

EXPERIMENTS ON THE NUTRITION OF ISOLATED ROOTS

Thesis

by

Fredrick Taylor Addicott

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## Summary of Thesis

### EXPERIMENTS ON THE NUTRITION OF ISOLATED ROOTS

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1. The history of plant tissue culture is outlined.
2. The technique of root cultures is reinvestigated from several aspects and some changes are incorporated for the present work.
3. The action of vitamin B<sub>1</sub> as a root growth hormone is examined, and its presence is found to be closely correlated with the growth rate, rate of cell divisions, and extent of meristem in isolated roots.
4. A second growth factor of pea roots present in yeast extract is found not to be among the microelements of plant nutrition nor among the amino acids.
5. Nicotinic acid is found to be the second growth factor of pea roots. With vitamin B<sub>1</sub> it is capable of supporting a higher rate of growth for pea roots than can yeast extract.
6. Evidence is presented to show that inositol, phosphate, and optimum hydrogen ion concentration may have promotive effects on the growth of pea roots.
7. A number of experiments are described which indicate that it may be impossible to establish a clone of pea roots.
8. Preliminary experiments on the culture of excised anthers are described, and the high activity of embryo extracts on anther growth is noted.

"Far too long, roots have lain hidden  
away in the ground." -- Vernon Quinn

## FOREWORD

This thesis is, in the main, concerned with the nutritional factors which are necessary for the unlimited growth of pea roots and the action of these factors in the root. A few experiments dealing with attempts to culture anthers will be mentioned. While studying cytology the writer became interested in tissue cultures because they afford such an excellent means by which to study the physiological factors in cell division. Before coming to this Institute the writer made some preliminary investigations on the growth of excised anthers in vitro, hoping that with these cultures it might be possible to study the physiology of the process by which nuclei previously dividing regularly by mitosis change and undergo meiosis. The results did not seem sufficiently promising to warrant extending the work as a thesis problem. It seemed more expedient to center the thesis work about a study of the physiology of root growth. In view of the numerous mitotic divisions in an actively growing meristem, a contribution to our knowledge of the growth factors of roots is likely to be of considerable value to the understanding of the physiology of nuclear divisions.



## ACKNOWLEDGMENTS

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## Chapter I

### INTRODUCTION

The possibilities of the culture of excised tissues and organs has long held the interest of biologists. Early attempts were made to culture isolated plant tissues before the turn of the century. Experiments with animal tissues followed soon after these, and favorable results were obtained with them almost from the very start. Techniques were soon developed by which a variety of animal tissues could be grown under sterile conditions for unlimited lengths of time. It was more than twenty-five years later, and only after numerous unsuccessful attempts, that it was possible to culture excised plant tissues indefinitely.

In recent years there have been several reviews of the work with plant tissue and organ cultures. (White, 54, 57, Harrison, 25, Fiedler, 17.) Of these Fiedler's is especially complete both as to mention of the early workers and to a detailed comparison of the methods and results of the later workers. It therefore seems unnecessary here to do more than outline the development of the study of plant tissue cultures, mentioning the more outstanding contributions, together with a few papers which indicate the breadth of the field.

Haberlandt (22) was the first to recognize the physiological possibilities of tissue cultures, although he was not the first

to work with excised plant tissues. Rechinger, and others, had earlier made observations on excised plant materials, but their experiments were not designed with any serious view towards maintaining the growth of their tissues. Haberlandt, however, was definitely interested in the nutritional relations of the tissues with which he worked and realized that the problem was one of great physiological significance. He and the other workers in his laboratory (Bobilioff-Preisser, Lamprecht, Theilmann) made numerous observations on various excised plant tissues, and while they were able to maintain cells alive over a period of months, they never observed divisions of isolated cells. White (54) believed that the greatest barrier to their success was the fact that they used differentiated tissues. Their materials (glandular hairs, epidermal cells, guard cells, palisade parenchyma, etc.) were all far past the embryonic stage. White considers that it was the fact that the cell walls of these materials were well developed which restricted their further growth. Undoubtedly this was a factor, but the primary difficulty was probably even deeper, in the protoplasm, and lay in the fact that the methods used failed to induce the nuclei to divide. It is probably not a coincidence that the only successful cultures of excised plant materials to date have been those of the actively dividing embryonic tissues. With our present knowledge it does not seem justified to preclude that it is impossible to induce growth and cell division in even the specialized tissues of Haberlandt's early experiments. A more complete understanding of plant nutrition and of growth hormones

may yet make it possible to induce dedifferentiation, followed by growth and cell division of mature plant cells. Studies of auxin induced swellings of which the histological works of Kraus, Hammer, Scott, and others are examples (12, 23, 30, 50) have already shown that the application of auxin to rather mature plant tissues may result in considerable cell division.

There were also many other workers between 1900 and 1933 in addition to those of Haberlandt's school. (See especially Fiedler's review.) Their results were likewise negative, and to all of them the criticism can be applied as before, that they attempted to culture mature tissues in a rather simple medium. An extreme example is one worker who tried to grow perianth tissues in solutions of only a single substance such as glycerol or sugar. (Kunkel, 31.) Since 1933 the work of Gautheret, Nobécourt, Bonner, and White in the field of culture of single tissues has met with considerable success. Gautheret (20), using the excised cambial tissue of woody plants, has been able to obtain some proliferation of cells, but his tissues grew very slowly. Nobécourt (39) has had similar results with excised carrot tissue. Bonner (5), using the parenchyma of bean pods, has been able to keep this tissue multiplying rapidly during several weekly transfers and with a considerable increase in both size and numbers of cells over the original explant. Bonner's work is of interest because he used in his culture medium preparations (as yet impure) of traumatin, the wound hormone. This was a clear advance beyond the previous workers, because by the use of this hormone he was able to induce growth and cell division in cells

which under ordinary circumstances would never divide again. The most recent work of White (63) indicates that there has now been obtained a close approach to a plant tissue culture in the strict sense of the term. On a medium in which the special growth factors are supplied as yeast extract he has maintained callus of Nicotiana growing through 40 weekly transfers. This, however, is not a culture of only a single tissue, as the callus parenchyma occasionally differentiates scattered xylem elements.

Even before Haberlandt's work Brown and Morris (13) had shown in 1890 that it was possible to culture excised embryos and that with due precautions mature plants could be reared from them. This work was continued and carried on by many workers. (Hannig, 24, Laibach, 32, La Rue, 33, Tukey, 52.) The culture of embryos became a very useful technique to biochemists (Ray, 41, Bonner and Bonner, 10.) studying the metabolism of plants as well as to breeders in circumventing the abortion of embryos of certain plants (cherry, peach, rose).

Brown and Morris considered the relation of the endosperm and embryo to be that of host and parasite, which is no doubt a quite legitimate interpretation. Since the culture of embryos presents no great difficulties, it became apparent that the nutritional relations of embryos could be readily studied. This has been done (Bonner and Axtman, 9, Kögl and Haagen-Smit, 28.) and will be discussed in more detail later (pps. 34, 35.). Suffice it here to mention that the endosperm (cotyledons in certain cases),

in addition to carbohydrates and amino acids in large amounts, furnishes the embryos with several substances of a hormonal nature. (See especially Went, 53.) The ease with which embryos could be cultured would suggest that their two principal parts, the shoot and the root, would be the next logical plant organs to attempt to culture. The most promising work in the field of plant tissue cultures has come as a result of the culturing of these meristematic regions. The work with buds has to date been only very limited and therefore will be mentioned first. White (55) in 1933 reported experiments in which he kept isolated buds of Stellaria media alive and growing for periods up to three weeks in sterile medium. The buds showed considerable growth and differentiation even though they did not develop internodes or anything resembling mature leaves or flowers. Bonner (unpublished) has obtained fairly well formed leaves from bud cultures, but these also did not develop internodes. It should be possible to learn much of the physiology of the bud if this work can be extended.

The earliest attempts to culture excised roots aseptically were likewise very promising, and they resulted in a continued interest in isolated roots on the part of many investigators, leading to a considerable advance in our knowledge of the physiology of the root. Robbins (42, 43) in 1922 published the results of experiments of several years previous, showing that the excised roots of various seedlings (corn, pea, cotton) could be kept alive and growing for some time in appropriate nutrients under sterile conditions. The medium contained inorganic salts with a sugar as

a source of carbohydrate, and in addition it was found that growth was considerably better if certain extracts were added to the medium. Robbins obtained his best results with a yeast autolysate. Kotte (29), also working on this problem, had considerable success with meat extract, "Nährstoff Heyden", and an amino acid mixture. These workers were followed by a group of investigators who studied one or another phase of root culture in more or less detail. Gautheret (20) made extremely careful and extensive observations on the cytological and morphological changes of isolated roots. Robbins, Bartley, and White (45) studied the growth of fragments of excised roots. Galligar (18, 19) studied the effect of initial root length, temperature, and various organic substances on the growth of excised roots. Loo and Loo (35, 36) found a beneficial effect of leaf extracts on the growth of root tips. The contributions of these investigators, as well as those of several others (see the reviews cited above), while of interest to workers studying roots, did not advance our knowledge of the specific factors involved in root growth greatly beyond the point to which it had been brought by Robbins.

It was White (56) who first obtained unlimited growth of excised tomato roots. He has maintained clones of these roots actively proliferating in culture since 1933. The medium which he used was a liquid one of salts, sugar, and a yeast extract. Since this paper appeared, investigators working with excised roots have concerned themselves primarily with determination and further study of the components of yeast extract which are active in the growth of roots.



## Chapter II

## MATERIALS AND METHODS

This thesis is essentially a continuation of an earlier work (Bonner and Addicott, 8.) in which the methods of root culture are carefully described. There are also available detailed descriptions of the technique of animal tissue culture, the principles of which apply equally well to plant work. (Cameron, 16, Buchsbaum and Loosli, 14, Parker, 40.) It therefore does not seem necessary to present an extended discussion of general culture methods here. However, it might be well to outline the technique used in this work, treating in detail only certain aspects which were especially studied during the course of these investigations.

Garden peas (Pisum sativum) of the variety Perfection from the Ferry-Morse Seed Company were used in almost every experiment. In a few cases the Alaska variety was used; since its root growth reactions were not detectably different from the Perfection, no special note has been made of the few isolated experiments in which it was used.

In order that conclusions resulting from experiments with the nutrition of excised roots are to have validity, it is essential that the roots be grown under sterile conditions. If contaminating organisms are present, the possibility that they will produce substances either toxic or promotive to growth is too great to allow the results of the culture to be given any consideration.

The precautions required for the sterile culture of roots are essentially the same as those that are regularly followed during pure culture work in bacteriology laboratories. However, it is advantageous to have a sterile chamber in which transfers can be made. It has been found most convenient to work in a small room which can be kept nearly free of air currents. An excellent room for such work was included in the second wing of the Kerckhoff Laboratories. Its special features included: steam jets in the ceiling, a drain in the floor, rounded corners of the ceiling and walls for easy washing, and a built-in concrete and tile table on three sides of the room. The steam is used to precipitate dust particles and spores which may be in the air. It was found that ordinary washable paint on the walls was unsatisfactory because of the ease with which molds grow on it under the conditions of high humidity in the room after steaming. This difficulty was remedied by the incorporation into the paint of a fungicide, such as Dowicide 6, which is a chlorinated phenol. Repeated washings with Lysol were, however, equally effective. It was found earlier that if a Lysol solution were repeatedly applied to painted or wooden surfaces and allowed to dry upon them that there remained a layer (probably a polymerized derivative of cresol, the active ingredient of Lysol) which was completely resistant to fungi.

It was found to be extremely difficult to sterilize the pea seeds completely without considerable reduction of the percentage of germination. Several methods of sterilization were tested during

the course of the investigations. Bromine water (La Rue, 34.) was found to be an effective agent and very simple to apply, but bromine proved to be so irritating to nasal membranes that it was very disagreeable to use in a small closed room. Toluene was found to be useless since short exposures to it did not sterilize the seeds and longer exposures killed them. Lysol was also found to be impractical over the range of dilutions in which it could be used. A thirty minute exposure of the seeds to a 10% Lysol solution retarded germination but failed to kill the bacterial spores. Mercuric chloride proved to be the most practical agent for the sterilization of the seeds. The most effective procedure was found to be as follows:

- 1) Wash 2 or 3 times with water to remove loose dirt.
- 2) 1-2 minutes in 95% alcohol.
- 3) 20-25 minutes in 0.1%  $\text{HgCl}_2$ .
- 4) Set out in sterile distilled water.

Even after this treatment occasionally a few bacteria appear during the first transfer, but the number of contaminants is only a very small percentage (usually less than 5%) of the sterile cultures. Longer treatment with either the alcohol or  $\text{HgCl}_2$  reduced the percentage of germination but had little effect on the number of contaminants. Some workers (Kögl and Haagen-Smit, 28) have followed the procedure of washing the pea seeds ten times with tap water before treating with alcohol, and when the seeds were removed from the  $\text{HgCl}_2$  they were washed five times with sterile distilled water. This was found to be of no

additional value to the method outlined above. It was thought also that the removal of broken or discolored seeds might eliminate a possible source of bacteria. While this undoubtedly removed a number of contaminated seeds, it did not greatly lower the percentage of infections. The schedule for sterilization given above stands as the most convenient and generally satisfactory of any that were tested.

The sterilized seeds are placed in 10 cm. Petri dishes and allowed to germinate in sterile distilled water. An effort is made to add only slightly more water than the seeds will use in swelling, otherwise germination is inhibited. After 2 days at 25° C. the roots project about 10 mm. and the shoot has not yet appeared. At this time the apical 5 mm. of the root is removed and placed in appropriate medium. Further transfers, if required for the experiment, are made at the end of each succeeding week by removing the apical 10 mm. and placing it in fresh medium.

The instruments necessary for this work are a scalpel and forceps. The long Bard-Parker handle number 7 with a number 10 detachable blade and ordinary dental forceps were found to be excellent because with them the worker can readily reach to all parts of a Petri dish without danger of infection. The instruments were always kept in 95% alcohol when not in use and were passed through the flame of an alcohol lamp immediately before use. As added precautions a sterile cloth 50 X 100 cm. was laid on the table, and the hands and forearms were kept moist with water during

the operations. If these steps were taken, contaminations were negligible.

Petri dishes were selected for the culture vessels for several reasons: They are very much more convenient to inoculate and to subculture from than are flasks. Petri dishes allow the medium to be spread out in a relatively thin layer which facilitates aeration. In flasks, where there is less surface per volume of liquid and in which the exchange of gases is more restricted, pea roots grew much more poorly than they do in Petri dishes. The shape of Petri dishes makes them easy to handle, stack, and wash. It has been found quite satisfactory to wash the dishes in a sulfuric acid-potassium dichromate cleaning solution followed by rinsing in tap and distilled water.

Experiments have shown that neither tap nor distilled water will support the growth of roots either alone or in combination with the standard salts; redistilled water must be used. For the preparation of this an all Pyrex still was used and was found to produce water of a very satisfactory purity for the root cultures. The composition of the medium, as regards major salts and sugar, was worked out (Bonner and Addicott, 8.) before the major part of these investigations began. It is shown in table 1. Care must be taken in dissolving the salts to see that the concentration never gets too high, since a precipitate, probably an iron phosphate, forms very readily. White (58) uses the precaution of making his medium in

TABLE I

## BASIC MEDIUM FOR EXCISED PEA ROOTS

Constituent	Concentration
$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	236 mg. per liter
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	36
$\text{KNO}_3$	81
KCl	65
$\text{KH}_2\text{PO}_4$	20
Fe-tartrate	1.5
Sucrose	4%

Note: More recent experiments (table 29) indicate that concentrations of  $\text{KH}_2\text{PO}_4$  as high as 1000 mg. per liter may give a considerable increase of growth over the concentration of 20 mg. per liter given here.

the form of three stock solutions and mixing these as required. It was found with the medium for pea roots that if the ferric tartrate were first carefully dissolved, the other salts could then be added in any way that might be desired without the formation of a precipitate. The medium was sterilized in cotton stoppered Erlenmeyer flasks by autoclaving at 15 pounds pressure for 20 to 30 minutes. Immediately before use it was poured into Petri dishes which had been dry sterilized in an oven for 2 hours at 180° C.

