two weeks old they were thinned to 15 per crock, and the crocks were divided into four lots of 5 each. One lot, a control, continued to receive standard nutrient solution. The second lot was supplied with nutrient solution containing adenine at a concentration of

0.1 mg. per liter (one part in ten million), the third lot containing adenine 0.01 mg. per liter, and the fourth lot with nutrient solution containing 0.001 mg. of adenine per liter. The plants remained in the greenhouse under conditions of long photoperiod. After five weeks of treatment the plants were harvested, and dry weights of roots and shoots determined. Table 23 shows that the increase of shoot dry weight brought about by addition of adenine to the nutrient solution is of a different order of magnitude from the variations in shoot dry weights produced by individual crocks. It is also clear that adenine at concentrations below 1γ per liter of nutrient solution suffices to increase the production of dry weight of Cosmos plants under the present conditions by approximately threefold. The promotive effect of adenine on the growth of Cosmos has been confirmed in ten separate experiments involving 1300 plants. Similar marked promotive effects of adenine on the growth of mustard (Brassica alba) and of cotton have also been observed in sand culture experiments. Adenine has also been found to promote the growth of Cosmos in soil.

Uric acid, which is structurally related to adenine, possesses but slight activity in the leaf test, see table 12. Uric acid, however, is capable of promoting the growth of Cosmos and mustard as is shown diagrammatically in figure 12. In sand culture experiments uric acid appears to influence the growth of leaves very materially just as does adenine, and it seems, therefore, possible that uric acid is converted in the intact plant to an active leaf growth substance - adenine or hypoxanthine. This view is somewhat strengthened by the fact that the growth promoting effect brought about by uric acid is of approximately the same

magnitude as that elicited by adenine. The fact that combinations of the two substances give a growth promoting effect approximately the same as that attributable to either substance alone, table 24, might also support the above view were it not for the fact that combinations of thiamin and adenine also are no more promotive of growth than either substance alone, see below.

Growth promoting effects of uric acid have been observed with pine seedlings (Pinus ramorata) grown in soil out of doors.

It might be expected that a combination of the growth promotive effects of thiamin, a root growth factor, and adenine, a leaf growth factor, should be possible. Such, however, is not the case, as is shown in table 25 for mustard and in table 26 for Cosmos. With mustard the plants which received both growth factors actually possessed a somewhat smaller dry weight than did the plants treated with either substance alone. A possible explanation of the non-additive effects of thiamin and adenine may be found in the fact that plants treated with adenine contain much more thiamin than plants not treated with adenine, table 27, and in fact, both Cosmos and mustard when treated with adenine contain nearly as much thiamin as similar plants treated with thiamin (8). It is known from investigations of the thiamin contents of different species of plants (18) that those species which contain relatively abundant thiamin (e.g. tomato) are little affected in their growth by additions of thiamin. Those species on the other hand which were found to respond to added thiamin were those with relatively small amounts of thiamin.

It is possible then that Cosmos or mustard plants treated with adenine produce so much thiamin that they are not limited in

their growth by the available amount of this substance. A similar case of the interaction of growth factors has been noted by Bonner and Bonner (14) in the case of isolated pea embryos. In this case pea embryos supplied with thiamin were found to synthesize much more ascorbic acid than pea embryos not supplied with thiamin. Although the interaction of growth factors has not been studied in detail it has been found that combinations of thiamin, nicotinic acid, adenine, estrone; and thiamin, nicotinic acid, adenine, vitamin B_6 exert no larger effect on the growth either of Cosmos or of mustard than does one only of the above substances (8).

At the same time that the above adenine and uric acid experiments were carried out similar experiments using the root growth factors, thiamin, nicotinic acid and vitamin B_6 were carried out (8). It is of interest to note that at least in some experiments the root growth factors just mentioned influenced the growth of the roots relatively more than that of the shoots, while adenine and uric acid on the other hand, influenced root growth relatively less than did the root growth factors. Thus the mean shoot/root ratio (as derived from dry weights) of the control plants from three representative experiments with Cosmos was approximately 7, while in the same experiments the shoot/root ratio of Cosmos plants treated with leaf growth substances was 10, and that of Cosmos plants treated with root growth substances 4. A similar relation obtains for numerous of the experiments with mustard. Exceptions to this generalization, however, were found as the experiment of table 23.

That adenine actually functions in these experiments primarily as a leaf growth factor is indicated by the fact that with Cosmos, applications of adenine result in a definite increase in

leaf area even before a general increase in the vigor of the treated plant has become marked. Figure 13 shows five representative leaves (the youngest fully mature leaf from each of five separate plants) from three series of plants, taken two weeks after growth factor application has been started. It may be seen that although the leaves from the thiamin treated plants are somewhat larger than those of the control, those of the adenine treated plants are considerably larger still. That the primary function of adenine in these experiments is as a leaf growth factor is also indicated by the fact that adenine has no activity as a root growth factor (13).

The marked promotive effects of the root growth factors thiamin, nicotinic acid, and vitamin B_6, of estrone, and of the leaf growth factors adenine and uric acid on the growth of plants might lead to the impression that the chemical specificity of the growth effects is small. Such, however, has been shown to not be the case (8). Experiments with Cosmos indicate that additions of numerous organic compounds to the nutrient medium are without substantial growth promoting influence, at least when the substances are applied in concentrations comparable to those in which the plant growth factors exert their influence. Among such substances inactive on the growth of Cosmos, but each active in one or more other physiological processes, were found urea, glutamic acid, and decane dicarboxylic acid (8).

Tomato was found not to respond to additions of adenine or uric acid. It is also known that it does not respond to additions of the factors necessary for the luxuriant growth of isolated tomato roots, thiamin, nicotinic acid, and vitamin B_6. Thiamin has been shown to be produced in great abundance by tomato,

and it would seem reasonable to suppose, therefore, that these other factors are produced by the tomato plant in amounts sufficiently large not to limit growth in the normal plant. Wheat also failed to respond in sand culture to any of the above mentioned growth factors (8). It would seem that these two much selected crop plants may themselves produce abundant supplies of the growth factors at present known.

This chapter may be summarized as follows:

(1) The growth of Cosmos and mustard plants in sand culture is shown to be promoted by additions of known leaf growth factors, adenine and uric acid.

(2) Adenine and uric acid appear to influence primarily leaf size. However, they both exert marked effects on the general vegetative growth of plants. These effects may be secondary to the influence on leaf growth.

(3) The effects of thiamin and adenine on growth of Cosmos and mustard in sand culture are not additive. It is suggested that this non-additive effect is due to the fact that plants treated with adenine contain nearly as much thiamin as plants treated with thiamin alone.

Table 23
***

Effect of adenine on the dry weight accumulation of Cosmos plants. Experiment started two weeks after planting of seed and harvested after a further five weeks. Fifteen plants per crock. Means of five crocks.

Treatment	Dry weight of shoots: gms. per crock (15 plants)	Dry weight of roots: gms. per crock (15 plants)
Control (nutrient only)	0.93 ± 0.07	0.106
Adenine 0.1 mg. per liter in nutrient	2.95 ± 0.23	0.437
Adenine 0.01 mg. per liter in nutrient	2.75 ± 0.15	0.404
Adenine 0.001 mg. per liter in nutrient	2.78 ± 0.12	0.346

Table 24
***

Effects of adenine and uric acid alone and in combination on the growth of Cosmos and mustard plants. Approximately 45 plants with each treatment. Treatment started two weeks after planting and experiment harvested after a further four weeks.

Treatment	Total dry weight in gms. per plant Cosmos	Mustard
Control (nutrient alone)	0.161	0.079
Adenine 0.01 mg./liter	0.226	0.169
Uric acid 0.01 mg./liter	0.198	0.169
Adenine 0.005 mg./liter + uric acid 0.005 mg./liter	0.226	0.161

Table 25
***

Effects of vitamin B_1 and adenine alone and in combination on the growth of mustard plants. One of two experiments yielding similar results. Treatment started two weeks after planting and plants harvested after a further four weeks. Fifty plants per series.

Treatment	Total dry weight per plant; gms.
Control (nutrient only)	0.079
Vitamin B_1 0.01 mg./liter	0.192
Adenine 0.01 mg./liter	0.169
B_1 0.005 mg./liter + adenine 0.005 mg./l.	0.123

Table 26
***

Effects of vitamin B_1 and adenine alone and in combination on the growth of Cosmos plants. Treatment started two weeks after planting and plants harvested after a further four weeks. Fifteen plants per series. One of three experiments yielding similar results.