It would seem of interest to indicate the method by which the proper concentration of the various constituents was determined, since this is the experimental method which forms the pattern for the determination of the optimum medium. For example, the optimal concentrations of each of several salts used in combination can only be determined by a series of successive approximations. Thus the concentration of  $\text{KNO}_3$  can be improved only to the point where some other salt becomes the limiting factor in the growth of the roots. After the concentration of this latter salt is improved, further work with the  $\text{KNO}_3$  becomes possible. It is by such a series of experiments that the optimum concentration of salts can be determined. As growth becomes increasingly greater through the addition of active growth factors to the medium, it may well become necessary to reinvestigate the salt concentrations. The cultures were allowed to grow in a

darkened cabinet of a room kept at 25° C. The roots were found to grow very much more uniformly under these conditions than they did in some of the earlier experiments in which it was necessary to keep them at room temperature.

For comparing the results of a group of similar experiments, the lengths to which the roots had grown in one week was found to be the most convenient measure. In most cases this was found to be very closely correlated with the dry weight of the root. (See table 15.) In the comparison of experiments of widely differing nature it was necessary to take into account the thickness of the root, the color, and the extent to which branch primordia may have grown out. In some cases it was necessary to make sections and study the internal anatomy of the roots before an understanding of the effects of certain substances could be reached.

As a standard test number, 20 roots were grown in each series, 4 per dish with 20 cc. of medium. In special series more roots were used. The statistical treatment of the data of the investigation has been of a simple nature. This has not been because the data could not be subjected to elaborate statistical analysis, but because the purpose of the experiments was to detect and study root growth factors. The significance of an experiment rested not on whether its results were statistically valid, but on whether the substance or condition tested in the experiment



would support growth. Therefore it was felt that the limited time available for this work would be better spent carrying on extensive cultures, testing possible growth factors, than in subjecting a few experiments to a detailed analysis. (The time necessary to make a careful statistical study of the results of one experiment is, if anything, greater than the amount of time it would take to carry the experiment on for another week. Furthermore, as is shown in table 14, the effect of a new growth substance is of such magnitude as to require no statistical aid in its detection.)

## Chapter III

VITAMIN B<sub>1</sub> AS A HORMONE OF ROOT GROWTH

Vitamin B<sub>1</sub> is essential to the growth of a wide variety of plants as well as animals. It is necessary for the propionic acid bacteria and for Staphylococcus, as has been shown respectively by Tatum, Woods, and Peterson (51), and Knight (26). Schopfer (49) and Burgeff (15) in 1934 found that it was required by certain molds. In the last year Köggl and Fries (27) have extended the list of fungi and the number of special substances required by individual species. Not all molds require an external source of vitamin B<sub>1</sub>. Robbins and Kavanagh (46) have confirmed the findings of Schopfer and Burgeff and have extended their studies to the intermediates of the vitamin (pyrimidine and thiazole). They have shown that some molds need only be given the pyrimidine part of the vitamin molecule and are apparently capable of synthesizing the thiazole part themselves.

Köggl and Haagen-Smit (28) in 1936 were the first to show that vitamin B<sub>1</sub> was beneficial to the growth of higher plants in their work on pea embryos, where they found the effects were most marked on the root system. In 1937 Bonner (6), working with pea roots, and Robbins and Bartley (44), simultaneously and independently working with tomato roots, have shown that it was an essential component of the yeast extract in which these roots had previously been grown. (See page 6.) White (60) has also worked with the vitamin B<sub>1</sub> nutrition of excised roots.

It is common knowledge that entire plants can be grown in inorganic nutrients without the addition of any organic compounds. Since excised roots cannot be grown without vitamin B<sub>1</sub>, it is obvious that this compound is a plant hormone as it must be supplied to the root from some other part of the plant. Bonner and Greene (11) have shown that vitamin B<sub>1</sub> is produced by leaves in the light and is transported to the roots where it affects growth in length.

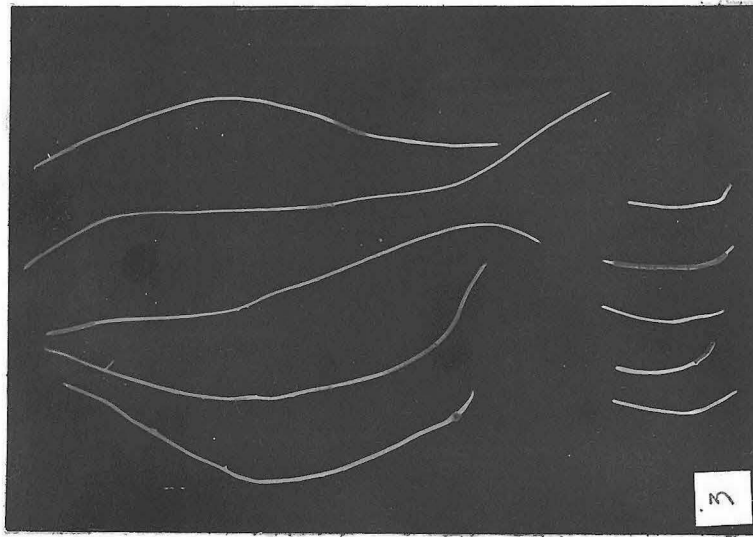
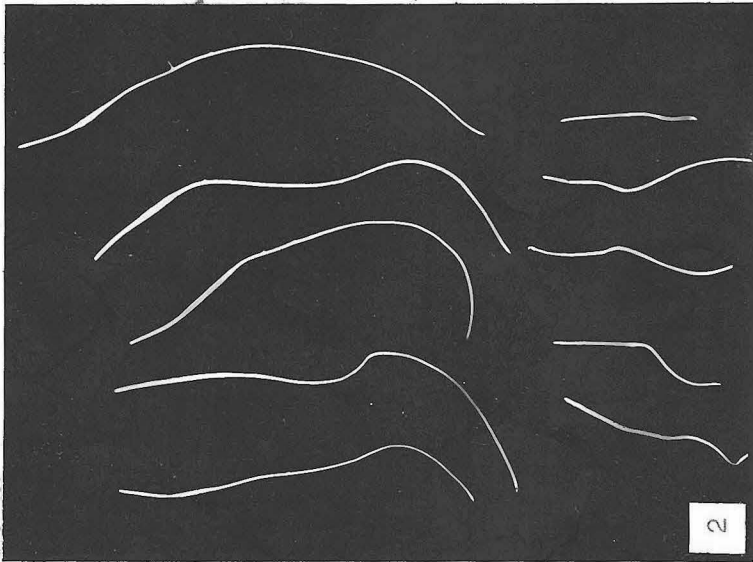
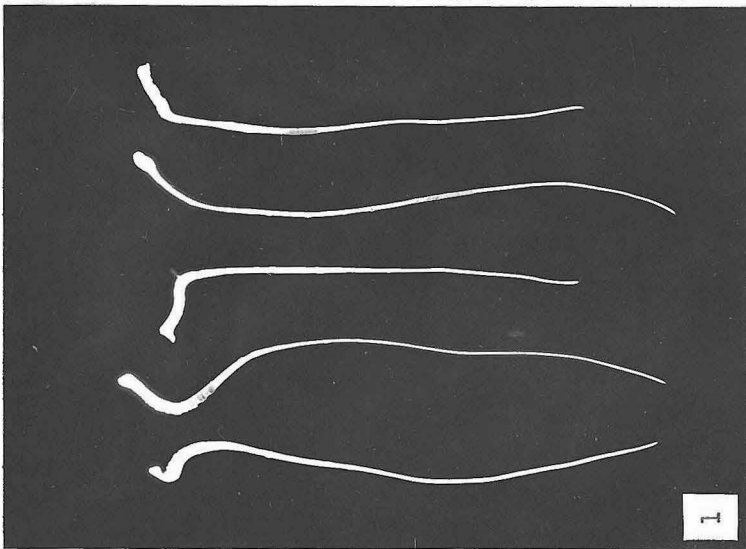
Vitamin B<sub>1</sub> affects not only the growth in length of excised roots, but the general morphology as well. The macroscopic differences between roots grown in the presence of excess vitamin B<sub>1</sub> and those which are deficient in the vitamin is so great as to suggest the possibility of considerable differences in the microscopic structures as well. This chapter describes an investigation of the action of vitamin B<sub>1</sub> on the meristem of isolated pea roots, a report of which is now in press. (Addicott, 1.)

The concentration of vitamin B<sub>1</sub> which was used was 0.1 mg. per liter. This is a more or less arbitrary selection since both earlier work (Bonner and Addicott, 8.) and a series run during these investigations (table 22.) indicate that vitamin B<sub>1</sub> is effective in root growth over a wide range of concentrations. If this is omitted from the medium for three weekly transfers, growth of the root ceases. (Table 2.) If vitamin B<sub>1</sub> is present in the medium, the growth rate remains practically constant during this same period. Consequently, for this work roots were studied at the end of each of the first three weeks in culture, since the effects

of deficiency of the vitamin were clearly marked in that time. At the end of each week the growth of the roots was measured and the tips fixed for cytological study. It should be added that vitamin B<sub>1</sub> was never used in the medium for the first week the roots were in culture. Bonner (7) has shown that the freshly excised pea root tip contains sufficient vitamin B<sub>1</sub> (and presumably other growth factors) to sustain growth of 45 to 60 mm. during the first week. The vitamin must be added if growth is to be maintained in later subcultures.

For cytological study root tips were fixed in Navashin's solution. In order to eliminate any variation which might be due to diurnal fluctuation of the rate of mitosis, roots were always fixed at noon. Dehydration was accomplished by means of the tertiary butyl alcohol method, and material was embedded in a rubber-paraffin mixture. Iron hematoxylin was used as the nuclear stain and fast green as the counter stain.

The growth habit of excised roots is indicated by figures 1-3 which are shadow photographs of roots at the end of the first, second, and third transfers. Roots after one week in culture show a characteristically swollen base, but at the end of succeeding transfers the root diameter is more uniform, although there is a gradual thickening towards the base. Figures 1-3 show also that the addition of vitamin B<sub>1</sub> to the culture medium results in sustained growth in length of the root. Roots supplied with the



Figures 1 - 3. Shadow photographs of roots at the end of each of the first three weeks in culture: 1. Roots at the end of the 1st weekly transfer, not supplied with vitamin B<sub>1</sub>. 2. Roots at the end of the 2nd transfer, upper row supplied with vitamin B<sub>1</sub>, lower row not supplied with vitamin B<sub>1</sub>. 3. Roots at the end of the 3rd transfer, upper row supplied with vitamin B<sub>1</sub>, lower row not supplied with vitamin B<sub>1</sub>.

vitamin are smooth and white with a slightly yellowish meristematic region when observed at the end of a transfer period. The roots not supplied with vitamin B<sub>1</sub> are short, brownish, and irregularly swollen.

The most striking microscopic differences between roots grown with and without the addition of vitamin B<sub>1</sub> are those to be observed in the meristematic region. Roots supplied with vitamin B<sub>1</sub> are essentially normal and possess an active and extensive meristem at the end of the third week. In roots which have not been supplied vitamin B<sub>1</sub> the meristem shows a very marked reduction in both extent and activity after three weeks, and a large proportion of the cells which were originally densely filled with cytoplasm are then highly vacuolated. These differences can be readily seen in the photomicrographs of this region which illustrate the effect of vitamin B<sub>1</sub> on the meristem of the roots. (Figures 4-6.)

Although vitamin B<sub>1</sub> exerts a marked effect on meristematic activity, roots deficient in the vitamin are nevertheless unaffected in certain other respects. Cell elongation and maturation of the cells arising from the meristem goes to completion. Since very few new cells are formed after the roots become deficient in vitamin B<sub>1</sub>, nearly all of the cells of these tips are mature. The walls of mature cells are well developed in roots cultured for three weeks without the addition of vitamin B<sub>1</sub>. Annular and spiral thickenings of the xylem vessels are laid down and have frequently

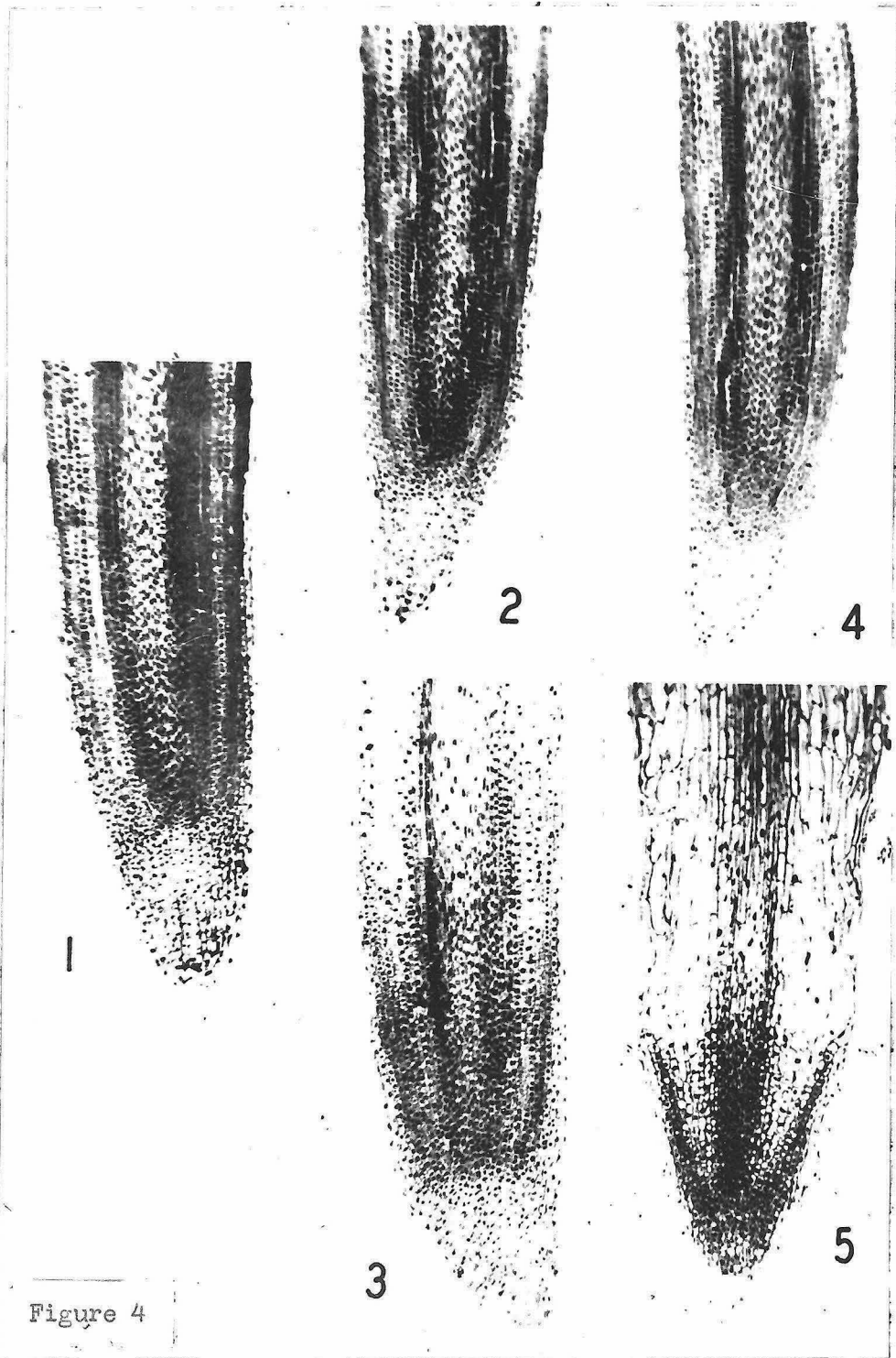
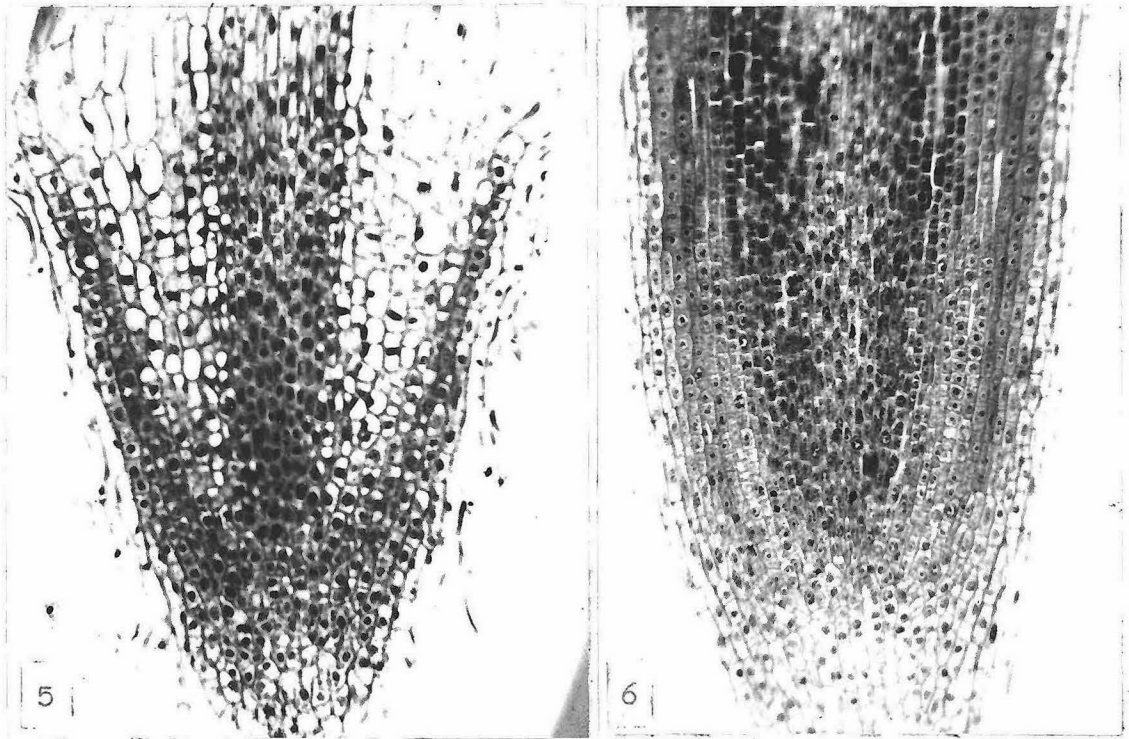


Figure 4

Figure 4. Photomicrographs of root tips at the end of the first three weeks in culture: 1. Root at the end of the 1st transfer, not supplied with vitamin B<sub>1</sub>. 2. Root at the end of the 2nd transfer, supplied with vitamin B<sub>1</sub>. 3. Root at the end of the 2nd transfer, not supplied with vitamin B<sub>1</sub>. 4. Root at the end of the 3rd transfer, supplied with vitamin B<sub>1</sub>. 5. Root at the end of the 3rd transfer, not supplied with vitamin B<sub>1</sub>. (Magnification 70X)



Figures 5 - 6. Photomicrographs of root tips at the end of the 3rd transfer: 5. Root not supplied with vitamin B<sub>1</sub>. 6. Root supplied with vitamin B<sub>1</sub> during the 2nd and 3rd transfers. (Magnification 160X)



been observed within a distance of a few cells from the short meristematic region. It is interesting to note that Kotte (29) in 1922 showed a figure of a similar condition in his pea root cultures. The unusual size of the vacuoles should also be mentioned since their development has attracted much attention, (e. g. Guilliermond, 21.). After roots have grown for three weeks without the addition of vitamin B<sub>1</sub> there are large vacuoles in the cells of the meristem in the region from which the cortical elements are derived. In these same meristems the cells from which the stelar elements are derived appear more like those in the roots supplied with vitamin B<sub>1</sub>. The vacuoles remain small in the cells of the meristem when vitamin B<sub>1</sub> is added to the culture medium. That the cells of roots deficient in vitamin B<sub>1</sub> are metabolically active is shown by the fact that they store starch abundantly. The deposition is greatest in the more proximal region of the roots, but starch grains, like the vacuoles, are conspicuously present even in the cells adjacent to the meristem. These points can be seen to a limited extent in the photomicrographs. (Figures 4-6.)

In order to obtain a quantitative relation between growth rate and meristematic activity of roots in culture, counts were made of mitotic figures to be found in the meristems of median longitudinal sections of roots subjected to different treatments. This count is a function of the mitotic frequency. The length of the apical meristem was also measured. The results, together with the growth rates of the corresponding roots, are summarized

in table 2. They are also presented graphically in figure 7 where, for ease of comparison, the growth rate, number of cell divisions, and length of the meristematic region of the roots at the end of the first transfer is taken as 100% in each case.

Thus it is seen that the presence of vitamin B<sub>1</sub> is not only correlated with the growth of the roots but with the number of cell divisions in the root tip and with the extent of the meristematic region as well. The effect of vitamin B<sub>1</sub> on growth seems to be quite independent of any effect that auxin might have, since cell elongation, differentiation, and maturation proceeded normally, as far as could be observed, in roots to which the vitamin was not supplied. The effect of vitamin B<sub>1</sub> as a hormone of root growth is correlated with the maintenance of normal meristematic activity.

TABLE 2

COMPARISON OF GROWTH RATE, CELL DIVISIONS, AND THE EXTENT OF THE  
MERISTEMATIC REGION IN EXCISED PEA ROOTS

	First Week	Second Week		Third Week	
	Minus vitamin B <sub>1</sub>	Minus B <sub>1</sub>	Plus B <sub>1</sub>	Minus B <sub>1</sub>	Plus B <sub>1</sub>
Growth Rate (mm. per wk.)	59.2	12.0	45.6	5.6	43.5
Number of Cell Divisions *	18.0	2.5	15.7	2.4	14.0
Extent of the Meristem (mm.) **	1.55	0.27	1.33	0.30	1.52

\* Because of the difficulty of identifying with certainty the early prophase and late telophase stages, only metaphase and anaphase stages were counted. The figures are a measure of the number of cell divisions per median longitudinal section.

\*\* These figures indicate the linear extent of apical meristematic activity in the tip of the root. The distance measured was from the tip of the meristem to the last observable figure which was definitely a part of the apical meristem.

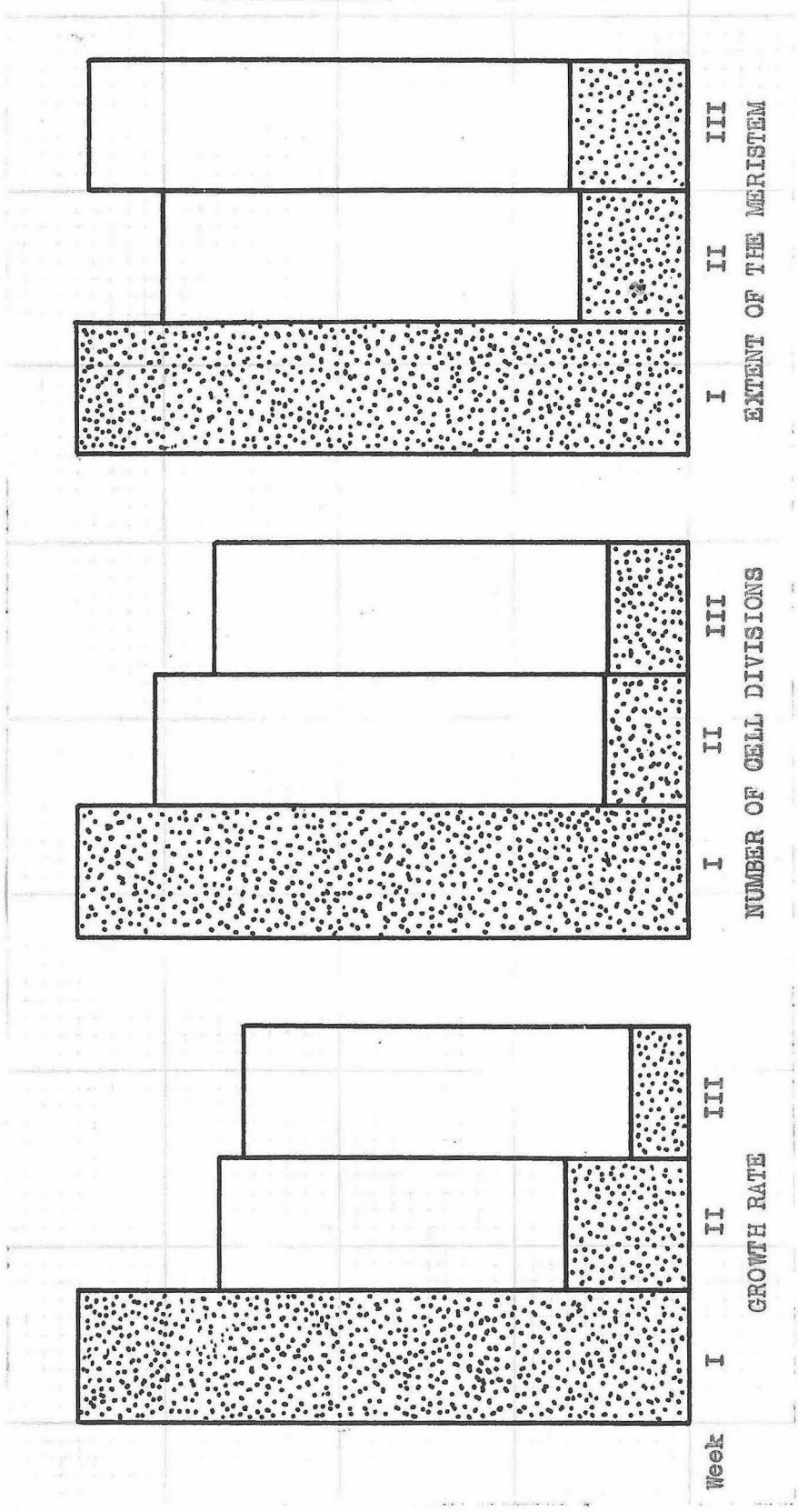


Figure 7. Diagram comparing the growth rate, number of cell divisions, and extent of the meristem of the root tips studied, from the data of table 2. In each of the three cases the value for the first week was taken as 100%. The stippled areas correspond to the roots not supplied with vitamin B<sub>1</sub> and are superimposed upon the clear areas representing roots to which vitamin B<sub>1</sub> was supplied. No roots were supplied with vitamin B<sub>1</sub> during the first week.

## Chapter IV

## ATTEMPTS TO FIND THE OTHER GROWTH FACTORS OF PEA ROOTS

As has been explained in the previous chapter, vitamin B<sub>1</sub> is capable of replacing yeast extract as a source of accessory growth substances during the first few weeks the root is in culture. If pea roots are grown for longer periods of time, it soon becomes evident that another factor present in the yeast extract is limiting to growth. After about the fourth week in culture, roots in medium containing only vitamin B<sub>1</sub>, in addition to the usual salts and sugar, show a decreasing growth rate which by the tenth week is close to zero. (Figure 8.) If yeast extract is present, growth remains constant at the initial rate of about 60 mm. per week. (Bonner and Addicott, 8.)

Other investigators had suggested (in the case of tomato roots) that the second growth factor might be among the microelements (Robbins, White, McClary and Bartley, 48, Robbins and Schmidt, 47, White, 61.), or among the amino acids of yeast extract (White, 59.). The preliminary experiment of Bonner and Addicott, who obtained a high rate of growth over six weekly transfers with an arbitrary mixture of amino acids added to nutrient containing vitamin B<sub>1</sub>, indicated that it might be profitable to investigate the amino acids very carefully for the effects on the growth of pea roots. The microelements were also investigated in order to obtain some definite evidence as to whether or not they might be the limiting factor in the growth of pea roots.

It would seem logical to look in the cotyledons for root growth factors since during germination these organs supply all the nutritional requirements of the root as well as the shoot. It is well known that a seed can germinate in distilled water and grow for some time simply on the materials that are stored in its cotyledons or endosperm. Consequently, several peas were ashed by incineration in a platinum crucible, and various concentrations of the ash were added to medium containing vitamin B<sub>1</sub>. These and the subsequent experiments discussed in this chapter were carried out on roots during their second transfer and always in the presence of vitamin B<sub>1</sub>. It would have been highly desirable to conduct these experiments with roots in the fourth or fifth transfer where the lack of the unknown factor is very apparent, but the time required to care for the roots over such a long period before making tests and the amount of glassware necessary prohibited such a procedure. It was necessary, therefore, to rely on the possibly small indications which would appear in the second week. As will be seen later, when the true second factor was tested under these conditions its effects were quite clear.

The results of the experiments with pea ash are shown in table 3. Under the test conditions pea ash was completely inactive as a growth factor. In high concentrations it was toxic, and as dilution progressed the growth approached that of the controls which received the standard salts. This is the type of reaction which is to be expected from an inactive substance and was met many times in the course of the investigations.

TABLE 3

EFFECT OF PEA ASH ON THE GROWTH  
OF EXCISED PEA ROOTS

Concentration of Ash mg. per liter of medium	Growth Expressed as percentage of roots receiving only standard salts
25	71
5	77
1	82*
0.1	87
0.01	99*
0.001	105*
0.0001	100

\*Average of 2 series.

As a supplement to pea ash two groups of accessory salts were employed. One was patterned after the supplementary salt solution used by White (61) and was modified only in order to make use of the elements in his list in the form in which they were available in this laboratory. (Accessory Salt Solution A, table 4.) The other was composed after a review of data on plant nutrition and salt accumulation (Miller, 37.) and included additional elements which were known to occur in plants, particularly the Leguminosae. (Accessory Salt Solution B, table 4.) The presence of an element in a plant is evidence that possibly, but not at all necessarily, the element may have some physiological function. These two solutions were incorporated in the medium in various combinations and concentrations, but no mixture or dilution was found in which the roots would grow to a greater extent than they would in vitamin B<sub>1</sub> alone with standard salts and sugar. (Table 5.) White found essentially the same situation in his experiments with tomato roots in which a mixture of accessory salts never gave better growth than did the controls.

In the hope that the negative results of the experiments with accessory salt solutions might be due to the presence of one or more salts in toxic concentration some experiments were made with single salts, also in the second week of culture. (Table 6.) These were likewise negative, additional zinc, manganese, copper, and tungsten being without significant effect. This list might have been extended and would have been had it not appeared in



TABLE 4

## ACCESSORY SALT SOLUTIONS

	Salt	Concentration mg. per liter
Solution A	ZnSO <sub>4</sub>	30.0
(after White, slightly modified)	Na <sub>2</sub> SiO <sub>3</sub>	27.0
	Al <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub>	107.0
	KI	15.0
	H <sub>3</sub> BO <sub>3</sub>	32.0
	NaCl	56.0
	MnSO <sub>4</sub>	44.0
	NiCl	4.0
	LiCl	3.9
	CoCl <sub>2</sub>	4.0
	Cu(NO <sub>3</sub> ) <sub>2</sub>	1.0
Solution B	TiO <sub>2</sub>	100.0
	SrCl <sub>2</sub> · 6 H <sub>2</sub> O	100.0
	Ba(CO <sub>3</sub> ) <sub>2</sub>	100.0
	Na <sub>3</sub> AsO <sub>4</sub> · 12 H <sub>2</sub> O	100.0
	CrO <sub>3</sub>	100.0
	Na WO <sub>4</sub> · 2 H <sub>2</sub> O	100.0
	CdCl <sub>2</sub> · 2 H <sub>2</sub> O	100.0

TABLE 5

EFFECT OF ACCESSORY SALT SOLUTIONS ON THE GROWTH  
OF EXCISED PEA ROOTS

Conc. Sol'n. A cc. per liter	Conc. Sol'n. B cc. per liter	Growth Expressed as percentage of roots receiving only standard salts
250	-	4
100	-	91
50	-	97
10	-	85
5	-	73
1	-	92
0.1	-	99
0.01	-	87
10	1	79
5	1	55
5	0.1	81
-	0.01	85

TABLE 6

EFFECT OF MICROELEMENTS, GIVEN AS SINGLE SALTS,  
ON THE GROWTH OF EXCISED PEA ROOTS

Salt	Concentration mg. per liter.	Growth Expressed as percentage of controls not given the salts.
$\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$	1	21
	0.1	102*
	0.01	100*
	0.001	106*
$\text{MnSO}_4$	10	35
	1	105*
	0.1	99*
	0.01	102*
$\text{Na}_2\text{WO}_4 \cdot 2 \text{H}_2\text{O}$	10	68
	1	94
	0.1	107
	0.01	102
$\text{ZnSO}_4$	10	78
	1	84
	0.1	85
	0.01	88

\* Average of two series.

other experiments that the limiting factor in the early weeks of culture is an organic substance and hence not to be found among the inorganic substances.