Treatment	Gms. dry weight per plant: Shoot	Root	Total
Control (nutrient only)	0.092	0.0146	0.108
Vitamin B_1 0.01 mg./liter	0.328	0.103	0.431
Adenine 0.01 mg./liter	0.277	0.0365	0.301
B_1 0.005 mg./liter + adenine 0.005 mg./liter	0.361	0.058	0.419

Table 27
***

Effect of adenine treatment on the vitamin B_1 content of Cosmos and mustard plants. Plants harvested when six weeks old, after four weeks of treatment.

Treatment	Vitamin B_1 content of leaves: Cosmos		Mustard	
	γ/gm.dry wt:	γ/plant	γ/gm.dry wt:	γ/plant
Control (nutrient only)	2.75	0.121	4.5	0.30
Vitamin B_1	5.00	0.690	8.4	1.43
Adenine	4.50	0.487	6.6	1.00

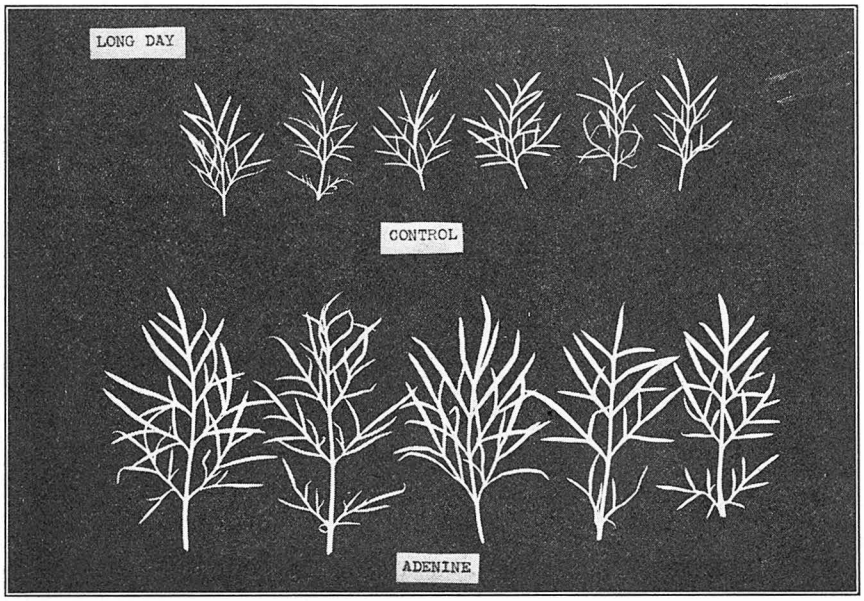


Figure 11.

Leaves from plants grown with and without adenine.
Control: leaves from Cosmos plants grown in washed sand and watered with Shive's nutrient solution, five weeks after germination.
Adenine: leaves from Cosmos plants grown under identical conditions but with the addition of 0.1 mg./l. of adenine to the nutrient solution.

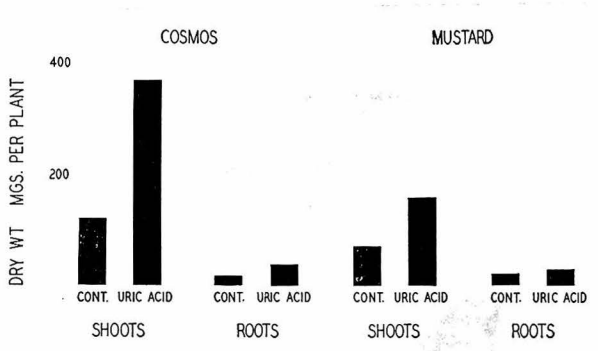


Figure 12.

Effect of uric acid (0.01 mg. per liter) on the growth of Cosmos and mustard plants. Experiments harvested approximately four weeks after beginning of treatment. Cosmos = 60 plants per treatment. Mustard = 45 plants per treatment.

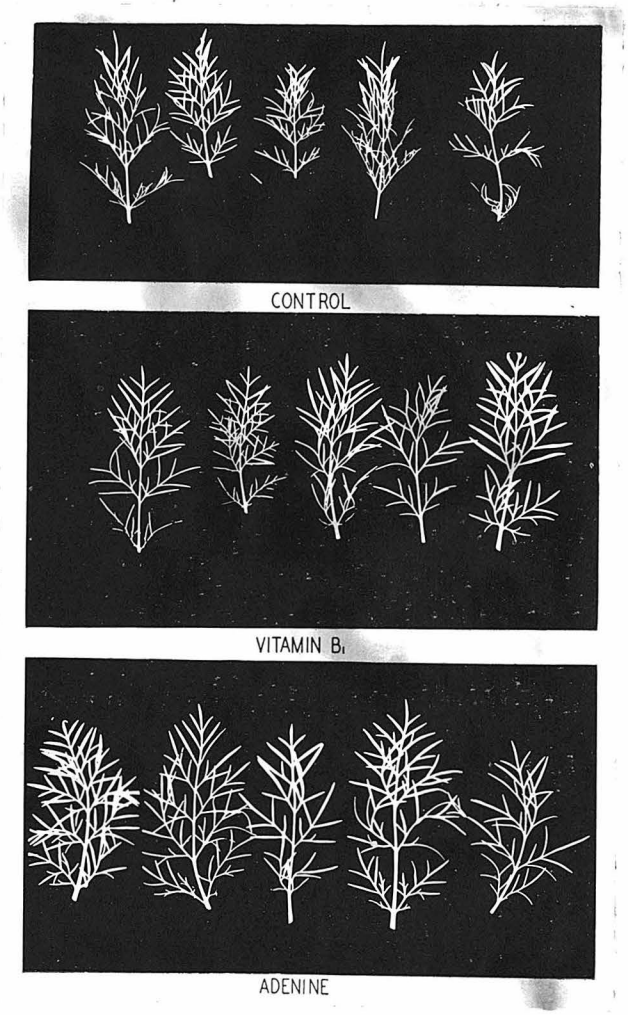


Figure 13.

Representative leaves from Cosmos plants subjected to various treatments. Youngest mature leaf from each of five plants in each series. Leaves taken approximately two weeks after beginning of treatment. Control= leaves from plants which received nutrient only. Vitamin B_1= leaves from plants which received 0.01 mg. of vitamin B_1 per liter of nutrient. Adenine= leaves from plants which received 0.01 mg. of adenine per liter of nutrient.

Chapter XII

***

Discussion

Pea diffusate, known to be rich in naturally occurring leaf growth factors, was selected as the crude source best suited for isolation of the factors limiting leaf growth in a normal intact plant. Fractionation of the pea diffusate established several classes of compounds as contributing to the activity of the crude material, one of which was shown to be a purine by the isolation and recognition of hypoxanthine, figure 14. In the course of testing other known purines for activity in leaf growth, the naturally occurring purine adenine, figure 14, was found to have an activity in the leaf test greater than that of hypoxanthine. A naturally occurring purine with activity greater than that of hypoxanthine would seem to cast some doubt upon the assumption that hypoxanthine, isolated from a source rich in naturally occurring leaf growth factors, represents the purine factor limiting leaf growth in a normal intact plant. That hypoxanthine may occur in relative abundance in the seed and seedlings of several members of the Leguminosae is seen from the report of its isolation (47). The isolation of adenine on the other hand from the seeds or seedlings of any legume has not been reported, though it has been reported from the leaves, seeds, and even pollen of other plants (47). The importance of adenine in many cellular constituents and processes (nucleic acids, and nucleosides) indicates that adenine may well occur in the seedlings of Pisum, but in all probability at high dilutions. Its isolation would, therefore, require a concentration greater than the four hundred times enrichment noted in the

isolation of hypoxanthine from pea diffusate - see chapter VIII. Fosse (36) has shown that uric acid metabolism in the Leguminosae proceeds in a manner similar to that of mammals and one might expect, therefore, that adenine metabolism would also proceed along similar lines, the first product of its metabolism being hypoxanthine produced by oxidative deamination. That hypoxanthine could arise in part by an in vivo metabolism of adenine during the preparation of the diffusate, and in part by an in vitro oxidative deamination of adenine during the course of fractionation seems possible. The conclusion, therefore, that the purine factor limiting leaf growth in an intact plant is hypoxanthine, may not be justified. The relative activities of the various purines in the leaf test were found to decrease in the order: adenine, hypoxanthine, xanthine, and uric acid with guanine totally inactive. This order is the probable sequence of adenine metabolism, i.e., adenine through an oxidative deamination is converted to hypoxanthine, which is in turn oxidized to xanthine. Xanthine is oxidized to uric acid which is then oxidized to allantoin (36). This might indicate that adenine is the active purine, and that the others are active only after synthesis into adenine, lowering of the apparent activity of the original purine being, therefore, correlated with the number of steps involved in this synthesis to adenine. A different basis for considering adenine the limiting purine factor in intact plants is found from a consideration of the possible mechanism involved in the limitation of leaf growth. For this purpose the role of other growth factors should be considered, particularly the relationship of growth factor requirements to cellular oxidation systems. No attempt

will be made to give a complete list of individual organisms requiring a particular factor. Rather a general survey of factors known to limit growth in one or more microorganisms or higher plants will be discussed.