Therefore it seemed apparent that even with the use of the purest chemicals available and the observance of great care in washing of glassware and the preparation of double-distilled water, there is present in the medium a sufficient supply of microelements to meet the requirements of roots growing at the rate of 60 to 80 or more mm. per week. (Figure 12.) As this figure shows, the growth rate never greatly exceeded 90 mm. per week, and it might be that under such conditions of growth the limiting factor is a microelement. There has not yet been an opportunity to test this possibility.

The search for the additional growth factor was next carried to the amino acids. Twenty, of the highest purity available (table 7), were used as a basis for selection in a long series of experiments. As before, it was necessary to test all the amino acids in the second week of culture. In addition to the amino acids being tested, the medium always contained vitamin B<sub>1</sub>, standard salts, and sucrose. Twenty roots were used as the standard number to test the effect of a single concentration or combination.

The roots varied in their response to single amino acids; in several cases no growth effect was found, and in only a few was there an appreciable effect of certain concentrations of particular acids. Table 8 shows the summary of the growth of pea roots in the presence of single amino acids. Only one acid,

TABLE 7

## AMINO ACIDS USED IN PEA ROOT EXPERIMENTS

- 
- dl-Alanine, residues, student preparation in these laboratories.
- d-Arginine, prepared by S. Fox of these laboratories.
- Asparagine, recrystallized 3 times.
- Aspartic acid·HCl, prepared by S. Fox of these laboratories.
- Cystein·HCl, prepared by S. Fox of these laboratories.
- Cystine, student preparation in these laboratories.
- Glutamic acid·HCl, C. P., Pfanstiehl.
- Glycine, I. Cornet of these laboratories.
- l-Histidine·HCl, Hoffmann-La Roche.
- dl-Isoleucine, Eastman.
- Leucine, natural, isolated by S. Fox of these laboratories.
- Lysine, Hoffmann-La Roche.
- dl-Methionine, Hoffmann-La Roche.
- d-Ornithine dichlorhydrate, Hoffmann-La Roche.
- dl-Phenyl alanine, Eastman.
- l-Proline, recrystallized by S. Fox of these laboratories.
- dl-Serine, C. P., Amino Acid Manufactures, University of California  
at Los Angeles.
- Tryptophane, C. P., Pfanstiehl.
- l-Tyrosine, prepared by S. Fox of these laboratories.
- d-Valine, Hoffmann-La Roche.

TABLE 8

EFFECT OF VARIOUS CONCENTRATIONS OF SINGLE AMINO ACIDS  
ON THE GROWTH OF PEA ROOTS IN THE PRESENCE OF VITAMIN B<sub>1</sub>

Acid	Growth										
	Expressed as percentage of controls given only B <sub>1</sub> .										
	Concentration (mg. per liter)										
	50	15	5	1.5	0.5	0.15	0.05	0.015	0.005		
Alanine				93*	86*	87*	83*				
Arginine					101	95	113*				
Aspartic Acid	58	67*	101*	81#	82*						
Cystein			45	96	91	96					
Cystine			31	112	99	111					
Glutamic Acid				113	117	89	95				
Glycine		62	74	100	75						
Histidine				101	92	107	96				
Isoleucine						113	97	91	124		
Leucine			86	98*	116*	108*	103				
Lysine		58	97	93	104						
Methionine				104*	100*	101*	110*				
Ornithine			85	99	95	100					
Phenyl-alanine		75	64	95*	94*	66					
Proline				117	84	119*					
Serine				97*	108*	106#	118#	117			
Tryptophane					125*	144	116*	88*			
Tyrosine				95	94	69	100				
Valine				89	98	107	110*	85			
				Concentration (mg. per liter)							
	100	50	10	5	1	.1					
Asparagine	62*	115	82*	111	87*	74*					

\* Average of 2 series. # Average of 3 series.

tryptophane, increased the growth of the roots over that of the controls by more than 20%. Seven amino acids were found to be without significant effect: alanine, aspartic acid, cysteine, lysine, ornithine, phenylalanine, and tyrosine.

As soon as the results of the tests of single amino acids began to accumulate to the extent that the optimum concentrations could be ascertained, experiments on the effects of the acids in combination were started. The procedure here was to take a group of five or six amino acids and vary the concentration of one while that of the others was kept at the optimum. It was hoped that in this way some combination promotive to growth might be discovered and utilized before each of the acids could be tested alone. The results of these series were of little positive significance and consequently are not recorded here in entirety. However, they were of value in that they indicated that three of the acids, arginine, cystine, and proline, had no beneficial effects when used in the presence of other amino acids. The results which showed this are in table 9.

There were ten amino acids remaining which had shown some indication, either singly or in combinations with 4 or 5 other acids, of exerting a promotive effect on the growth of pea roots. The effect of each of these acids was then studied as its concentration was varied in the presence of the other 9, all kept at the optimum concentration. The results are summarized in table 10, which shows that three more acids, histidine, methionine,

TABLE 9

EFFECTS OF CERTAIN AMINO ACIDS IN COMBINATION WITH OTHER  
AMINO ACIDS ON THE GROWTH OF PEA ROOTS

Acid	Growth				Remarks
	Expressed as percentage of controls given only vitamin B <sub>1</sub>				
	Concentration*				
	10X	1X	0.1X	0	
Arginine	94	116	68	120	Tested in the presence of glutamic acid, proline, serine and valine.
Cystine	101	102	-	108	Tested in the presence of arginine, glutamic acid, proline, serine, and valine.
Proline	90	92	85	86	Tested in the presence of arginine, glutamic acid, serine and valine.

\* 1X equals the optimum concentration found in the previous tests. (Table 8) The other amino acids were at concentrations shown by this table to be optimum.



TABLE 10

EFFECT OF COMBINATIONS OF TEN SELECTED AMINO ACIDS  
ON THE GROWTH OF PEA ROOTS \*

Acid	Concentration			Value of
	10 X	1 X	0.1 X	1 X in mg. per liter
Asparagine	97	92	119	1.0
Glutamic Acid	117	113	90	0.5
Glycine	132	138	155	0.5
Histidine	94	94	99	0.15
Iso-leucine	92	112	92	0.015
Leucine	82	116	112	0.5
Methionine	68	104	79	0.05
Serine	70	82	83	0.05
Tryptophane	79	130	117	0.5
Valine	107	115	120	0.05

\* Growth is expressed as the percentage of the length of the control roots which were given all the amino acids (concentration 1 X) except the one being studied.

and serine, can be eliminated. There remain seven acids all of which show promotive effects on growth in the presence of the others. They are listed in table 11, together with the concentrations which should be optimum for the growth of pea roots. It is interesting to note that this is quite a different combination of amino acids than was found by White to be optimum for tomato roots. (Table 12.)

This combination of amino acids was developed by working with roots all of which were in their second week of culture. In this transfer the effect of the absence of the second growth factor from yeast extract is hardly noticeable. Consequently, it was essential to test the effects of the combination in later transfers in order to determine if it were really capable of supporting growth indefinitely. By the 6th and 7th weeks, roots grown with only vitamin B<sub>1</sub> as an accessory factor show a considerable decrease in growth rate, and it is at this time that the effect of the second factor should be most clear. Consequently, 250 roots were selected at the end of a first transfer, divided into two lots and placed in a medium containing vitamin B<sub>1</sub>, standard salts, and sucrose. The first lot was given the amino acid combination; the second lot did not receive it. The root tips were transferred weekly to fresh medium, and it was possible to keep the series in culture for ten weeks. By that time the losses which occur at each transfer had accumulated to the extent that it was impracticable to continue with the few

TABLE 11

AMINO ACIDS OPTIMUM FOR THE GROWTH  
OF PEA ROOTS

Acid	Concentration mg. per liter
Asparagine	0.1
Glycine	0.05
Glutamic	0.5
Isoleucine	0.015
Leucine	0.5
Tryptophane	0.5
Valine	0.05

TABLE 12

AMINO ACIDS ESSENTIAL FOR OR BENEFICIAL  
TO THE GROWTH OF ROOTS

Tomato Roots *	Pea Roots
-	Asparagine
Glutamic Acid	Glutamic Acid
-	Glycine
Histidine	-
Isoleucine	Isoleucine
Leucine	Leucine
Lysine	-
Phenyl-alanine	-
Proline	-
Serine	-
-	Tryptophane
Valine	Valine

\* White

remaining roots. The summary of the measurements of lengths appears in table 14, and graphically in figure 8. Shadow photographs of the roots at the end of the tenth week are shown in figure 9. Each root in this figure started the week as a tip 10 mm. long. Five mm. of the tip was removed for cytological study before the root was photographed. The lengths shown, therefore, are 5 mm. more than the roots actually grew. It is clear from the data that the amino acid combination does, at least in a certain stage (4th to 7th week), significantly increase the growth of the roots above that of the vitamin B<sub>1</sub> controls. However, it is equally clear that the amino acids only suffice to make the decline in growth rate less abrupt and that they are completely unable in this combination to supplement vitamin B<sub>1</sub> and support the continued growth of the roots at the initial rate. Thus it is apparent that the amino acid combination is not the second growth factor for pea roots.

## Chapter V

## NICOTINIC ACID AS THE SECOND GROWTH FACTOR OF PEA ROOTS

It was apparent from the work reported in the preceding chapter that the likelihood of the unknown growth factor being among the microelements or amino acids was very remote. Attention was turned, in consequence, to an extension of previous work with the growth effects of various physiologically active substances. This earlier work had been done with roots which were in their first week of culture so there was good reason to reinvestigate the effects of these substances in later transfers. The most important result was that nicotinic acid was found to have a promotive effect on root growth. The other substances tested will be discussed in a later chapter (VI).

It is not surprising that nicotinic acid together with vitamin B<sub>1</sub> should affect the growth of higher plants; they are known to be essential to the growth of several microorganisms. Knight (3', 4', 5') has found that these two substances are growth factors for Staphylococcus aureus. His work has been confirmed by Koser, Finkle, Dorfman, and Saunders (9') and Kögl and Wagtendonk (6'). Nicotinic acid is also necessary to the growth of Staphylococcus albus (Koser, Finkle, Dorfman, Gordon, and Saunders, 8'), of Bacillus proteus (Fildes, 2'), of the

dysentery bacillus (Koser, Dorfman, and Saunders, 7<sup>1</sup>) and of the diphtheria bacillus (Mueller, 10<sup>1</sup>). It is interesting to note that in all cases where it was investigated nicotinic acid amide was found to be as active as the acid itself.

The experiments which are reported below are the first in which the essential nature of nicotinic acid in root growth of higher plants is established. It has been noted (Bonner, 1<sup>1</sup>) that nicotinic acid exerts a promotive effect upon the shoot growth of isolated pea embryos. It is probable that this effect can be attributed to the action of the substance upon root growth.

A series of preliminary experiments with the effect of various concentrations of nicotinic acid on the growth of excised roots gave indications of a positive effect on root growth. Table 13 shows the effect of the acid in the second transfer, and in the presence of vitamin B<sub>1</sub>, salts, and sucrose. It is seen that there is a wide range of concentrations over which nicotinic acid shows activity. 0.5 mg. per liter was taken as a satisfactory concentration and used in all subsequent experiments.

To determine whether nicotinic acid was able to support growth at the initial rate in later transfers as well, it was incorporated in some of the cultures of the series which was testing the amino acid combination. This series had been started with a

TABLE 13

DETERMINATION OF THE OPTIMUM CONCENTRATION OF NICOTINIC ACID  
FOR THE GROWTH OF PEA ROOTS\*

Nicotinic Acid Concentration in mg. per liter.	Growth Expressed as percentage of control roots receiving only vitamin B <sub>1</sub> .
5.0	76
1.0	127
0.5	131
0.1	110
0.05	122

\*Test run on standard roots in the second transfer.



TABLE 14

GROWTH OF PEA ROOTS WHEN SUPPLIED VITAMIN B<sub>1</sub>  
OPTIMUM AMINO ACIDS AND NICOTINIC ACID\*

Accessory Factors	Growth				
	Average per week in mm. - standard error				
	Weeks				
	2 and 3	4 and 5	6 and 7	8 and 9	10
Vitamin B <sub>1</sub>	39.7±0.9	30.4±1.0	10.4±1.0	1.8±0.8	0.7±0.4
Vitamin B <sub>1</sub> plus Amino Acids	42.2±0.7	38.5±0.9	18.2±2.0	2.5±1.4	0.9±0.9
Vitamin B <sub>1</sub> plus Amino Acids plus Nicotinic Acid	-	55.8±3.5	57.4±4.8	64.5±1.8	60.4±6.3

\* Test started with 250 roots.

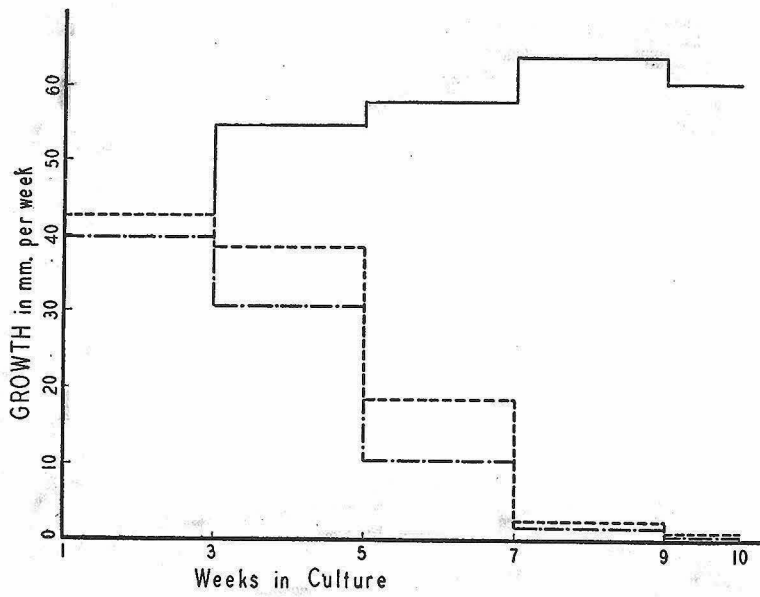
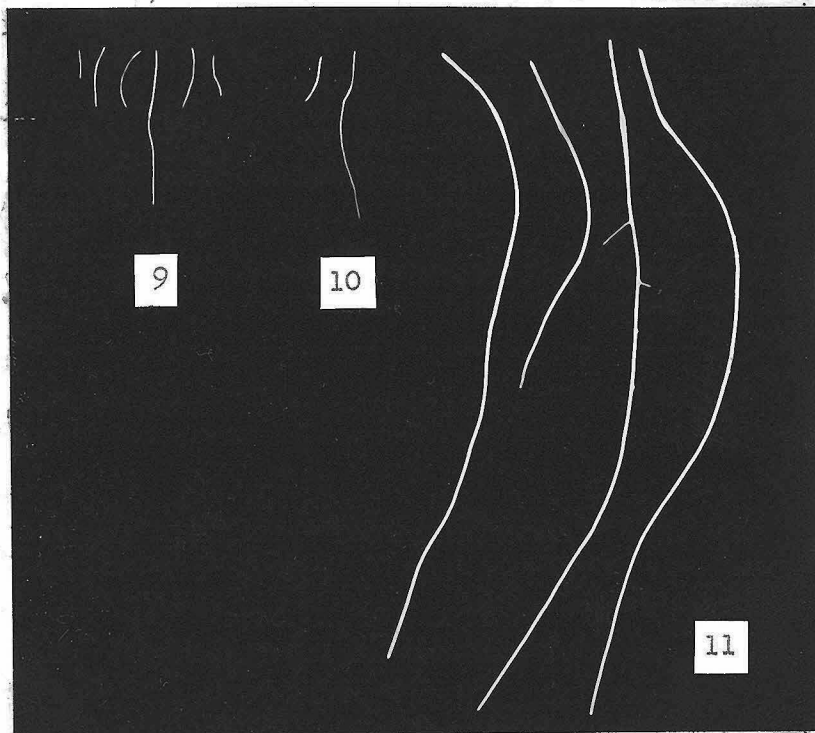


FIG. 8. Growth of isolated pea roots with and without nicotinic acid in addition to vitamin B<sub>1</sub> and the optimum mixture of amino acids: Solid line, nicotinic acid plus vitamin B<sub>1</sub> plus amino acids; dotted line, vitamin B<sub>1</sub> plus amino acids; dot and dash line, vitamin B<sub>1</sub> alone. Nicotinic acid was introduced into the medium of part of the series which had been cultured in vitamin B<sub>1</sub> and amino acids at the end of the third week. No accessory growth substances are added to the roots during the first week.



Figures 9 - 11. Shadow photographs of roots at the end of the 10th transfer: 9. Supplied vitamin B<sub>1</sub>. 10. Supplied vitamin B<sub>1</sub> and amino acids. 11. Supplied vitamin B<sub>1</sub>, amino acids and nicotinic acid. (5 mm. have been removed from the tip of each root for cytological study)

large number of roots so that it might be maintained in culture for two or three months. To a portion of the roots which had been receiving vitamin B<sub>1</sub> and amino acids, nicotinic acid was added at the end of the third week. The series was then continued to the tenth week with three types of medium, (a) vitamin B<sub>1</sub> alone, (b) vitamin B<sub>1</sub> plus amino acids, (c) vitamin B<sub>1</sub> plus amino acids plus nicotinic acid (each containing the usual salts and sucrose). The results are shown in table 14 and figure 8; shadow photographs of the roots at the end of the tenth week are shown in figures 9-11. It is clear that the addition of nicotinic acid enables the roots not only to grow at the initial rate but actually at a rate some 50% higher than during the first three weeks. This increase of growth rate during the first few weeks is characteristic of roots growing in medium containing both vitamin B<sub>1</sub> and nicotinic acid and may be due to the presence of some substance inhibitory to growth which is diluted each week until its effect is lost. In the tenth week roots receiving nicotinic acid showed no signs of suffering from nutritional deficiencies. As the shadow photographs indicate, the roots are uniformly thick and long. Roots suffering from nicotinic acid deficiency are thin but rather uniform in diameter. Nicotinic acid affects not only growth in length but also deposition of solid material. In table 15 is shown data of experiments in which roots were dried and weighed after each transfer. Roots clearly deficient in nicotinic acid (4th to 6th transfers) show a low growth rate and low dry weight. Roots supplied nicotinic acid show high

TABLE 15

COMPARISON OF MEASUREMENTS OF LENGTH AND DRY WEIGHT AS  
INDICATORS OF THE GROWTH OF PEA ROOTS IN VITRO

Week in culture		Medium		
		Vitamin B <sub>1</sub>	Vitamin B <sub>1</sub> plus nicotinic acid.	Vitamin B <sub>1</sub> plus nicotinic acid plus amino acids
3	Length	37.3	57.5	60.3
	Weight	1.04	1.91	2.19
4	Length	24.1	64.1	66.8
	Weight	0.73	1.89	2.22
5	Length	23.0	77.1	70.4
	Weight	0.49	2.17	2.02
6	Length	14.8	78.0	78.4
	Weight	0.39	2.02	2.18
12	Length	-	80.8	74.4
	Weight	-	2.69	2.48
20	Length	-	73.7	74.9
	Weight	-	2.58	2.75

Note: Each figure is the average of 30 to 90 roots.  
Length in mm. Weights in mg.

growth rate and high dry weight. These promotive effects of nicotinic acid continue through the 20th transfer. Table 15 also shows that in the presence of nicotinic acid the optimum amino acid combination had no pronounced effect on the growth of the roots in the third to sixth transfers either as to length or dry weight.

Up to this point no statistical analysis of the results of an experiment has been presented because it was felt that time necessary for such calculations would be better spent if devoted to growing more roots. However, it might be of interest to indicate the nature of data on which the averages presented in the various tables are based. The manner in which the data were collected and averages made is shown in table 16. To test the reliability of the means of such samples the standard error of the mean was calculated for each part of the experiment described just above. (Table 14.) The method of calculation is shown in table 17. While the standard errors are of value in the comparison of one or two of the averages in table 14, they are not needed to make obvious the effects of nicotinic acid in later transfers. For most of the work, particularly the preliminary experiments, the averages of the root lengths were considered to give a sufficient qualitative indication of the effectiveness of the substance being tested because the ability to facilitate growth in length is the most striking characteristic of the second factor for which search was being made. The final test of the second growth factor is in determining whether it, with

TABLE 16

SAMPLE PAGE OF DATA:

MEASUREMENTS OF ROOT LENGTHS FOR SIXTH WEEK  
OF AMINO ACID-NICOTINIC ACID SERIES

Vitamin B <sub>1</sub> *		Vitamin B <sub>1</sub> plus Amino Acids	Vitamin B <sub>1</sub> plus Amino Acid plus Nicotinic Acid
5	10	32 mm.	56mm.
8	3	23	68
21	5	32	70
11	29	15	88
31	15	49	76
7	28	17	72
40	10	34	53
11	11	15	57
25	16	18	24
19	21	5	16
14	9	22	15
10	8	38	31
7	13	42	56
5	8	30	26
43	25	5	48
12	13	5	
12	5	4	15-756
7	11	25	
22	17	10	Average: 50.4
8	13	10	
21	7	16	
<hr/>		14	
42	- 616	47	
		25	
Average: 14.7		25	
		26	
		26-584	
			Average: 22.5

\* All media received standard salts and sucrose.

TABLE 17

SAMPLE PAGE OF DATA: COMPUTATION OF STANDARD ERROR OF THE MEAN

FOR A VITAMIN B<sub>1</sub> PLUS AMINO ACID SERIES

IN THE SIXTH AND SEVENTH WEEKS

Length	f	d	d <sup>2</sup>	Formulae
0	3	19	361	$\sigma_M = \frac{\sigma}{\sqrt{N}}$
1	1	18	324	
2	2	17	289	
3	3	16	256	
4	1	15	225	$\sigma = \sqrt{\frac{\sum f d^2}{N-1} - c^2}$
5	4	14	196	
7	1	12	144	
8	3	11	121	
9	2	10	100	$\sigma_M = \sqrt{\frac{\sum f d^2}{N(N-1)} - \frac{c^2}{N}}$
10	3	9	81	
11	1	8	64	
14	2	5	25	
15	2	4	16	<p>M - mean.  N - number of measurements.  f - frequency of any measurement.  d - deviation of a measurement from the assumed mean.  c - mean minus assumed mean.  <math>\sigma</math> - standard deviation of the sample.  <math>\sigma_M</math> - standard error of the mean.</p>
16	1	3	9	
17	1	2	4	
18	1	1	1	
19	-	0	0	
21	1	2	4	
22	1	3	9	
23	1	4	16	
25	4	6	36	
26	1	7	49	
27	1	8	64	
29	1	10	100	<p>Calculations</p> <p>M = 18.2  N = 53.0  c = -0.8  fd<sup>2</sup> = 11,525</p>
30	1	11	121	
32	2	13	169	
34	1	15	225	
38	1	19	361	
42	1	23	529	
43	1	24	576	
44	1	25	625	
47	2	28	784	
49	1	30	900	
51	1	32	1024	
				$\sigma_M = \sqrt{\frac{11,525}{53 \times 52} - \frac{0.64}{53}}$ $= \sqrt{4.19 - 0.012}$ $\sigma_M = 2.04$



vitamin B<sub>1</sub> can support growth at the initial rate for an indefinite number of subcultures. White (62) believes that undiminished growth through ten weekly transfers is ample demonstration that a medium can support the growth of roots indefinitely. The writer is also of this opinion. Therefore, whenever a substance or combination of substances gave indication of being effective in promoting root growth, rather than subject the data to statistical analysis the experiments were simply conducted for a greater number of transfers. It is evident from table 14 and figure 8 that statistics could hardly point out more clearly the effect of nicotinic acid on the growth of pea roots.

To round out the evidence for nicotinic acid as a growth factor of pea roots it would be very desirable to culture the roots for a period of several months or years in the completely known medium of salts, sucrose, vitamin B<sub>1</sub>, and nicotinic acid. When roots branch freely in culture such a procedure is perfectly feasible, as White has shown with tomato roots (56) and Bonner (unpublished) with radish and flax. However, it is unfortunate for the work with pea roots that they do not branch readily in culture. While pea roots in culture grow at a somewhat higher rate than do tomato roots, they rarely form branches during the period of a weekly transfer, and any branches which do appear are not capable of giving rise to a clone in any of the media so far tested. (See chapter VII.) Consequently, when it is desired to carry a series of pea roots for several months in culture it is necessary to

start with a large number, since even with the greatest of care losses may occur at each transfer through injury or infection. Nearly 500 roots were selected for an experiment to determine whether vitamin B<sub>1</sub> and nicotinic acid, as the only accessory growth factors, could support the growth of roots for a period of many weeks. The previous experiment (table 14 and figure 8.) had shown that amino acids alone with vitamin B<sub>1</sub> would not support growth for ten weeks, but that if nicotinic acid were added, growth at the end of a ten week period was higher than at the start. The possibility still existed, however, that the amino acids were necessary in addition to nicotinic acid. The roots were therefore equally divided into cultures which contained amino acids and those which did not contain them. All media contained vitamin B<sub>1</sub>, nicotinic acid, standard salts, and sucrose. This series is still in culture, although the number of roots remaining is less than 100. The lengths to which the roots grew during each of the first 22 weeks are shown in table 18 and diagrammed in figure 12. Shadow photographs of the roots at the end of the 20th transfer appear in figures 14 and 15. It is seen that the total growth of the average root of this series was 1632.4 mm. in length and 49.7 mg. in dry weight. (Table 19.) The weekly growth rate in length for the entire period was 74.2 mm. Thus it is clear that nicotinic acid can supply the second growth factor for pea roots. When provided with vitamin B<sub>1</sub> and nicotinic acid pea roots will grow at a higher rate than in the optimum concentration of yeast extract.

TABLE 18

GROWTH OF PEA ROOTS DURING 20 WEEKLY TRANSFERS IN A MEDIUM  
OF NICOTINIC ACID AND VITAMIN B<sub>1</sub>

Week	Growth in mm. per week	
	Vitamin B <sub>1</sub> plus nicotinic acid	Vitamin B <sub>1</sub> plus nicotinic acid plus amino acids
2	-	49.9
3	-	44.9
4	-	72.3
5	-	79.2
6	71.5	75.4
7	85.1	76.8
8	87.5	91.8
9	89.8	87.9
10	73.2	81.4
11	73.8	66.9
12	80.8	74.4
13	81.0	78.3
14	73.0	71.5
15	65.3	67.5
16	48.2	63.3
17	52.8	74.6
18	75.1	84.9
19	83.4	81.9
20	73.7	74.9
21	73.6	69.0
22	86.7	79.1

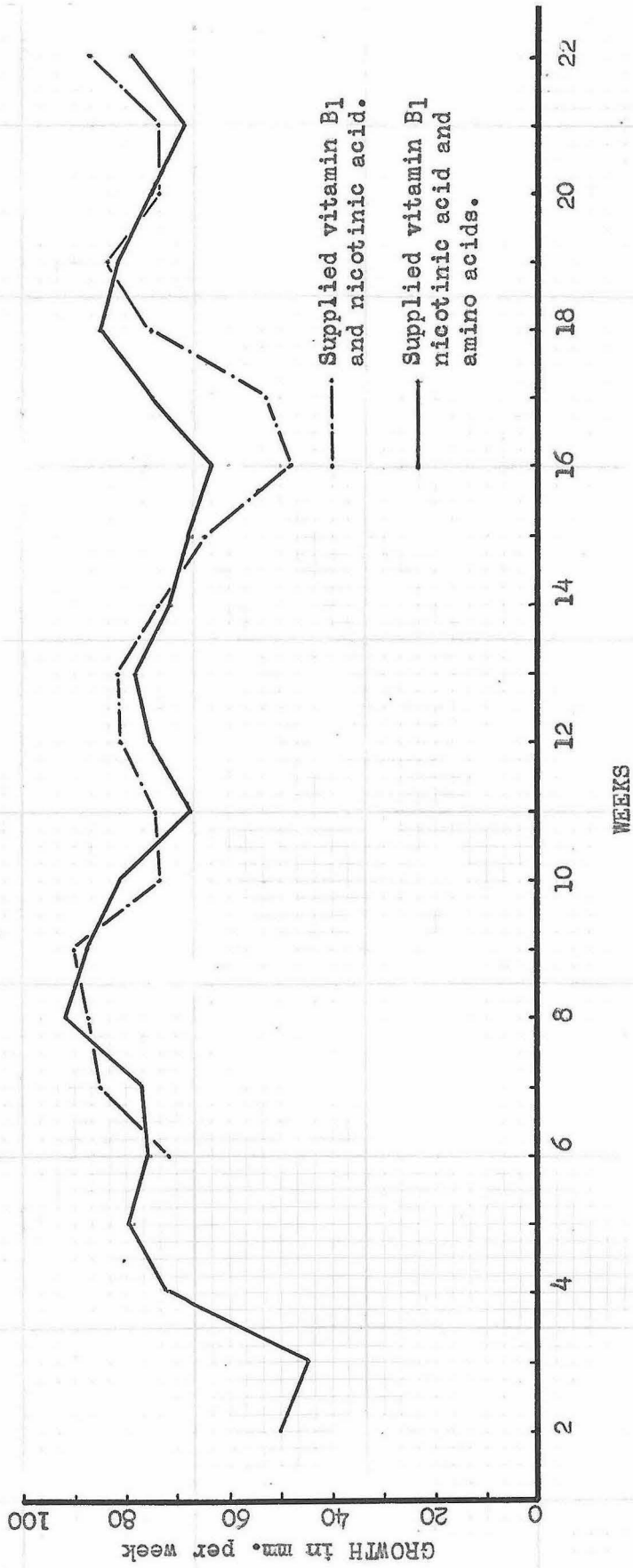
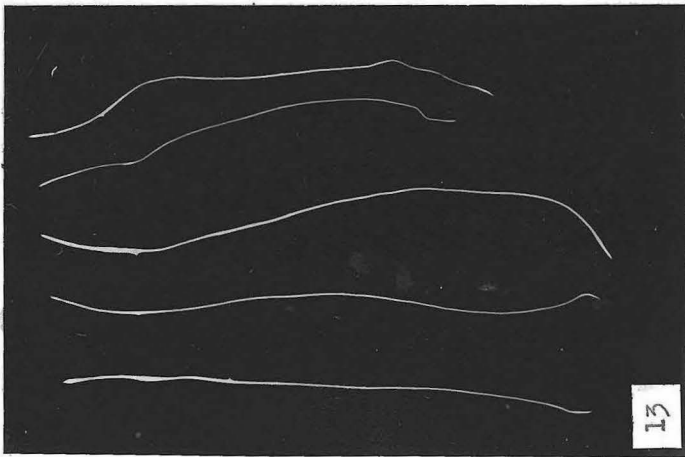
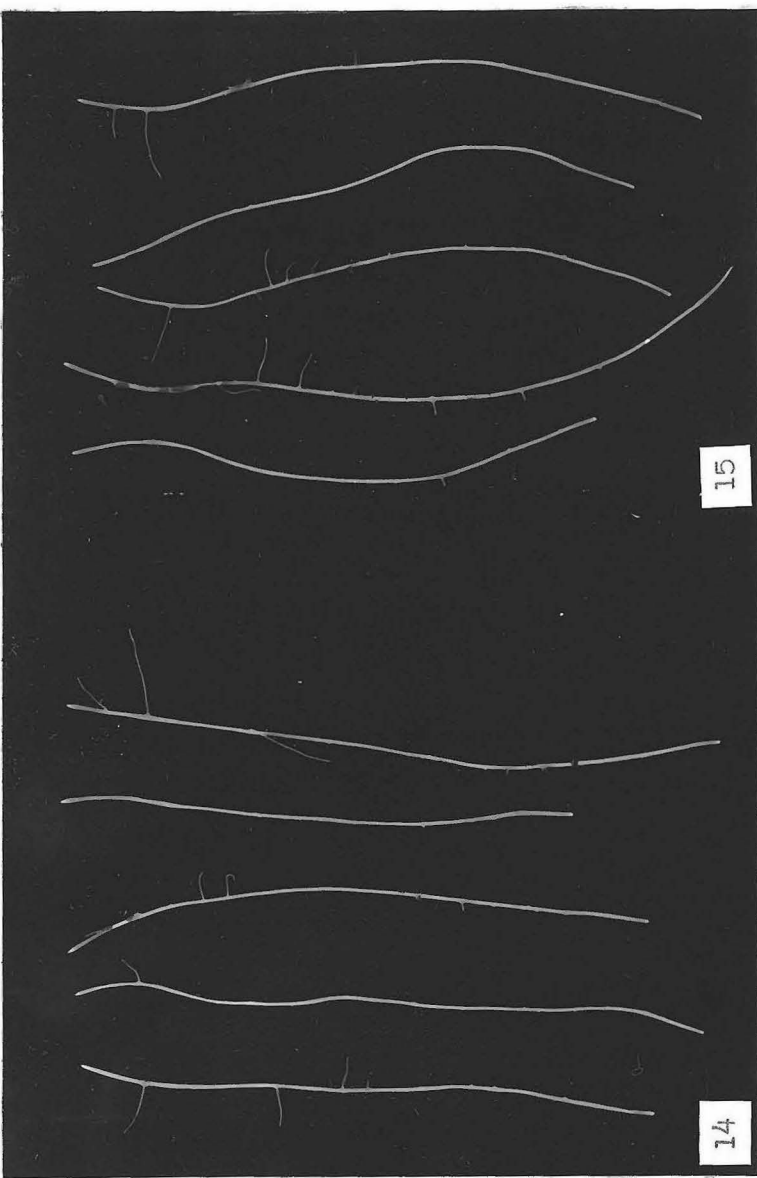


Figure 12. Effect of nicotinic acid and vitamin B<sub>1</sub> on the growth of pea roots with and without the addition of amino acids.