Cozymase is known to be an accessory growth factor for Haemophilus influenzae and allied types (63a). The constituent part of cozymase, nicotinic acid amide, is necessary for the growth of several microorganisms (48). In many cases nicotinic acid is utilized equally well as in the growth of pea roots (1), or even better as for example by the diptheria bacillus (70).

Lactoflavin (vitamin B_2) requirements are often encountered, as for example in the case of lactic acid bacteria (76). Heme-like requirements have also been reported (64,67).

Thiamin is required by many different organisms, and in varying stages of degradation. The intact molecule is required for example by the parasitic fungus Phytophthora (84). An organism less fastidious which can use equally well the two halves of the thiamin molecule, the thiamin pyrimidine and the thiamin thiazole, figure 15, is Phycomyces blakesleeanus (27,92,95). Pea roots are capable of utilizing even less intact portions (15). The mold Absidia ramosa has been reported to require both components for rapid development (94), but can grow slowly in the presence of the pyrimidine alone. Still others develop as rapidly in the presence of one component alone as when the whole thiamin molecule is supplied. This is true of Mucor ramannianus which needs only the thiazole constituent (71), and also of the yeast Rhodotorula (93). A very important fact in regard to the requirements for these constituent parts of the thiamin molecule is, that though they appear

to be requirements for the particular constituent per se, they in reality are an indirect requirement of the organism for thiamin itself.

The growth factors enumerated above are ones whose limitation of growth is understandable on the basis of limiting a specific cellular reaction and these factors account for a large proportion of the total number of known growth factor requirements. The requirement for factors as biotin (55-57), pantothenic acid (116-119) and ascorbic acid (28,67) are of course well known. These substances in all probability are important in some biological oxidation (38,75). At the present time, however, their function in cellular economy is too obscure to permit of further discussion here. Other chemically well-defined growth factors whose role in cellular economy is even more obscure include auxin (113), cholesterol (28), estrone (55-91), vitamin B_6 (68,96), pimelic acid (69), and traumatic acid (16,33,34).

Modern developments in the field of cellular metabolism have tended to give a unified concept of biological oxidations. Disregarding the special processes, there is one mechanism operative throughout all metabolism, aerobic or anaerobic, i.e., a shift of metabolic hydrogen (4,75,103). Under aerobic conditions this shift terminates in water, under anaerobic conditions in lactic acid or alcohol. The characterization of metabolism as a hydrogen shift toward oxygen implies that carbon dioxide, the second product of oxidation, does not arise through a direct oxidation of carbon atoms, but rather by catalytic decarboxylation. Recent developments have further emphasized that phosphorylation processes are not limited to the first attack upon hexoses, but that phosphorylation reactions dominate almost the entire field of anaerobic reactions (75), thus

dominating the initial reactions common to both respiration and fermentation.

Three main classes of systems must, therefore, be concerned, and are found in cellular oxidations, i.e. hydrogen transporting systems, decarboxylation systems, and phosphorylation systems. The enzyme systems involved in the initial dehydrogenation of hexoses or their breakdown products are known to have phosphopyridine nucleotide prosthetic groups, figure 14, (35,104-106). By removal of hydrogen from the substrate, this prosthetic group called codehydrogenase is reduced. It has been shown that this reduced codehydrogenase is reoxidized in fermentation by such metabolites as pyruvic acid or acetaldehyde, or in respiration it is reoxidized by diaphorase. Diaphorase, having the probable prosthetic group alloxazine adenine dinucleotide (39,107), figure 15, is in turn oxidized by the cytochrome system which is in turn oxidized via cytochrome oxidase by oxygen. As mentioned earlier, a growth factor requirement for diphosphopyridine nucleotide (cozymase) is known. The limitation of growth is then explained (experimental proof for this has been obtained - 63b) on the basis that the organism in question is unable to carry out the initial dehydrogenation of the foodstuffs. More common than the requirement for the entire coenzyme, is the requirement for its constituent nicotinic acid amide or its derivative nicotinic acid. Though direct proof is lacking, the requirement for these two factors is interpreted as being actually a requirement for the codehydrogenase. On this basis the organism is unable to synthesize nicotinic acid or acid amide, but can, given this one factor, synthesize the codehydrogenase. The limitation of growth by these factors is then explained on the same

basis as the limitation of growth by codehydrogenase deficiency, i.e. inability to carry out the initial dehydrogenation of the foodstuffs.

Lactoflavin is known to be a constituent of the prosthetic group of diaphorase (39,107). Though direct proof again is lacking it seems logical to assume that the requirement for lactoflavin is actually a requirement for diaphorase. Cytochrome is known to be a heme compound. Heme-like requirements are, therefore, understandable on the grounds of cytochrome requirement, and the aerobic growth requirement of Haemophilus influenzae for a heme-like substance has been shown by Lwoff (64) to probably be a cytochrome requirement. That fewer cases of lactoflavin and heme requirements are found in microorganisms than for nicotinic acid amide might be expected. Although the dehydrogenase is necessary for the initial removal of hydrogen from the foodstuffs the oxidation of the reduced dehydrogenase may go through other channels, as in many types of fermentation. It might be expected, therefore, that facultative anaerobes are limited by lactoflavin and heme-like substances only under aerobic conditions. Haemophilus influenzae is an excellent example. Aerobic growth of this organism requires the entire codehydrogenase and a heme-like substance. Anaerobic growth on the other hand requires only the codehydrogenase (64).

Decarboxylations center almost entirely upon the enzyme system carboxylase, the prosthetic group of which has been shown to be thiamin pyrophosphate (62), figure 15. Abundant examples of thiamin requirements are found, and experimental proof has been obtained by Horowitz and Heegaard (46) that in pea roots at least,

thiamin requirements are actually requirements for cocarboxylase. Growth requirements for thiamin are, therefore, understandable on the basis of metabolism inhibition through lack of a decarboxylation system.

Hypoxanthine has at present no known function in cellular oxidations. Adenine on the other hand is known to be important in biological oxidation systems and could limit growth by a mechanism similar to that described for nicotinic acid amide, and thiamin. Adenine could limit metabolism by being an essential component in, 1) a dehydrogenase system and, 2) a phosphorylation system. Codehydrogenase and codiaphorase are both nucleotides, and thus require adenosine phosphate in their synthesis. Organisms are known to require either the intact thiamin, thiamin pyrimidine, or thiamin thiazole. Just as these organisms require either thiamin pyrimidine or thiamin thiazole for thiamin, and thiamin for cocarboxylase, leaves might require adenine for codehydrogenase, or codiaphorase. Leaves with adenine synthesis insufficient for codehydrogenase synthesis would require additional adenine to fulfill their requirements for this factor. Limited adenine synthesis would also affect the third main system involved in biological oxidations mentioned earlier, i.e. phosphorylations. Adenosine phosphates are known to be an important phosphorylation system and are of the utmost importance not only in the initial stages of carbohydrate oxidation, but also in the production of cocarboxylase by phosphorylation of thiamin. At present it is impossible to make any prediction as to which of these mechanisms is operative in leaf growth. An attempt has been made, however, to indicate the possible mechanisms through

which adenine could limit growth in a manner similar to the mechanism known for other growth factors. These mechanisms do not, of course, rigorously establish adenine as the purine factor active in leaf growth in the intact plant. In view of the fact that adenine shows the highest activity in the leaf test of any of the purines tested, and since the mechanism of its growth limitation is readily explainable on a basis similar to that of other known growth factors of the intact plant, adenine would seem to be the purine leaf growth factor of the intact plant. Hypoxanthine would then show the same relationship to adenine as thiazole and pyrimidine to thiamin.

The possible role of organic acids in leaf growth will be reconsidered in connection with the role of inorganic salts. It might be well to first consider their activity as measured in terms of the different tests. Dicarboxylic acids were found active in the leaf test, the most active being acids containing five to ten carbon atoms. The structure of the naturally occurring active acid has not yet been established, but appears not to be one of the common dicarboxylic acids. In the excised pea embryo test and in the green plant test, dicarboxylic acids were found inactive. In the excised pea embryo and in the green plant, therefore, a factor other than dicarboxylic acids appeared to be limiting leaf growth, which was shown to be adenine in the presence of potassium nitrate.

The role of inorganic salts in leaf growth should next be considered. Potassium nitrate was found the most active inorganic salt tested. Also active in the leaf test were calcium and sodium nitrate, the activities of calcium nitrate and potassium nitrate being nearly additive. Ammonium salts were found totally inactive. In the excised pea embryo test potassium nitrate was found to exert an influence upon leaf growth, and while not demonstrated in the

present work, it is known that the presence of nitrate in the nutrient affects leaf growth of green plants grown in sand culture (29,41). It has of course been generally observed that an abundance of nitrogen in the soil gives rank growth of foliage. The striking morphological response of certain plants given nitrate has often been reported, thus the most striking result obtained by fertilizing apple trees with nitrate is increased leaf growth (Verner, 100). Similar effects have been noted upon other plants as cotton (45), tobacco (5), and tomato (29). A common subject of investigation for many years has been the relative effects of ammonia and nitrate salts as the external source of nitrogen upon morphological development. The effects of nitrate and ammonia on culture of tomato observed by Clark (29) are of particular interest in connection with the present work. Clark grew tomatoes in sand culture, and divided them into two main series; "nitrate" plants receiving calcium nitrate as the sole source of external nitrogen, and "ammonium" plants receiving ammonium sulfate at the same molar concentration of nitrogen as the nitrate plants. The nitrate plants showed, after one month, much more luxuriant growth than the ammonium plants; the differences as found by Clark are shown in table 28, taken from his paper. Total nitrogen in the leaves of ammonia plants expressed as percentage dry weight was higher than in the nitrate plants. Protein nitrogen was very much higher in the leaves of the ammonium plants than in the leaves of the nitrate plants, as well as the total soluble nitrogen. Considering the total soluble nitrogen, ammonium, amino, asparagine, and glutamine nitrogen were all markedly higher in the leaves of the ammonium plants than in the leaves of the nitrate plants. Only nitrate and total inorganic nitrogen was higher in

the nitrate leaves. The unknown soluble organic nitrogen was nearly the same in the two series indicating that the increase in total soluble nitrogen in the leaves of the ammonium plants was due mainly to amino and amide nitrogen. One further difference between the two series was found in the concentration of organic acids. On the basis of milliequivalents in 100 grams of dry leaf tissue, Clark found 153 in the nitrate plants, 72% of which could be accounted for as oxalic, malic and citric acids. Only 71 were found in the ammonium plants, 8% of which could be accounted for as oxalic, malic and citric acids. The concentration of unknown acids is, therefore, slightly higher in the ammonium series.

Considering leaf growth alone, one striking morphological difference between the nitrate and ammonium plants is the greatly increased leaf growth of the nitrate plants. This effect is probably not due directly to amino acid or amide synthesis. Amino acids and amides, which are known to be active in the leaf test, occurred at higher concentrations in the leaves of the ammonium plants. The effect may be due to increased adenine synthesis, since the increased adenine concentration sufficient to markedly increase leaf growth is far too small to be detected in Clark's gross analyses. Against this possibility, however, are a) that the adenine precursor may be an amino acid, or that in any case the precursor is probably in the form of reduced nitrogen which would be in equivalent or greater abundance in the ammonium plants; b) that an effect greater than the effect of nitrate alone can be obtained with adenine in the excised pea embryo test; c) that the total amount of growth observed in leaf sections floated upon a potassium nitrate solution is many times greater than that obtainable from similar sections floated upon an adenine

solution. It must be emphasized that these arguments do not rule out the possibility that the increased growth caused by nitrate may be due to increased adenine synthesis; they do show, however, that other possible mechanisms should be considered. Assuming that this effect of nitrate upon leaf growth is not due primarily to reduced forms of nitrogen, two possibilities only are left, 1) that nitrate itself is directly responsible, and 2) that the effect of nitrate is due to some secondary reaction during the course of nitrate reduction. How nitrate could act directly is difficult to understand, but the possibility at present exists. As it is known that many plants are able to accumulate large quantities of nitrate without injury, and that some typical accumulating plants are without exceptionally large leaves, as for example celery (79), the theory of direct action seems less likely. The mechanism of nitrate reduction is still obscure (72). The two important facts that have been established are that nitrate is first reduced to nitrite, and this reduction can take place in the dark. Whether nitrate reduction takes place under the conditions of the leaf test is not determined. Zaleski (74) showed the probable reduction of nitrate in the dark in detached leaves floated upon sucrose solution. His evidence is inconclusive, however, nor does the work of others (6,101) establish nitrate reduction as occurring under the conditions of the leaf test. The catalysis of nitrate reduction in bacteria by a carbon monoxide sensitive iron system (nitrate reductase) has been reported by Quastel (80). Whether this system acts directly upon the nitrate, however, has not been determined, making it difficult to decide whether the nitrate reduction takes place by oxidation of a specific enzyme or by oxidation of a

specific metabolite. Nitrate reduction, being strongly endothermal, requires an abundance of carbohydrate. Hamner (41) has studied the effect of nitrate addition to tomato plants previously depleted of nitrate. He found that in plants with high carbohydrate content in the dark, nitrate was detectable in the tops four to six hours after nitrate application, with nitrite appearing about two hours later. Respiration showed a marked increase subsequent to the appearance of nitrate in the tops. He further observed that after fourteen hours the leaves of nitrate treated plants were appreciably enlarged. With low carbohydrate reserve, the same phenomenon was found, but considerably delayed. It cannot be determined from his data, however, whether the delayed response in leaf growth was due to a longer time required for the nitrate to arrive in the tops, or whether it was delayed nitrate reduction. As mentioned earlier, Clark (29) found a higher concentration of organic acids present in the nitrate plants than in the ammonium plants. This is of interest in view of the fact that organic acids, as discussed earlier, are known to be active in promoting leaf growth. It is, therefore, possible that the action of nitrate is through the production of a specific acid, this acid being produced during the course of nitrate reduction, indirectly through increased carbohydrate metabolism, or directly through oxidation of a specific precursor. The origin of this acid is probably dependent upon which of the possible mechanisms of nitrate reduction discussed above is operative.

At the present time, therefore, the role and mechanism of organic acids and inorganic salts in leaf growth must be left in abeyance. In summarizing it can only be said that there is the possibility that nitrate as well as organic acids act

directly in promoting leaf growth. There is the second possibility on the other hand, that the nitrate after reduction or during the course of reduction gives rise to secondary substances active in leaf growth.

In conclusion, known leaf growth factor requirements should be considered in relation to the known growth factor requirements of the other organs. That the growth factor requirements of leaves are different from those of the other organs is obvious. Leaves require adenine, inorganic salts, and probably an organic acid. Stems require auxin, biotin and caulocaline, while roots require thiamin, nicotinic acid and vitamin B_6. This in itself is not surprising if mutual inter-dependence of the organs is to be found. Considering the point of origin of these various growth factors, the leaf is known to be the seat of synthesis for factors controlling the growth of all other gross organs in the plant. As shown diagramatically in figure 16, auxin precursor is produced in the leaf, from which it is transported to the terminal growing bud. The growing bud then converts this precursor into active auxin controlling stem and bud growth in conjunction with secondary factors. Thiamin produced in the leaf regulates root growth, and florigen produced in the leaf controls floral initiation. Leaves even produce substances controlling their own growth. Auxin produced in the leaf controls vein and petiole growth. The factor produced in older leaves affecting the growth of younger leaves (Gregory 37) is tentatively assumed to be adenine, thus placing adenine on the list of known phytohormones. Leaves controlling the growth of so many organs might well be expected to have their growth in turn controlled by at least one other organ, thus leaving the plant not entirely at the mercy of the caprices of the leaves. Roots can affect leaf growth as shown by

the increased leaf growth of Cosmos in nutrient sand culture when given thiamin. Thiamin is totally inactive in leaf growth, indicating that increased leaf growth is a secondary phenomenon correlated with increased root growth. The present work indicates that this factor present in the roots affecting leaf growth is an inorganic salt, nitrate. Nitrate salts are, therefore, tentatively suggested as the factor present in the roots and affecting leaf growth. Stem growth, wisely enough, is not controlled solely by the leaf, but must have in addition a factor present in the roots, caulocaline. A rough outline of the system might then be: leaf growth controlled by the roots, and root growth by the leaves, while stem growth is controlled cooperatively by roots and leaves. The value of such a system to the plant is seen in the fact that increased root growth gives increased leaf growth, the two of them then together giving increased stem growth and a normally proportioned plant. Or increased leaf growth gives increased root growth and the two again together give increased stem growth, thus preserving the symmetry of the normal plant. Reductions in growth rates would act in the reverse but analogous manner. The original and individual symmetries would not be due to relationships as those just described but might be due to the relative dependence of the various organs upon each other, i.e., upon the autotrophy of the different organs, ultimately determined by the genotype.