Figures 13 - 15. Shadow photographs of roots at the end of the 2nd and 20th transfers: 13. Roots at the end of the 2nd transfer, supplied with vitamin B<sub>1</sub>, intact. 14. Roots at the end of the 20th transfer, supplied with vitamin B<sub>1</sub> and nicotinic acid, apical 10 mm. removed for subculture. 15. Roots at the end of the 20th transfer, supplied with vitamin B<sub>1</sub>, nicotinic acid and amino acids, apical 10 mm. removed for subculture.

TABLE 19

COMPARISON OF TOTAL LENGTH GROWN AND TOTAL DRY WEIGHT  
FORMED BY EXCISED PEA ROOTS UNDER OPTIMUM CONDITIONS  
OVER A PERIOD OF 22 WEEKS\*

	Total	Average per week
Length (mm.)	1,632.4	74.2
Dry Weight (mg.)	49.68	2.26

\* Calculated on the basis of the data in tables 15 and 18.

## Chapter VI

## EFFECTS OF MISCELLANEOUS FACTORS ON THE GROWTH OF PEA ROOTS

Source of Carbohydrate: At the start of the experiments reported in this thesis it had been assumed that the type and concentration of sugar as previously developed (4% sucrose, Baker's C. P. ) would be satisfactory. However, these earlier experiments were all made with roots in the first transfer, and since the interest had shifted to the growth of the roots in later transfers, it seemed advisable to reinvestigate the carbohydrate requirements of the roots. The experiments corroborated the previous work in that sucrose was found to be superior to either dextrose or fructose or an equimolar combination of the two. Table 20 shows these results, as well as those from a concentration series with sucrose. Since there appears to be activity in a fairly wide range of concentrations for roots in the second transfer, from 2 to 6%, 4% was retained as an effective concentration. It also seemed desirable to examine the effects of purity of the sugar, since there is some reason to believe that even C. P. preparations may have considerable impurities. Through the kindness of J. L. Webb of these laboratories it was possible to obtain recrystallized dextrose and sucrose of his preparation. Table 21 shows the results of this experiment. It is seen that brown (impure) sugar is very toxic, and commercial white sugar is not completely free of growth inhibiting substances. However, recrystallized sucrose is not significantly better than C. P.

TABLE 20

EFFECT OF SUGARS ON THE GROWTH OF EXCISED PEA ROOTS  
IN THE SECOND TRANSFER

Vitamin B <sub>1</sub>	Sugar*	Growth Expressed as percent of the roots receiving vitamin B <sub>1</sub> and 4 % sucrose. Averages of two series.
-	-	18
-	4% Sucrose	101
Present	-	18 #
Present	4% Sucrose	100
Present	20% Sucrose	28
Present	10% Sucrose	93
Present	8% Sucrose	94
Present	6% Sucrose	105
Present	2% Sucrose	106
Present	1% Sucrose	69
-	4% Dextrose	38
Present	4% Dextrose	24#
Present	2% Dextrose	42
Present	1% Dextrose	57
Present	4% Fructose	12
Present	2% Fructose plus 2% Dextrose	17

\* Sucrose and dextrose were Baker's C. P. grade. Fructose was Pfanstiehl's C. P. Special.

# Average of four series.



TABLE 21

EFFECT OF SUGAR PURITY ON THE GROWTH OF EXCISED  
PEA ROOTS IN THE SECOND TRANSFER

Vitamin B <sub>1</sub>	Sugar	Growth Expressed as percent of roots receiving B <sub>1</sub> and 4% C. P. Sucrose	Source of Sugar
Present	4% C. P. Sucrose	100	Baker
Present	4% Brown Sugar	20	C & H, package
Present	4% Sucrose	83	Commercial, cubes
Present	4% Recrystallized Sucrose	102	Prepared by J. L. Webb of these laboratories
—	4% Recrystallized Sucrose	67	"
Present	4% Recrystallized Dextrose	57	"
—	4% Recrystallized Dextrose	128	"
Present	2% Recrystallized Dextrose	94	"

sucrose, so that the use of the latter grade seems quite justified. The toxic effects of dextrose seen in table 20 seem to be largely eliminated when the sugar is recrystallized. The unexpected result of greater growth with dextrose when vitamin B<sub>1</sub> is absent has no obvious explanation. It seems unwise to speculate on the reason for this effect before more experiments are performed.

Vitamin B<sub>1</sub> Concentration: It also seemed advisable to reinvestigate the vitamin B<sub>1</sub> concentration, since that used in the cultures had been determined by a single series of experiments sometime previously. Table 22 shows the results of this determination. It is seen that there is a very wide range of effective concentrations, from 10 mg. per liter to 0.0001 mg. per liter. This is similar to the result obtained in the previous experiment and indicates that the concentration of vitamin B<sub>1</sub> which has been in use, 0.1 mg. per liter, should be quite satisfactory.

Effect of Theelin on Root Growth: It has been found (Bonner and Axtman, 9, K $\ddot{u}$ gl and Haagen-Smit, 28.) that theelin has a beneficial effect on the growth of pea embryos. Experiments were therefore designed to test its possible effect on isolated roots. Four concentrations of theelin were used in the presence of vitamin B<sub>1</sub> and nicotinic acid. As table 23 shows, no significant effect was found, although the roots were maintained in these media for 4 weeks. It is possible that if nicotinic acid were present in the medium theelin would likewise have no effect on the growth of embryos.

TABLE 22

EFFECT OF VITAMIN B<sub>1</sub> ON THE GROWTH  
OF PEA ROOTS IN THE SECOND TRANSFER

Concentration of Vitamin B <sub>1</sub> mg. per liter.	Growth mm. per week
10	39.5
1	47.4
0.1	41.4
0.01	40.7
0.001	42.2
0.0001	44.6
0.00001	28.5
0.0	29.0

\* Average of 2 series.

TABLE 23

EFFECT OF THEELIN IN THE PRESENCE OF VITAMIN B<sub>1</sub> AND NICOTINIC ACID  
ON THE GROWTH OF EXCISED PEA ROOTS

Concentration in terms of saturation*	Growth			
	Expressed as percentage of controls receiving only vitamin B <sub>1</sub> and nicotinic acid as accessory growth substances.			
	Week			
	2	3	4	5
0.1	89	97	90	95
0.01	90	96	90	100
0.001	92	104	91	98
0.0001	89	80	87	95

\* For example 0.1 saturated indicates that 0.1 of the amount of theelin is present that is necessary to saturate the solution.

Effect of Inositol on Root Growth: Inositol, the yeast growth factor, bios I, had been tested in earlier experiments and found to be inactive on the growth of roots during the first transfer. The possibility that inositol, like vitamin B<sub>1</sub>, might not be limiting to growth in the first transfer, led to the investigation of this substance in later transfers. In the presence of vitamin B<sub>1</sub> alone inositol did have a slight effect during the second transfer. (Table 24.) The experiment was repeated in the presence of both vitamin B<sub>1</sub> and nicotinic acid, and it was found that the effect persisted in the presence of nicotinic acid. It has not yet been possible to follow the action of inositol in later transfers than the second.

Vitamin C and the Growth of Pea Roots: It may be of interest here to report the results of analyses of excised pea roots for vitamin C, which were made as a routine part of class work in the course of Plant Chemistry. The extraction method used was that of Muslin and King (38), and the extract was titrated with 2,6-dichlorophenol-indophenol (Bessey and King, 2.). The samples studied were: (a) initial tips just as they were removed from the young seedling prior to the first culture, (b) roots at the end of the 1st transfer after the tip had been removed for subculture, and (c) roots at the end of the 19th week after the tips had been removed for subculture. It was found (table 25) that there was from 0.1 to 0.2 mg. per gm. fresh weight of vitamin C in these samples. The interesting result was, however, that there was found to be approximately

TABLE 24

EFFECT OF INOSITOL ON THE GROWTH  
OF EXCISED PEA ROOTS

Concentration in mg. per liter	Growth	
	Expressed as percentage of controls not receiving inositol.	
	In the presence of vitamin B <sub>1</sub>	In the presence of vitamin B <sub>1</sub> and nicotinic acid.
60	91	-
30	89	-
10	105	98
1.0	113	112
0.1	-	114
0.01	-	118

TABLE 25

THE ASCORBIC ACID (VITAMIN C) CONTENT  
OF EXCISED PEA ROOTS

	Milligrams per gram of fresh weight	Milligrams per root
Initial Tips (as excised first from the seedling)	0.137	0.0012
First Week Bases (after 10 mm. of the tips had been removed for subculture)	0.108	-
Nineteenth Week Bases (after 10 mm. of the tips had been removed for subculture)	0.176	0.0033

three times as much vitamin C in a root after the 19th weekly transfer as there was in the initial tip. It is thus quite clear that the roots are capable of synthesizing vitamin C sufficient to meet the needs of unlimited growth at a rate of 74.2 mm. per week.

An experiment was also run to determine whether the roots would respond to additional vitamin C in the presence of vitamin B<sub>1</sub> and nicotinic acid. The results are shown in table 26. The roots which received high phosphate show no effect of additional vitamin C on growth. In the low phosphate series there is an apparent effect of vitamin C at the lowest concentration when compared with the controls receiving no vitamin C. However, the controls were not only relatively short, they were also all quite brown at the end of the 5th week, whereas all the other roots were white. This was taken to indicate that by some oversight toxic substances were present in the flask in which the medium for these roots was prepared, resulting in the poorer growth. If the usual growth rate of roots in similar transfers and under the same conditions is taken as the value for the controls (about 75 mm. per week), no effect of added vitamin C is found either at low or high phosphate concentrations.

Phosphate and the Growth of Pea Roots: There seemed little reason to reinvestigate the inorganic constituents of the medium since the earlier experiments (Bonner and Addicott, 8.) showed that there was quite a wide range of concentrations through which the growth of the roots did not vary to any great extent. However, there was



TABLE 26

EFFECT OF VITAMIN C AND HIGH  $\text{KH}_2\text{PO}_4$  CONCENTRATION  
ON THE GROWTH OF ISOLATED PEA ROOTS

Vitamin C concentration in mg. per liter	Growth			
	in mm. per week			
	Week*			
	5		6	
	High $\text{KH}_2\text{PO}_4$	Low $\text{KH}_2\text{PO}_4$	High $\text{KH}_2\text{PO}_4$	Low $\text{KH}_2\text{PO}_4$
1000	0	0	-	-
100	14.9	3.4	-	-
50	83.2	46.9	67.2	16.6
10	94.4	73.5	96.9	78.4
0	106.8	47.8	98.9	57.4

\* These roots had been carried for the 4 previous weeks in high and low phosphate.

Vitamin C was sterilized by autoclaving with the medium.

High  $\text{KH}_2\text{PO}_4$  equals 1000 mg. per liter.

Low  $\text{KH}_2\text{PO}_4$  equals 20 mg. per liter.

one anomolous result, namely, that the highest concentration of  $\text{KH}_2\text{PO}_4$  which was tested gave better growth than the others. There remained, therefore, the possibility that still higher concentrations might give higher growth rates. Consequently, the  $\text{KH}_2\text{PO}_4$  concentration series was repeated over a wider range, using roots in the second transfer in the presence of vitamin  $\text{B}_1$ . The results of this experiment appear in table 27. It is seen that during the second culture roots receiving more than 40 times as much  $\text{KH}_2\text{PO}_4$  as was earlier thought to be optimum grew to be 20% longer.

The possibility arose that this promotive effect of  $\text{KH}_2\text{PO}_4$  might be due to an effect of the salt on the pH of the medium. Buffers were therefore included in the medium, MacIlvaine's  $\text{Na}_2\text{HPO}_4^-$  citric acid buffer being selected as having constituents least likely to produce injurious effects. As is shown in table 28, when 100 cc. of buffer were used per liter of medium there was no significant difference between the growth rate of the roots at the optimum pH and that of the unbuffered controls. However, if only 10 cc. of buffer were used per liter of medium, the growth rate at certain hydrogen ion concentrations was clearly higher, more than 30% in one case. (pH 5.4.) Both of these amounts of buffer were sufficient to hold the pH within 0.1 of a unit for an entire week. The unbuffered controls, however, usually change about 2 units in the same time. They start the week near pH 5, and at the end of the week the medium is at pH 7 or higher.

TABLE 27

EFFECT OF PHOSPHATE ON THE GROWTH  
OF EXCISED PEA ROOTS

Concentration of $\text{KH}_2\text{PO}_4$ mg. per liter	Growth Expressed as percentage of the roots receiving 12 mg. per liter
10,000	19
1,000	105
500	120**
100	108**
20	104*
0	44*

\*\* Average of 3 series.

\* Average of 2 series.

TABLE 28

EFFECT OF HYDROGEN ION CONCENTRATION ON THE GROWTH  
OF EXCISED PEA ROOTS

pH	Growth	
	Expressed as percent of unbuffered controls.	
	100 cc. of buffer* per liter	10 cc. of buffer per liter
2.7	0	-
3.4	4	-
3.8	37	-
4.0	-	63
4.1	84	-
4.3	78	-
4.4	91	90
4.5	99	121
4.6	89	125
4.8	83	-
4.9	-	111
5.2	44	-
5.4	-	137
5.6	21	-
6.7	2	-
7.2	3	-

\* Mac Ilvaine's  $\text{Na}_2\text{HPO}_4$  - citric acid buffer was used; the medium contained standard salts, sucrose and vitamin  $\text{B}_1$ .

TABLE 29

EFFECT OF  $\text{KH}_2\text{PO}_4$  ON THE GROWTH OF EXCISED PEA ROOTS IN  
THE PRESENCE OF VITAMIN  $\text{B}_1$  AND NICOTINIC ACID

Concentration mg. per liter	pH Start of Week	pH End of Week	Growth*			
			1	2	3	4
1000	4.5	6.0	122	108	116	124
500	4.7	6.2	118	94	104	118
100	4.6	6.9	105	101	102	108
20	4.9	7.2	100	100	100	100

\* Expressed as percent of controls receiving the standard concentration of  $\text{KH}_2\text{PO}_4$ , 20 mg. per liter.

After nicotinic acid had been found to be a growth factor of roots, the effects of  $\text{KH}_2\text{PO}_4$  were tested again in the presence of both vitamin  $\text{B}_1$  and nicotinic acid and over a period of four transfers. As table 29 shows, the higher concentrations are definitely promotive to growth, although they are also not well buffered.

It would seem likely that in these experiments there are two independent effects, one of phosphate and the other of hydrogen ion concentration. However, it might be that the promotive effects of  $\text{KH}_2\text{PO}_4$  result from the partial buffering of the medium at very high concentrations. There has not yet been an opportunity to test effects of these two factors, pH and high phosphate concentration, in combination, nor to determine if the effects would hold in later transfers than the fourth. It seems apparent, however, that we are dealing here with variations in the medium which, although they may be important, are not essential to the growth of excised roots.

## Chapter VII

## ATTEMPTS TO ESTABLISH A CLONE OF PEA ROOTS

In most of the work with root cultures the original source of material has been the young seedling, germinated under sterile conditions. If experiments are continued for any period of time, it soon becomes a very laborious procedure to sterilize and germinate seeds for each experiment. It is especially time consuming when it is necessary to maintain the roots for one or more transfers in order to deplete them of any growth factors which were present at the time of excisement. It would be extremely convenient, once a medium has been found which will support root growth indefinitely, to establish a clone from a single root and to maintain this clone as a source of material for all experiments. It would not only be convenient from the point of time saved, but it would render the material more uniform so that experiments would give more significant results with the same number of roots.

White (56) found no difficulty in establishing a clone of tomato roots, and Dr. Bonner has had equal success in these laboratories with radish and flax; but to date, although the nutrition of excised pea roots can be considered to be well understood, these roots still do not branch freely. This is rather surprising because the primordia of secondary roots are formed regularly by primary roots in culture, even in advanced transfers. As figures 14 and 15 show, roots in the 20th week in culture have formed

several primordia, of which some have grown out to a limited extent. However, under the optimum conditions for growth of primary roots (standard salts, sucrose, vitamin B<sub>1</sub>, and nicotinic acid) branching will not continue. The situation is essentially as outlined in figure 16. The top row shows primary roots which have been in culture for some weeks. The tips of these can be subcultured indefinitely without loss of growth rate. If the decapitated bases are allowed to remain in culture media for a second week, the branch roots will grow out to about 30 mm. If the tips of these secondaries are removed and placed in the optimum medium containing vitamin B<sub>1</sub> and nicotinic acid, they will continue to grow at the rate of about 30 mm. per week. Occasionally they will form side branches, but if these tertiaries are subcultured, they will grow a few mm. and then stop entirely without forming any detectable branch primordia. It should be noted that the secondary branches start and remain very much thinner than the primaries from which they come, and that tertiary branches are still thinner than secondary ones. The lower part of the figure shows the results when cultures are made starting with roots from seedlings which have been growing for one or for two weeks. The secondaries of such seedlings are about the diameter of primary roots that have been in culture for more than one week; they have essentially the same habit and rate of growth and can be subcultured indefinitely. Tertiaries from these secondaries will grow at a rate of about 30 mm. per week. They are smaller in diameter than the secondaries and are comparable in



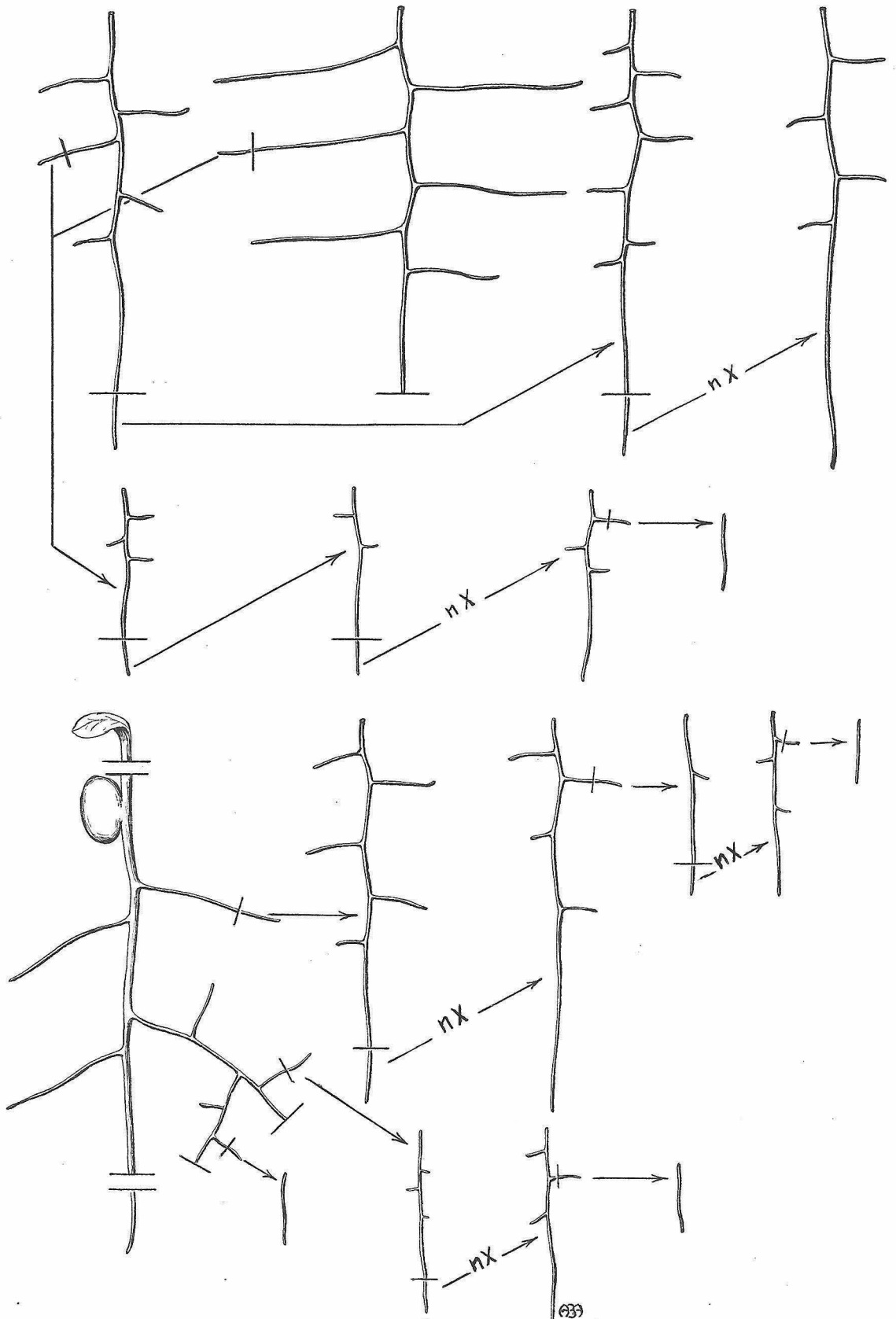


Figure 16. Diagram showing the results of attempts to establish a clone of pea roots. explanation in text.

every way to secondaries from cultured primaries. The branches of these tertiaries will grow only a few mm. if subcultured. Even if the tertiaries and quaternaries are allowed to form on the seedling it does not change their later habit of growth.

It is clear also that the insignificant growth of branch pea roots is not due to apical dominance, since the removal of the tip of a root does not alter the potentialities of its branches, either as to later growth or as to their ability to branch again. Apical dominance enters only in cultures of rapidly growing roots, where, if the root remains intact, a branch will not grow out until the tip has grown some distance beyond the branch primordium. The lack of branch growth may in some cases be correlated with high auxin concentrations. Dr. van Overbeek (unpublished), in analyses of excised primary roots of the same age which did and did not form branches, has found that roots lacking branches had a much higher auxin content than did roots with branches. It also seems reasonably certain that limited branch growth is not due to an insufficiency of auxin. The application of concentrations of 200 to 0.2 gamma of indole-acetic acid per liter was without effect on branching. The higher concentrations showed toxic effects on the roots, with typical auxin swellings. Roots in the lower concentrations branched to no greater extent than did the controls. Other growth factors, including yeast extract and higher concentrations of vitamin B<sub>1</sub> and nicotinic acid, were without effect on branching. In addition, neither vitamin B<sub>6</sub>, vitamin C, theelin, inositol, nor any of the amino acids had any

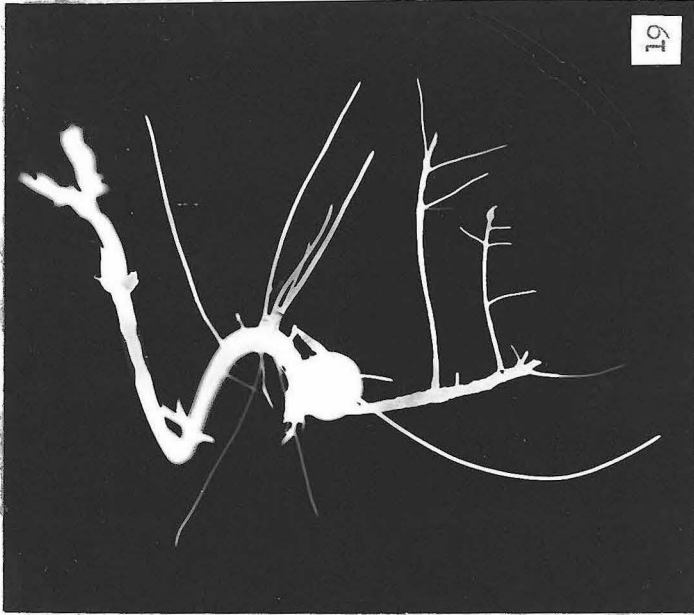
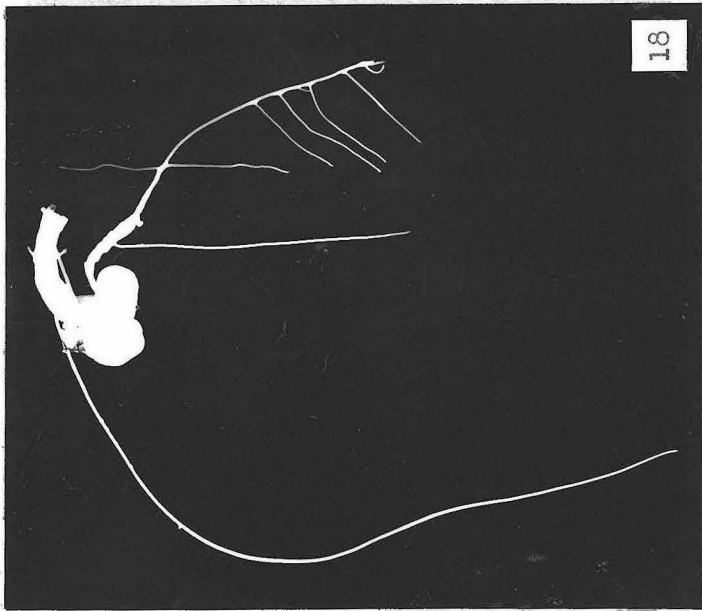
noticeable effect on the branching of pea roots.

All of the foregoing results can be summarized as follows: The ability of a given pea root both to form branches and to form branches that grow is directly correlated with the diameter of the root and not with any nutritional factor yet investigated. Thus the branches from the thick primary root of a seedling grow in culture at the rate of 70 to 80 mm. per week and branch rather freely. The branches from the slightly thinner primary roots which have been in culture for a week or more grow at a lower rate and branch less freely. The thinnest roots grow hardly at all and form no branches.

That the inability of branch roots to grow depends primarily on their diameter rather than on a deficiency of any specific growth factor is also strongly suggested by the following experiment which shows that even the pea cotyledon and shoot themselves cannot supply substances causing pea roots to branch indefinitely. Seedlings were placed in the liquid medium optimum for root growth, both in the light and in the dark. Every few days the root systems were trimmed so that all of the root growth factors from the seed and shoot could be concentrated in forming branches on only one or two roots. These conditions should be optimum for the formation of roots, and if the pea roots are capable of indefinite branching, it should appear here. The results of this experiment can be seen in figures 17 and 18. The quarternary roots had been growing for 13 days at the time the photographs were taken and showed a growth rate, in every case, of less than 10 mm. per week. No further branch primordia were detectable.



Figure 17. Shadow photograph of a 5 week old seedling grown in vitro in the light. Quarternary branches are to be seen on the decapitated tertiaries. The secondary branches present grew out in the 13 days since the root system had been trimmed.



Figures 18 - 19. Shadow photographs of seedlings grown in vitro in the dark: 18. Four week old seedling showing growth of the quaternary branches in 13 days, and one tertiary branch and an adventitious root which had grown out in the week since the root system had last been trimmed. For the sake of clarity the shoot had been removed just before photographing. 19. Three week old seedling showing quaternary branch roots and the numerous adventitious roots which had grown from the base of the shoot during the 5 days since the root system had been trimmed.

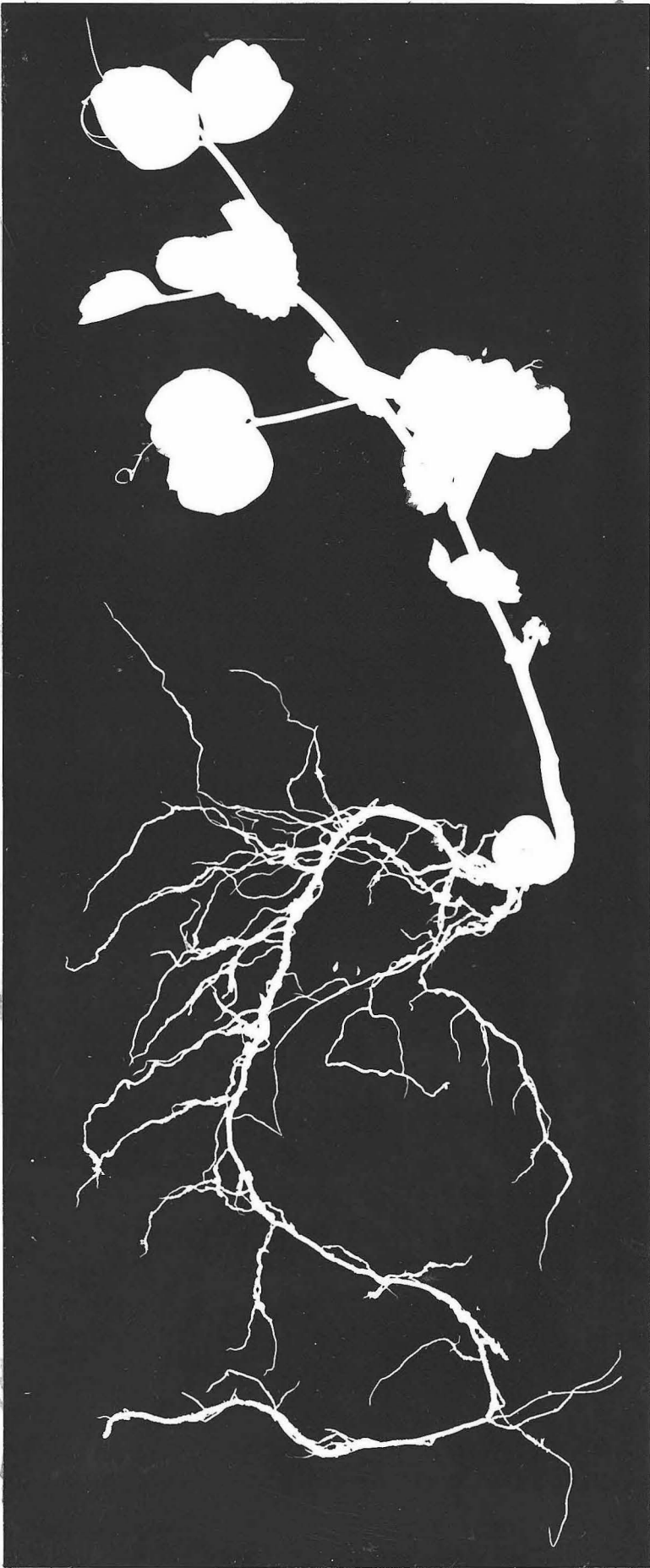


Figure 20. Intact pea seedling 4 weeks old;  
grown in garden soil in the greenhouse.

Figure 19 was taken 5 days after the tertiaries were decapitated and all other branches removed. It shows, in addition to the slight growth of the quaternaries, numerous adventitious roots at the base of the shoot. Both in the light and in the dark this tendency to form additional secondary roots rather than branch roots was observed. The removal of these adventitious roots did not seem to have any effect on the growth of the quaternary roots. Figure 20 shows a seedling germinated at the same time as the others, four weeks previously, and which had been grown in garden soil in the green house. There are some tertiary branch roots, but no quaternaries are to be found on such seedlings.

The evidence above supports the hypothesis that pea roots are incapable of indefinite branching. This does not necessarily imply that the root system of the pea is a very restricted one. Primary, secondary, and tertiary roots could, in the course of a few weeks, form a very extensive system (figure 20) which, together with quaternary roots, if they are formed, should be capable of absorbing the water required by a bushy variety of pea such as Perfection. The inability to establish a clone of pea roots appears to be due not to any deficiency in the culture medium but rather to an inherent limitation of the peas used.