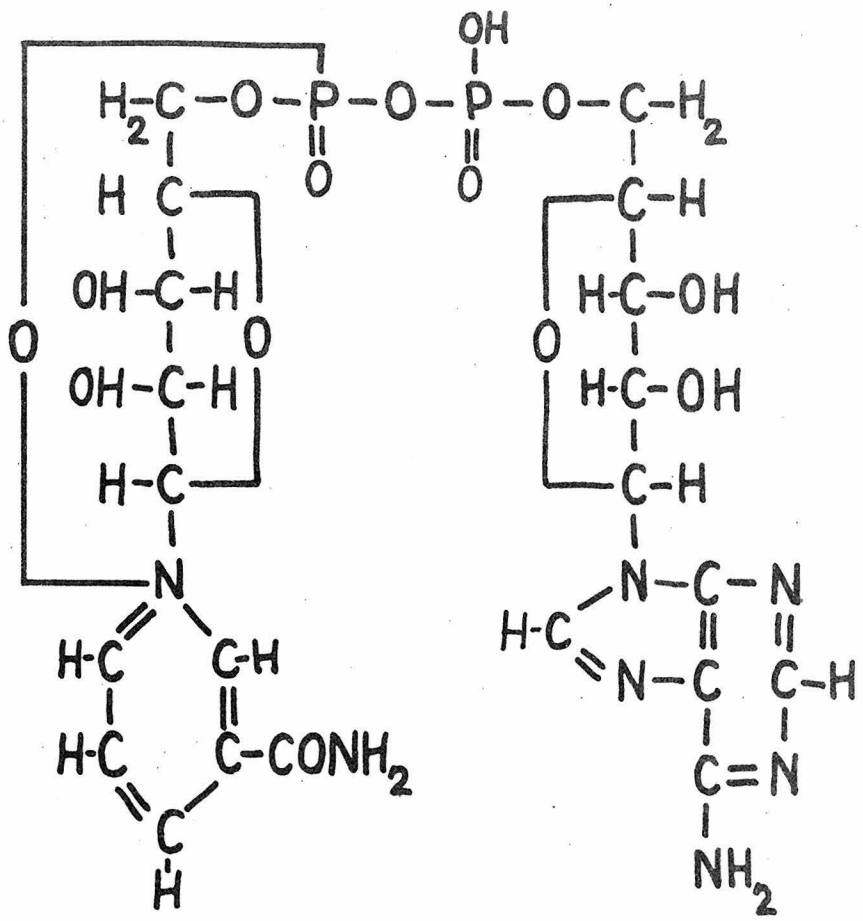
Table 28.

***

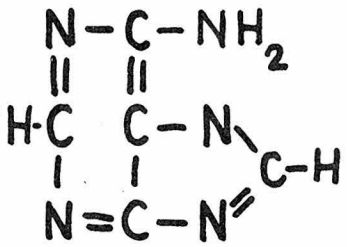
The wet and dry weights of tomato plants receiving only ammonium sulfate or calcium nitrate as the external source of nitrogen, from Clark (29).

Solution	Average fresh weight of one plant		Average dry weight of one plant			Dry weight expressed as % of fresh weight	
	Leaves	Stems	Leaves	Stems	Roots	Leaves	Stems
	gm.	gm.	gm.	gm.	gm.	%	%
Nitrate	94.4	175.6	10.1	12.5	1.8	10.7	7.1
Ammonium	38.7	53.7	4.1	4.1	2.2	10.6	7.6

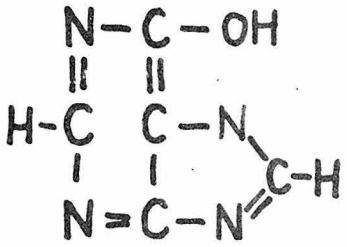
Diphosphor-pyridine-dinucleotide



Adenine



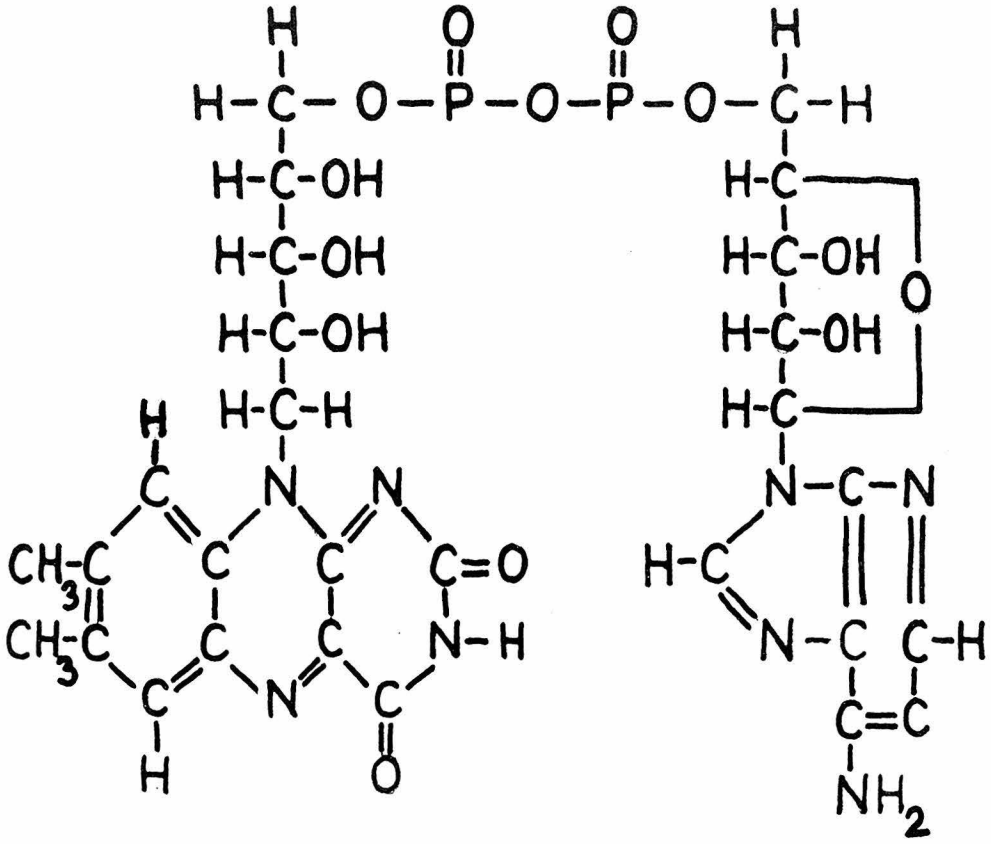
Hypoxantine*



*(Hypoxanthine)

Figure 14.

Alloxazine - adenine - dinucleotide



Thiamin-pyrophosphate

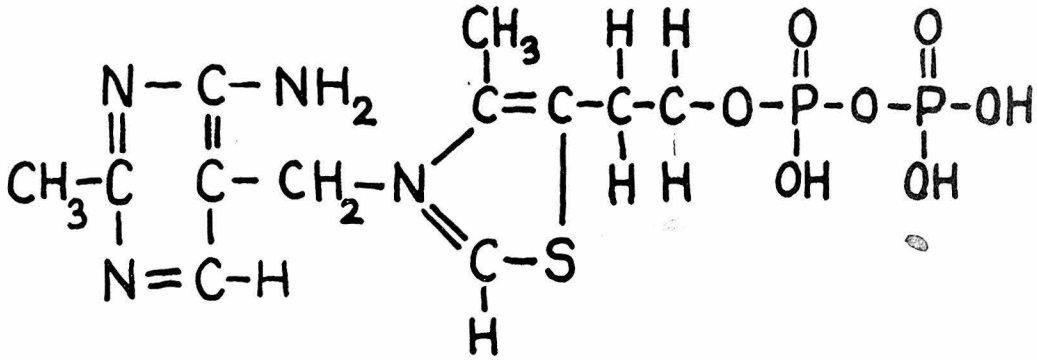


Figure 15.

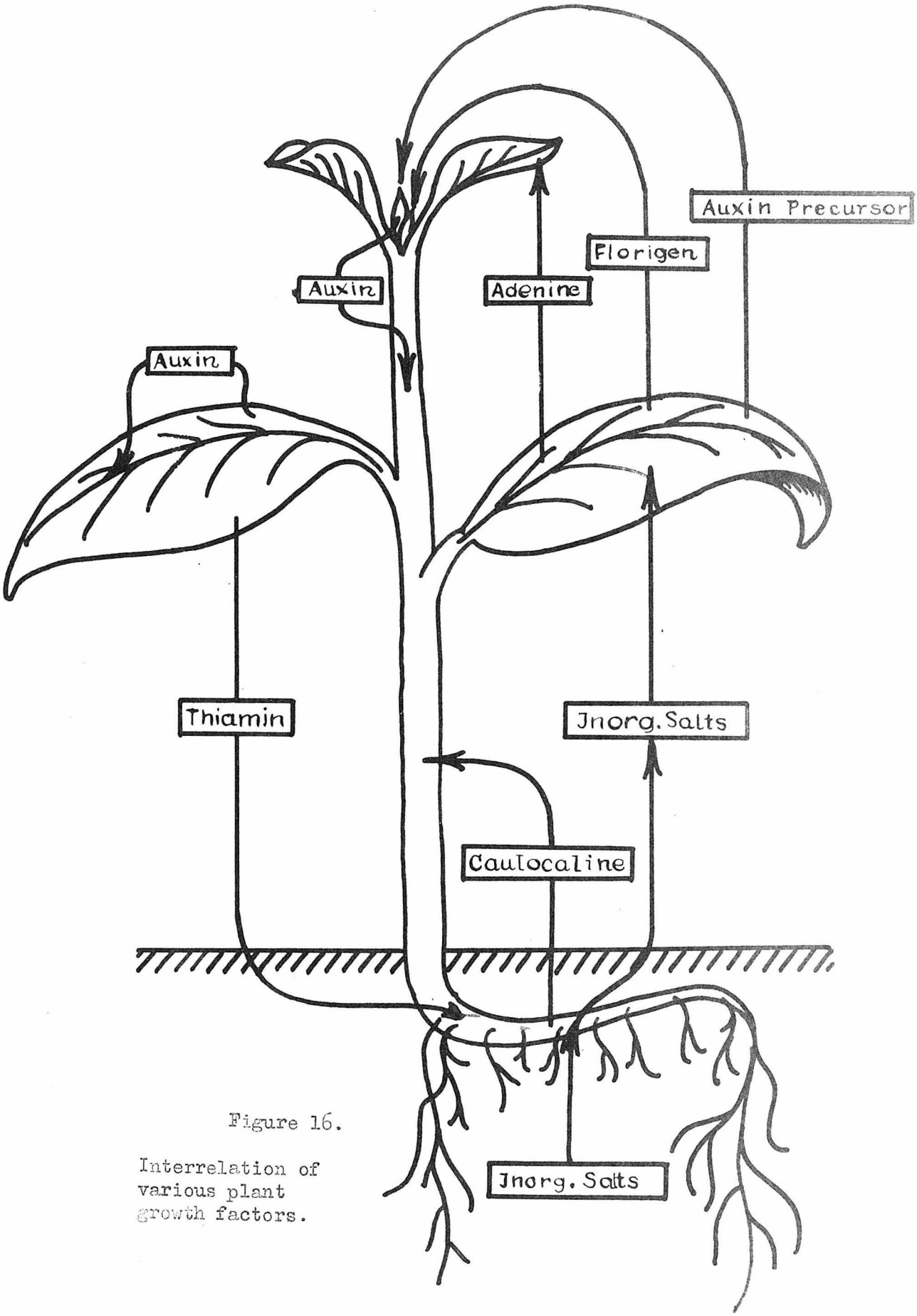


Figure 16.

Interrelation of various plant growth factors.

Conclusions

***

Detailed summaries have been included at the end of each chapter so that only the most significant facts concerning leaf growth factors need be outlined here.

(1) A satisfactory quantitative bio-assay for leaf growth factors is as follows: Circular disks ca. 19.5 mm^2 in area are cut from young first foliage leaves of Raphanus. Twenty such disks are floated on the solutions to be tested and allowed to grow on these solutions for 30 hours at 25° C. The total wet weight of all 20 sections from a single solution is then determined by direct weighing in a standard manner. Additional methods that may be used are measurement of leaf growth in excised embryos, and growth of excised immature leaves.

(2) Leaf growth factors are widely distributed in various natural products and extracts. The standard crude extract with which the leaf test is calibrated is a seed diffusate of Pisum sativa. This pea diffusate is more active as measured in the leaf test than any of the other seed diffusates prepared, or of any of the leaf extracts prepared with the exception of a young palm leaf extract. Urines are very active, cow urine being 100 times more active than the standard pea diffusate preparations.

(3) Fractionation, and isolation in part, of the active constituents of the pea diffusate shows inorganic salts, organic acids, and the purine hypoxanthine contributing to the activity of the crude material. Study of pure crystalline substances shows three general classes of substances active at high dilutions promoting leaf growth, i.e. inorganic salts, organic acids, and purines. Leaf growth factors appear, therefore, to be multiple in nature. The factor limiting leaf growth in many different species of plants, however, appears to be a

purine. The purine with highest leaf promoting activity is adenine, and it is suggested that adenine is the purine factor limiting leaf growth in normal intact plants.

(4) Adenine in the presence of potassium nitrate largely replaces the effect of crude pea diffusate in promoting leaf growth in excised pea embryos and in immature excised leaves. Adenine too exerts a marked promotive effect upon the vegetative growth of plants in sand culture. Since adenine is known to be without promotive effect upon root growth, and since the leaves of such adenine treated plants are much larger than the controls, the marked increased general vigor of such plants is thought to be a secondary phenomenon connected with increased leaf growth. Adenine should, therefore, be included in the list of phytohormones.

(5) Histological studies indicate that in Nicotiana tabacum leaves the nature of the response to pea diffusate is one of cell enlargement. This enlargement appears most marked in the epidermal and probably palisade cells, giving rise to pronounced intercellular air space formation.

Literature cited.

***

1. Addicott, F. and J. Bonner. Nicotinic acid and the growth of isolated pea roots. Science 88: 577-578. 1938.

2. Avery, G.S. Structure and development of the Tobacco leaf. Amer. Jour. Bot. 20: 565-592. 1933.

3. ------. Differential distribution of a phytohormone in the developing leaf of Nicotiana, and its relation to polarized growth. Bull. Torrey Bot. Club. 62: 313-330. 1935.

4. Barron, E.S.G. Cellular oxidation systems. Physiol. Rev. 19: 184-239. 1939.

5. Beaumont, A.B., C.J. Larsinos, P. Piekenbrock and P.R.Nelson. The assimilation of nitrogen by tobacco. J. Agr. Res.43: 559-567. 1931.

6. Björkstén, Johan. Zur Kenntnis der Synthese von Eiweisstoffen und ihrer Bausteine bei höheren Pflanzen. Biochem. Zeit. 225: 1-80. 1930.

7. Bonner, D.M. Activity of the potassium salt of indole-3-acetic acid in the Avena test. Bot.Gaz. 99: 408-411. 1937.

8. ------, and J. Bonner. On the influence of various growth factors on the growth of green plants. Amer. Jour. Bot. 27: 38-42.1940.

9. Bonner, J. Vitamin B_1, a growth factor for higher plants. Science 85: 183-184. 1937.

10. ------. Unpublished work.

11. ------, and F. Addicott. Cultivation in vitro of excised pea roots. Bot. Gaz. 99: 144-170. 1937.

12. ------ and G. Axtman. Growth of plant embryos in vitro. Preliminary experiments on the role of accessory substances. Proc. Nat. Acad. Sc. 23: 453-457. 1937.

13. ------ and P.S. Devirian. Growth factor requirements of four species of isolated roots. Amer. Jour. Bot. 26: 661-665. 1939.