The evidence accumulated in the study of this problem indicates that there is something in the anatomy of the pea roots which prevents them from forming large branches. It will not be possible to reach a complete understanding of the limited branching of pea roots until the histology of branch formation can be carefully studied.



## Chapter VIII

## DISCUSSION

From the evidence presented, it is clear that vitamin B<sub>1</sub> and nicotinic acid are the only accessory substances necessary to the unlimited growth of pea roots. That the growth of roots is optimum with only these two substances cannot as yet be definitely stated. But the growth rate of these isolated roots (74.2 mm. per week) is very close to that of the roots of intact etiolated seedlings under the same conditions during the first two weeks of growth (72.7 mm. per week). It may be that several factors, among them phosphate, hydrogen ion concentration, and inositol, might have additional effects on growth in later transfers than it has yet been possible to study. However, the principal purpose of the nutritional phase of the work has been accomplished with the discovery of nicotinic acid as the second growth factor of pea roots.

There is a point of discrepancy between earlier experiments and the results reported here, that is, with the reaction of the roots to amino acids. Bonner and Addicott (8) were able to obtain what appeared to be unlimited growth in an arbitrary mixture of amino acids with vitamin B<sub>1</sub>. However, when pains were taken to develop a mixture which would be optimum both as to identity and concentration of amino acids, it would not support growth of the roots. Nevertheless, the latter mixture did have the ability to increase



the growth rate of roots supplied with vitamin B<sub>1</sub> over that of the roots which received only the vitamin. Both this effect and the success of the earlier arbitrary mixture might be ascribed to impurities. It is known that commercial preparations of at least two amino acids contain appreciable amounts of other substances. L-leucine preparations have from 2 to 8% methionine in them, and commercial asparagine contains such quantities of vitamin B<sub>1</sub> that it must be carefully recrystallized before it can be used in the Phycomyces assay for vitamin B<sub>1</sub>. Although all the amino acids used were the purest possible to obtain, it seems not improbable that they might contain substances similar to nicotinic acid which therefore gave the inconsistent effects noted above.

The question of the nature of the action of vitamin B<sub>1</sub> in the root is far from settled. However, there can be no doubt but that in the study of roots cultured with and without vitamin B<sub>1</sub> during the first three transfers, that vitamin B<sub>1</sub> was the factor limiting mitotic activity in the roots. The nature of the physiological reactions which link it with cell divisions are not completely known. However, as is well known for yeast, in the pea root vitamin B<sub>1</sub> has recently been shown to act as a part of carboxylase. (Heegaard, unpublished.) It would be of considerable interest to determine in what manner mitosis is connected with the action of this enzyme.

Still less is known of the mechanism of the action of nicotinic acid in the root since it has not yet been possible to study even

the anatomical effects of nicotinic acid in detail. However, roots suffering from nicotinic acid deficiency are quite different in appearance from those suffering from vitamin B<sub>1</sub> deficiency. The former become very thin but remain smooth and more or less white; this is in contrast with the swellings, irregular outline, and brownish tone of roots deficient in vitamin B<sub>1</sub>. Preliminary observations of sections indicate that the absence of nicotinic acid does not affect cell elongation, wall deposition, or the storage of starch. As is the case in vitamin B<sub>1</sub> deficiency, the lack of nicotinic acid is correlated with the absence of cell divisions in the meristem. Preliminary examination showed no cells in division in nicotinic acid deficient roots. The principal difference observed has been that the roots become thick in the absence of vitamin B<sub>1</sub> and that they become thin in the absence of nicotinic acid. However, since the material studied was not strictly comparable, the nicotinic acid deficient roots having come from the 10th transfer and the vitamin B<sub>1</sub> deficient roots never having been taken from later than the 3rd transfer, the differences observed may be related to the length of time the roots had been in culture. Further data must be obtained before any definite statement can be made regarding the similarities and differences of the effects of nicotinic acid and vitamin B<sub>1</sub> on the anatomy of the root. It is not known whether nicotinic acid is active in the root as such or whether it is converted by the roots to some active form. The preliminary work of Bonner and Buchman (unpublished) indicates that nicotinic

acid amide is as active as nicotinic acid on the growth of pea roots. This would support the idea that either they are both readily converted by the root to an active form or else that they are both equally active. The first interpretation seems the most likely since nicotinic acid amide is known to be the active part of two dehydrogenase systems. The possible action of nicotinic acid through a dehydrogenase system in the root has not yet been investigated.

It should be of considerable general interest that it is now possible to culture roots indefinitely in completely known media. In these cultures we have an organ of a higher plant growing and differentiating in a very simple substrate. To a certain degree the culture of plant parts has now surpassed the culture of animal tissues and organs. The simplest medium in which animal tissues can be cultured indefinitely is very complex at best. Pea roots require only salts, sucrose, vitamin B<sub>1</sub>, and nicotinic acid. From the aspect of the physiology of the plant, the substances which the root requires from the shoot are thus much more simple than might first have been supposed. It is a striking fact that roots when supplied inorganic salts and three relatively simple organic substances are capable of synthesizing all of the hormones, proteins, enzymes, and other substances both simple and complex that are necessary for the formation of living material and for indefinite growth. The growth involves not only increase of root material but active and continued cell divisions. The study of roots in vitro can only be said to have begun; it

should be a fertile field for the physiologist, the biochemist, and the cytologist who is interested in the reactions of actively growing organs.

It is hoped that it will be possible in the future to multiply many fold the experiments reported here. As they stand they do little more than indicate the direction in which future research might go in order to reach a more complete understanding of the physiology of the root and its relation to the plant as a whole.

## SUMMARY

1. The history of plant tissue cultures is outlined.
2. The technique of the aseptic culture of excised roots is described. It is reinvestigated from several aspects and certain changes are incorporated for the present work.
3. The action of vitamin B<sub>1</sub> as a growth hormone of roots was found to be through an effect on meristematic activity rather than on cell elongation, which is the primary effect of the auxins.
4. Cell elongation, differentiation, and maturation were found to proceed normally, as far as could be observed, in roots to which vitamin B<sub>1</sub> was not supplied.
5. A second growth factor of pea roots present in yeast extract was found not to be among the amino acids or microelements of plant nutrition.
6. A mixture of amino acids optimum for the growth of pea roots in the presence of vitamin B<sub>1</sub> was found to increase the growth over that of roots supplied vitamin B<sub>1</sub> alone, but it would not support growth indefinitely.
7. Nicotinic acid was found to be capable of acting as the second growth factor of pea roots. It, with vitamin B<sub>1</sub>, can support the growth of pea roots at a rate nearly 50% higher than can yeast extract.

8. Evidence is presented to show that inositol, optimum hydrogen-ion concentration, and very high phosphate concentration may have promotive effects on the growth of pea roots in vitro.

9. Attempts to establish a clone of pea roots are described. Experiments are presented which indicate that it may be impossible to establish such a clone.

## APPENDIX

## THE CULTURE OF EXCISED ANTHERS

To the writer's knowledge there have been no previous attempts to culture excised anthers, and yet if more were known of their nutrition and particularly of the nutrition of sporogenous cells, it might throw an interesting light on the processes of mitosis and meiosis. It was with this hope in mind that preliminary experiments with the culture of anthers were started. It was not anticipated that such experiments might have the direct result of making it possible to culture sporogenous cells. But it was hoped that they would indicate the direction that further research should take in order to make some concrete contribution to the physiology of cell divisions.

It was decided to use the anthers of the nasturtium, Tropaeolum majus. This plant has large flowers and can be kept blooming for extended periods of time. Furthermore, the chromosomes are reputed to be favorable material for cytological observations. The seeds of as pure a variety as can be obtained on the market, Double Golden Gleam, were kindly supplied by the Bodger Seed Company of El Monte. Young flower buds were removed from the plants at the time when the spur had just become obvious as a protuberance of 0.5 mm. or less. The anthers of these buds were from 1 to 2 mm. long. If the buds were taken at any earlier stages, the anthers were so small and delicate as to make it almost impossible to move them without injury, even with delicate

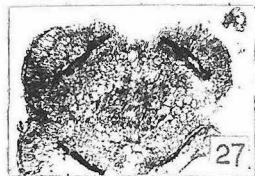
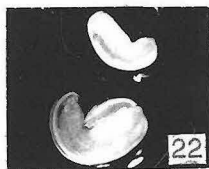
forceps. Yet it would be very desirable to work with even younger anthers in which the cells are still actively dividing; The necessary methods were not obvious at the time these experiments were performed, but it is now believed that it would be possible to handle anthers as soon as they could be distinguished by the unaided eye.

The methods used in this work are essentially the same as those required for the sterile culture of roots. The instruments and methods of sterilization of medium and vessels are the same. It was found to be convenient to sterilize the buds with bromine water. Exposure of from 10 to 20 minutes in a solution of 10% saturated bromine water was sufficient to sterilize the flower buds before the anthers were excised. A number of types of culture vessels were tested, both of soft and pyrex glass, among them being test tubes, Petri dishes, shell vials, and Erlenmeyer flasks. If anything, growth was better in soft glass as far as could be observed. Shell vials were selected because it was found that each culture would run for several weeks, and a cotton stoppered vessel was therefore desirable. Also, the shorter length of the vials made the explant more readily accessible. The medium contained the standard salts developed for pea roots. (Table 1.) Growth was found to be better with sucrose as a source of carbohydrate than with dextrose. Also agar medium seemed to be slightly better than a liquid one. No effect of light on the cultures was noted. Presumably this was because there was an adequate supply of carbohydrate in the medium. The anthers were



removed from the bud with considerable care and placed on the surface of agar slanted in the vials. The experiments were followed photographically. A simple camera utilizing 35 mm. motion picture film was mounted on the tube of a microscope, and a single lens, a low power objective, was used, giving a final magnification of 3.7 times. Eastman Sound Track Positive film was used as being inexpensive and one of the finest grained films obtainable.

Under most of the nutritional conditions studied the anthers enlarged 30 to 40%; in a few cases the enlargement was something over 100%. However, it is not believed that this enlargement was accompanied by any cell divisions. Sections were made of anthers frozen with CO<sub>2</sub> in order to determine the nature of the growth reaction. Figures 25 to 27 show photographs of these sections. It appears that the growth of the anthers is primarily due to a cell enlargement of the non-sporogenous tissues. There is found at the same time a considerable deposition of cell wall materials, as can be readily seen in the photographs. Furthermore, it was invariably observed that during two weeks of culture the sporogenous tissue of the anthers degenerates to the point that it is impossible to distinguish any of its cells. These observations were of a most cursory nature and should be supplemented by a study of carefully fixed and stained paraffin sections before any definite statements can be made as to the nature of the growth of the anthers.



Figures 21 - 27. Photomicrographs of the growth of excised anthers of Tropaeolum in vitro: 21, 23. Anthers at the start of culture. 22. Anthers of figure 21 after two weeks on medium containing 0.1% seedling extract. 24. Anthers of figure 23 after seven weeks on medium containing 1.0% seedling extract. 25. Cross section of an anther at the start of culture. 26 - 27. Sections of anthers after two weeks in culture. (Magnification: figures 21 - 24, 3.7  $\times$ ; figures 25 - 27, about 36  $\times$ )

After the rough limits of the cultural conditions had been determined, attention was devoted almost entirely to the effects of various growth promoting substances and preparations. The previous experiments with sugars, agar, etc., had all been done with 0.01% yeast extract in the medium. A concentration series with yeast extract disclosed, however, that this material was ineffective in the presence of sucrose. The anthers grew equally well (about 30-40% in two weeks) in concentrations ranging from 0.5% down to 0. Likewise, yeast autolysate, a filtrate of bakers' yeast which had been allowed to autolyse at 45° C, was without additional effect. Several water extracts of nasturtium plants in various stages of development were tried. The extracts were prepared by grinding the material in a mortar with a small amount of water and then sterilizing it by passage through a Seitz filter. It was interesting that none of these crude extracts proved to be toxic. Growth in them was as good or better than that in any previous series. In fact, extracts of germinating seeds and young seedlings gave growth of about 100% increase in linear dimensions, which means approximately an 8 times increase in volume. (See figures 21-24.) This was by far the most promising result of any of the cultures of nasturtium anthers and should certainly be investigated further. The activity of these embryo extracts in the growth of anthers is interesting especially in the light of the fact that Bonner, Haagen-Smit, and Went (3) and Bonner and Haagen-Smit (4) have found an excellent source of leaf growth factors in the diffusate of germinating seeds. Anthers are but

modified leaves, and it would certainly be worthwhile, in addition to investigating the action of extracts and diffusates, to test the activity of the known leaf growth factors on the growth of anthers.

A few pure substances which were known to possess growth promoting properties of one type or another were also tested. Vitamin B<sub>1</sub> was tried in the presence of sucrose but was without effect. Auxin in concentrations from  $5 \times 10^{-4}$  to  $5 \times 10^{-6}$  gm. per liter, seemed to cause the anthers to be somewhat warty and distorted in shape. Lower concentrations seemed to have little effect. Dibenzanthracene, one of the most potent carcinogenic substances known, was also used. Applied in a lanolin paste of concentrations from 1 part in 5,000 to 1 part in 500,000, it appeared to be without effect. It would be of great interest to extend this list by considerable proportions, particularly with regard to what has recently been found of the leaf growth factors and flowering substances.

After coming to these laboratories, it was impossible to do any great amount of work with anthers because of the necessity of concentration on the problems of root cultures. However, there were available pips of Convallaria majalis, the Lily of the Valley. This is a plant similar to tulip and hyacinth, in that the flower stalk which appears in the spring is formed during the preceding summer. It is also similar in that a cold treatment is necessary before the pip will start to grow in the spring. After an appropriate cold treatment, the plants can at any time be brought into

full bloom in less than three weeks by placing them at 25° C.

Only a very few anther cultures were made using Convallaria and these with negative results. However, some interesting observations were made on the pollen mother cells of this plant which might be of interest at this time. It was found that the sporogenous tissue of Convallaria anthers is at the pollen mother cell stage as the pips are sold commercially after cold treatment. When they are placed under moist conditions at 25°, leaves and flower stalk start to elongate and meiosis takes place during the second day. This process was followed in vitro by placing buds of pips, both those which had received cold treatment and those which had not received it, on various culture media. The anthers were smeared and examined at intervals to determine the condition of the sporogenous cells. It was found that in all the cultures anthers from cold treated pips started meiosis, and many of the pollen mother cells completed the second division. However, under the same cultural conditions the pollen mother cells from non-cold treated pips showed no indication of any meiotic divisions. It is clear then that we are dealing here with a condition in which the process of meiosis is not only limited by temperature, as has long been known, but, in addition, the stimulus of moderate temperature following cold treatment is actually necessary to initiate meiosis.

A brief summary of the attempts to culture excised anthers follows:

1. A technique for the sterile culture of anthers has been described.
2. Anthers of Tropaeolum majus (nasturtium) have been kept alive in vitro for 7 weeks, whereas in the flower they are withered in something less than 3 weeks from the time the bud first appears.
3. Under the conditions of culture described here the anthers do not mature and dehisce. The growth which has been observed was the result of cell enlargement of the non-sporogenous tissues.
4. In all the cultures of Tropaeolum which have been observed, growth has been accompanied by a degeneration of the sporogenous tissue in which the cell identity has become obliterated.
5. In media containing embryo and seedling extracts anthers have increased their linear dimensions by as much as 100%.
6. Cold treated Convallaria undergo meiosis in the second day after being placed in warm conditions.
7. In excised buds of cold treated pips the pollen mother cells will undergo meiosis, although mature pollen grains have not yet been obtained. If the pips have not received a cold treatment, the pollen mother cells of isolated buds do not start to divide.

Prospectus: The outlook for further and more successful work with the culture of anthers appears far from hopeless. In view of the preliminary experiments mentioned here, the following are suggested as being promising of better results:

1. The incorporation of refinements in technique, making it possible

to start cultures with very young anthers in which the cells are still actively dividing, thus increasing the chances of carrying on the anthers in an embryonic condition.

2. Application of knowledge and technique found in studies of the growth hormones of other plant organs, of which the root and the leaf are now becoming well understood.

3. A better knowledge of the physiological relations of the anther in situ is essential to any thorough understanding of the reactions of excised anthers.

4. In order to evaluate any results of work with excised anthers, the sporogenous tissues should be observed by careful cytological techniques for the duration of the experiments.

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