14. ------ and D. Bonner. Ascorbic acid and the growth of plant embryos. Proc. Nat. Acad. Sc. 24: 70-75. 1938.

15. ------ and E.R. Buchman. Syntheses carried out in vivo by isolated pea roots I. Proc. Nat. Acad. Sc. 24: 431-438. 1938.

16. ------- and J. English. A chemical and physiological study of traumatin a plant wound hormone. Plant Physiol. 13: 331-348. 1938.

17. Bonner, J. and J. Greene. Vitamin B_1 and the growth of green plants. Bot. Gaz. 100: 226-237. 1938.

18. ------ and -------. Further experiments on the relation of vitamin B_1 to the growth of green plants. Bot. Gaz. 101: 491-500. 1939.

19. Borsook, H. and J. Dubnoff. Personal communication.

20. Bottomley, W. The significance of certain substances for plant growth. Ann. Bot. 28: 531-539. 1914.

21. ------. Some accessory factors in plant growth and nutrition. Proc. Roy. Soc. London B. 88: 237-247. 1914.

22. Boysen-Jensen, P. Über die Leitung des phototropischen Reizes in Avena-keimpflanzen. Ber. d. bot. Ges. 28: 118-120. 1910.

23. ------. La transmission de l'irritation phototropique dans l'Avena. Bull. Acad. Roy. Denmark. No. 1: 3-24. 1911.

24. ------. Über die Leitung des phototropischen Reizes in der Avena-Koleoptile. Ber. d. bot. Ges. 31: 559-566. 1913.

25. Brown, H. and G. Morris. Researches on the germination of some of the Gramineae Part I. Jour. Chem. Soc. London. 57: 458-520. 1890.

26. Brunner, G. Beiträge zur Entwicklungs Physiologie der Kiefern-keimlinge. Jahrb. wiss. Bot. 76: 407-440. 1932.

27. Burgeff, H. Pflanzliche Avitaminose und ihre Behebung durch Vitaminzufuhr. Ber. d. bot. Ges. 52: 384-390. 1934.

28. Cailleau, R. Le Cholestérole et L'Acide Ascorbique facteurs de croissance pour le Flagellé Tétromitidé Trichomonas foetus Riedmüller. Compt. rend. Soc. Biol. 127: 861-1421. 1938.

29. Clark, H.E. Effect of ammonium and nitrate nitrogen on the composition of the tomato plant. Plant Physiol. 11: 5-24.1936.

30. Darwin, C. The power of movement in plants. London. 1880.

31. Dietrich, K. Über die Kultur von Embryonen ausserhalb der Samens.Flora, 17: 379-414. 1924.

32. Duggar, B.M. Biological effects of Radiation. New York. 1936. Chapter XXI. The effects of light intensity upon seed plants.

33. English, J., J. Bonner and A.J. Haagen-Smit. The wound hormones of plants II. The isolation of a crystalline active substance. Proc. Nat. Acad. Sc. 25: 323-329. 1939.

34. -------. The wound hormones of plants. IV. Structure and synthesis of a traumatin. Jour. Amer. Chem. Soc. 61: 3434-3436. 1939.

35. v. Euler, H., H. Albers and F. Schlenk. Über der Co-Zymase. Z. physiol. Chem. 237: I. 1935.

36. Fosse, R. Uréogenèse et Métabolisme de l'azote purique chez les Végétaux. Compt. Rend. Acad. Sc. Paris. 208: 865-868. 1939.

37. Gregory, F.G. Studies on the energy relations of plants II: Effect of temperature on increase in area of leaf surface, and in dry weight of Cucumis sativus. Ann.Bot. 42: 469-507. 1928.

38. Gyorgy, P., D. Melville, D. Burk and V. du Vigneaud. The possible identity of vitamin H with biotin and Co-enzyme R. Science 91: 243-245. 1940.

39. Haas, E. Isolierung eines neuen gelben Ferments. Biochem. Zeit. 298: 378-390. 1938.

40. Haberlandt, G. Kulturversuche mit isolierten Pflanzenzellen. Sitzungsb. Akad. Wiss. Wien Math. Naturwiss Kl.III. 69-92. 1902

41. Hamner, K.C. Effects of nitrogen supply on rates of photosynthesis and respiration in plants. Bot. Gaz. 97: 744-764. 1936.

42. ----- and J. Bonner. Photoperiodism in relation to hormones as factors in floral initiation and development. Bot. Gaz. 100: 388-431. 1938.

43. Hannig, E. Über die Cultur von Cruciferen-Embryonen. Bot. Zeitung 62: 45-80. 1904.

44. Hayward, H.E. Personal communication.

45. Holley, K.T., T.A. Pickett and T.G. Dulin. A study of ammonia and nitrate nitrogen for cotton I. Influence on absorption of other elements. Ga. Sta. Bull. 169. 1931.

46. Horowitz, N.H. and E. Heegaard. Unpublished work.

47. Klein, G. Handbuch der Pflanzenanalyse Spezielle Analyse III. Page 19 and 405, Springer, Wien. 1933.

48. Knight, B.C.J.G. The nutrition of Staphylococcus aureus; nicotinic acid and vitamin B_1. Biochem. Jour. 31: 731-737. 1937.

49. Kögl, F. and A.J. Haagen-Smit. Über die Chemie des Wuchsstoffs. Proc. Kon. Akad. v. Wetensch., Amsterdam. 34: 1411-1416. 1931.

50. ------, A.J. Haagen-Smit and H. Erxleben. Über ein Phytohormon der Zellstreckung. Reindarstellung des Auxins aus menschlichen Harn. Z. physiol. Chem. 214: 241-261. 1933.

51. ------, H. Erxleben and A.J. Haagen-Smit. Über ein Phytohormon der Zellstreckung. Zur Chemie des Krystallisierten Auxins. Z. Physiol. Chem. 216: 31-44. 1933.

52. Kögl, F. and H. Erxleben. Über die Konstitution der Auxine a und b. Z. Physiol. Chem. 227: 57-73. 1934.

53. ------, A.J. Haagen-Smit and H. Erxleben. Über ein neues Auxin ("Heteroauxin") aus Harn. Z. Physiol. Chem. 228: 90-103. 1934.

54. ------, A.J. Haagen-Smit and H. Erxleben. Über den Einfluss der Auxine auf das Wurzelwachstum und die chemische Natur des Auxins der Gras-Koleoptilen. Z. Physiol. Chem. 228: 104-112. 1934.

55. ------ and A. J. Haagen-Smit. Biotin und Aneurin als Phytohormone. Z. Physiol. Chem. 243: 209-227. 1936.

56. ------ and N. Fries. Über den Einfluss von Biotin, Aneurin, und meso-inositol auf das Wachstum verschiedener Pilzarten. Z. Physiol. Chem. 249: 93-110. 1937.

57. ------ and Benno Tönnis. Über das Bios-Problem. Darstellung von Krystallisierten Biotin aus Eigelb. Z. Physiol. Chem. 242: 43-74. 1936.

58. Koser, S.A., A. Dorfman and F. Saunders. Nicotinic acid as an essential growth-substance for dysentery bacilli. Proc. Soc. Exptl. Biol. Med. 38: 311-313. 1938.

59. Kotte, W. Kulturversuche mit isolierten Wurzelspitzen. Beit. Allgem. Bot. 2: 413-434. 1922.

60. -----. Wurzelmeristem in Gewebekultur. Ber. d. Bot. Ges. 40: 269-272. 1922.

61. Linnossier, D. Les Vitamines et les Champ gnons. Compt. Rend. Soc. Biol. Paris. 82: 381. 1919.

62. Lohmann, K. and Ph. Schuster. Untersuchungen über die Cocarboxylase. Biochem. Zeit. 294: 188-214. 1937.

63 a. Lwoff, A. and M. Lwoff. Studies on codehydrogenases I. Nature of growth factor "V". Proc. Roy. Soc. London, B. 122: 352-359. 1937.

63 b. ----- and -----. Studies on Codehydrogenases II. Physiological function of growth factor "V". Proc. Roy. Soc. London, B. 122: 360-373. 1937.

64. ----- and -----. Rôle physiologique de l'hémine pour Haemophilus influenzae Pfeiffer. Ann. Inst. Pasteur. 59: 129-136. 1937.

65. ----- and I, Pirosky. Détermination du facteur de croissance pour Haemophilus ducreyi. Compt. Rend. Soc. Biol. 124:1169-1171. 1937.

66. Lwoff, M. Recherches sur la nutrition des trypanosomides. Ann. Inst. Past. 51: 55-116. 1933.

67. Lwoff, M. L'hématine et l'acide ascorbique, facteurs de croissance pour le flagellé Schizotrypanum cruzi. Compt. Rend Acad. Sc. Paris. 206: 540-542. 1938.

68. Möller, E.F. Vitamin B_6 (Adermin) als Wuchsstoff für Milchsäure bakterien. Z. Physiol. Chem. 254: 285-286. 1938.

69. Mueller, J.H. Studies on cultural requirements of bacteria X. Pimelic acid as a growth stimulant for C. diphtheriae. Jour. Biol. Chem. 119: 121-131. 1937.

70. -----. Nicotinic acid as a growth accessory substance for the diptheria bacillus. Jour. Bact. 34: 429-441. 1937.

71. Müller, W. and Schopfer, W.H. L'action de l'aneurine et de ses constituents sur Mucor ramannianus Möll. Compt. Rend. Acad. Sc. Paris. 205: 687-689. 1937.

72. Nightingale, G.I. The nitrogen nutrition of green plants. Bot. Rev. 3: 85-174. 1937.

73. Noller, C.R. Organic Synthesis. Vol. XV. John Wiley and Sons, New York. 1935.

74. Onslow, M.W. The principles of plant biochemistry. page 281. Cambridge. 1931.

75. Oppenheimer, C. and K.G. Stern. Biological oxidations. Nordemann. New York. 1939.

76. Orla-Jensen, S., N.C. Otte and A. Snog-Kjaer. Der Vitaminbedarf der Milchsäure bakterien. Zentr. Bakt. Parasitenk. Infekt. II. 94: 434-447. 1936.

77. Paál, A. Über phototropische Reizleitung. Ber. d. bot. Ges. 32: 499-502. 1914.

78. -----. Über phototropische Reizleitung. Jahrb. wiss Bot. 58: 406-458. 1919.

79. Platenius, H. Carbohydrate and nitrogen metabolism in the celery plant as related to premature seedling. Cornell Univ. Agr. Expt. Sta. Mem. 140. 1931.

80. Quastel, J.H. Effect of CO on biological reduction of nitrate. Nature 130: 207. 1932.

81. Ray, S. On the nature of the precursor of the vitamin C in the vegetable kingdom. I. Vitamin C in the growing Pea seedling. Biochem. Jour. 28: 996-1003. 1934.

82. Robbins, W.J. Cultivation of excised root tips and stem tips under sterile conditions. Bot. Gaz. 73: 376-390. 1922.

83. Robbins, W.J. Effect of autolyzed yeast and peptone on growth of excised corn root tips in the dark. Bot. Gaz. 74: 59-79. 1922.

84. ------. Thiamin and growth of species of Phytophthora. Bull. Torrey Botan. Club. 65: 267-276. 1938.

85. ------ and W. E. Maneval. Further experiments on growth of excised root tips under sterile conditions. Bot. Gaz. 76: 274-287. 1923.

86. ------ and Mary Bartley. Vitamin B_1 and the growth of excised tomato roots. Science 85: 246-247. 1937.

87. ------ and M. Schmidt. Vitamin B_6, a growth substance for excised roots. Proc. Nat. Acad. Sc. 25: 1-3. 1939.

88. ------ and M. Schmidt. Further experiments on excised tomato roots. Amer. Jour. Bot. 26: 149-159. 1939.

89. ------ and F. Kavanagh. Vitamin B_1 or its intermediates and growth of certain fungi. Amer. Jour. Bot. 25: 229-236. 1938.

90. Sachs, Julius. Stoff und Form der Pflanzenorgane I & II. Arb. bot. Inst. Würzburg 2. 452-488. 1880. 689-718. 1882.

91. Scharrer, K. and W. Schropp. Die Wirkung von Follikelhormon-Krystallisaten auf das Wachstum einiger Kulturpflanzen. Biochem. Zeit. 281: 314-328. 1935.

92. Schopfer, W.H. Versuche über die Wirkung von reine-kristallisierten vitaminen B auf Phycomyces. Ber. d. botan. Ges. 52: 308-312. 1934.

93. ------. L'action des constituants de l'aneurine sur les levures (Rhodotorula rubra et flava). Compt. Rend. Acad. Sc. Paris. 205: 445-447. 1937.

94. ------. L'aneurine et ses constituants, facteurs de croissance de Mucorinees, (Parasitella, Absidia) et de quelques espèces de Rhodotorula. Compt. Rend. Soc. Biol. 126: 842-844. 1937.

95. ------ and A. Jung. L'action des produits de désintegration de l'aneurine sur Phycomyces. Le second facteur de croissance des Mucorinees. Compt. Rend Acad. Sc. Paris. 204: 1500-1501. 1937.

96. Snell, E.E. and W.H. Peterson. Growth factors for bacteria X. Additional factors required by certain lactic acid bacteria. Jour. Bact. 39: 273-285. 1940.

97. Stauffert, Inge. Vergleichend anatomisch-physiologische Untersuchungen an Chelidonium majus, und seiner laciniaten Mutationsform. Verh. Nat. Med. Ver. Heidelberg. 18: 138-182. 1938.

98. Thimann, K.V. and F.W. Went. On the chemical nature of the root-forming hormone. Proc. Kon. Akad. v. Wetensch. Amsterdam. 37: 456-459. 1934.

99. Tukey, H. Artificial culture methods for isolated embryos of deciduous fruits. Amer. Soc. Hort. Sc. 32: 313-322. 1934.

100. Verner, L. Effect of nitrate fertilization on apple trees. Proc. Amer. Soc. Hort. Sc. 30: 32-36. 1933.

101. Vickery, B.H., G. Pucher, A. Wakeman and C. Leavenworth. Chemical Investigations of the Rhubarb plant. Conn. Agr. Expt. Sta. Bull. 424. 1939.

102. Vyvyan, M.C. Studies of the rate of growth of leaves by photographic method. Ann. Bot. 38: 60-103. 1924.

103. Warburg, O. Chemische Konstitution von Fermenten. Ergebn. Enzymf. Vol. 7: 210. 1938.

104. ----- and W. Christian, and H. Griese. Wasserstoff übertragendes Co-Ferment, seine Zusammensetzung und Wirkungs-weise. Biochem. Ztschr. 282: 157-205. 1935.

105. ----- and W. Christian. Gärungs-Co-Ferment. Biochem. Ztschr. 285: 156-158. 1936.

106. ----- and -----. Pyridin, der Wasserstoff übertragende Bestandteil von Gärungsfermenten. Biochem. Ztschr. 287: 291-328.1936.

107. ----- and -----. Bemerkung über gelbe Ferments. Biochem. Ztschr. 298: 368-377. 1938.

108. Went, F.W. On growth-accelerating substances in the coleoptile of Avena sativa. Proc. Kon. Akad. v. Wetensch. Amsterdam. 30: 10-19. 1926.

109. -----. Wuchsstoff und Wachstum. Rec. Trav. bot. Neérl. 25: 1-116. 1928.

110. -----. Specific factors other than auxin affecting growth and root formation. Plant Physiology 13: 55-80. 1938.

111. -----. Transplantation experiments with peas. Amer. Jour. Bot. 25: 44-55. 1938.

112. -----. The dual effect of auxin on root formation. Amer.Jour. Bot. 26: 24-29. 1939.

113. -----, and K. V. Thimann. Phytohormones. Macmillan. 1937.

114. White, P.R. Potentially unlimited growth of excised tomato root tips in a liquid medium. Plant Physiol. 9: 585-600. 1934.

115. Wildiers, E. Nouvelle substance indespensable au développement de la Levure. La Cellule 18: 313-331. 1901.

116. Williams, R.J., C. Lyman, G. Goodyear, J. Truesdail and D. Holaday. "Pantothenic Acid" a growth determinant of universal biological occurrence. Jour. Amer. Chem. Soc. 55: 2912-2927. 1933.

117. ------ and R.T. Major. The structure of Pantothenic Acid. Science 91: 246. 1940.

118. Wolley, D.W. Biological responses to the constituent parts of Pantothenic Acid. Jour. Biol. Chem. Soc. 130: 417-419. 1939.

119. ------, H. Waesman and C.A. Elvehyem. Nature and partial synthesis of the chick antidermatitis factor. Jour. Amer. Chem. Soc. 61: 977. 1939.