VIRUS INFLUENCE ON PIGMENTS IN DARK-GROWN AND LIGHT-GROWN PLANTS

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Three issues were investigated; first, the influence of virus on pigment production in diseased plants; second, the increase of virus as indicated by a resulting change in carotene concentration; third, the function of pigments other than chlorophyll in photosynthesis in young potato leaves. Results on the first issue demonstrated an increase in carotene and xanthophyll concentration, and a decrease in chlorophyll concentration in diseased relative to normal plants. Evidence on the second issue suggested a close relationship between virus increase and carotene concentration. Data on the third issue indicated a small and possibly significant photosynthesis due to leaf carotenoids.

PREFACE

This investigation represents a study of pigment development in virus diseased plants. An inquiry into this sequence is made through the use of young potato plants grown in the dark, and studied for alteration in pigment concentrations under both light and dark conditions.

The present work has been supported by facilities provided by McMaster University through the Department of Biology. The provision of funds in the form of a Research Assistantship by this institution made possible the present work.

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I. INTRODUCTION

1. Early Period of Discovery

Recognition of some aspect of plant virus disease, which occupied a small group of virologists between 1900 and 1940, sets the stage for the present work. Generally speaking, the early period was occupied with the study of symptoms and the discovery of new viruses. By 1940 increasing attention was being given to the virus as a chemical unit. Plant physiology and biochemistry were just beginning to give the pathologist fragmentary evidence which he could use to interpret the nature of virus interference. Some of these developments the author will use to indicate his reason for choosing his problem; some will serve to construct a picture of the general field within which this problem has a place.

The first recognized demonstration of virus existence was made by Iwanowsky (16) in 1892, who proved that sap from a diseased plant could induce recognizable symptoms in a healthy plant. Using similar transfer techniques, others discovered tobacco and potato viruses in tobacco and potato plants respectively. As a result the pathogen was classified as tobacco mosaic or potato leaf roll to designate its natural host and symptom. The conclusion that host specificity (virus

in potato plants only) represented a genetic or evolutionary connection between virus and natural host was complicated by Allard's (1) transfer of tobacco mosaic virus to potato. Closely related to this study of symptoms is Holmes' (15) work in 1928. Using a symptom as an indicator, he observed a direct relationship between concentration of local lesions which develop on the inoculated leaves of Nicotiana glutinosa.

While Holmes started an interest in virus as an infective unit that caused a visible symptom, Helen Perdy and Mr. Beale (26) were developing an interest in the chemical properties of the unit. Their discovery that tobacco mosaic virus protein later in 1935 established a new line of investigation. Although biochemistry was still young, it came to be the virologist's hope for an early solution to his problems.

When Smith (27) established in 1929 the importance of the aphid Myzus persecae as a vector of potato leaf roll, a third line of investigation was begun. This topic is considered below from an interest in relating the properties of animal and plant virus. Later findings of Black (3) in 1950 and Maramorasch (20) in 1951 indicated that aster yellows and clover club-leaf viruses had undergone an incubation period in the insect vector in which virus concentration had increased 100 fold. Furthermore, clover club-leaf virus had been transmitted from one generation of insect vectors to another through 21 generations without loss

of infectivity. It is possible that new developments on this topic might indicate more clearly the exact degree of relationship between plant virus and animal. Furthermore, the alterations in normal physiology in virus infected plant and animal tissue, due to plant and animal virus respectively, may show related features.

In summarizing the developments during this 40 year period one finds that two distinct approaches to virus research had developed. One continued the work begun by Iwanowsky, Allard, Holmes on discovery and description of virus symptoms with more attention being given to the modification of tissue and cell structure, that is symptom. The other followed Stanley's lead in the direction of chemical analysis. Often this led to biochemical studies of virus reproduction (24) or utilization of cell products such as amino acids, purines, and pyrinidines in virus synthesis (34) or an analysis of the virus as a complex molecule (36). The problem undertaken here arises from work done following the first approach on modification of tissue and cell structure. However, its definition and procedure places it in the category of the second approach as described above. The remainder of the introduction will trace the development of and necessity for investigation such as that considered here.

2. Modifications of Tissue and Cell Structure

The early studies on mosaic diseases brought out two

factors which are generally accepted by those who have studied the histology and cytology of these diseases. The first is modification in cell structure of the leaf, and second, modifications of the chloroplasts. It is out of interest in an apparent contradiction in the interpretation of these histological and cytological symptoms that the author has chosen to work on a virus causing mosaic symptoms.

A mosaic symptom is commonly understood as an irregular patchwork of yellow or yellowish green in diseased areas contrasting with the green of healthy areas. Beijerinck (4) in 1899 began the first studies on modification of cell structure. He claimed that the transition from diseased to healthy was abrupt rather than gradual. Melches (21) in 1913 made similar studies on both tomato and potato. His observations indicated that the diseased yellow areas were generally thinner (90 mic.). This change in thickness was found to be the result of shortening of the palisade cells to a degree at which they appeared cuboidal in form. He also noticed a reduction in the size and number of chloroplasts in both the palisade and spongy parenchyma tissue.

However, the significance of this condition was doubtful when some plants were found to recover from their symptoms under the influence of sun and age. In addition it was known that mature plants did not develop severe mottle, only the young growing leaves became severely mottled. Goldstein (14) in 1926 suggested that a virus toxin could

hinder the completion of histog-nesis in so far as differentiation was concerned. This opinion allowed for the development of symptoms in young growing leaves, and at the same time did not rule out the possibility that these same leaves could later recover from the effects of a virus toxin. The influence of sun and normal growing conditions could, in this case, assist the young diseased leaf to recover. Recent literature is beginning to support in part Goldstein's original suggestion. His toxin would now be considered a pre-virus molecule found following the invasion of the first infective virus and before the formation of a second duplicate of the first. Presumably the pre-virus might constitute the block to normal metabolism which in the diseased state releases metabolites such as amino acids, pentose sugars, and phosphates for virus duplication.

The state of evidence to date is so fragmentary that conclusions regarding the causes of symptom development are still hypothetical. In the absence of a better term, "inhibition" became a part of the virologists vocabulary to describe the effect of virus on normal metabolism and development. Cook (9) was among the first to use this term with reference to modifications of the chloroplasts. Others had already observed a reduction in the size and number of chloroplasts in diseased tissue. Some were of the opinion that chloroplasts were being destroyed. Cook accepted the opposite point of view from his observations. He could find no evidence that chloroplasts

were destroyed nor injured. Cook's photomicrographs of sectioned leaves showed chloroplasts in cells of the healthy plant to be large and the markings well defined as compared with chloroplasts from the corresponding cells of a diseased plant which were small and with poorly defined markings. He made it clear that these results could be found only in young leaves of a diseased plant; chloroplasts in the chlorotic areas of older leaves were always very nearly or quite normal in appearance.

Cook and Goldstein appear to have arrived at similar conclusions. Cook wanted to substantiate his description of the effect of virus invasion as an inhibition of development rather than a destruction. Goldstein accepted the effect as an inhibition and suggested that a toxin of virus might be the cause. The present work relies upon Cook's evidence for much of its argument. Theoretical conclusions, such as that proposed by Goldstein, are of interest and are considered later under the topic of Discussion.

Cook produced enough evidence to show that symptom development depended on the age of the plant before inoculation. He criticized his predecessors on the basis that they had not considered the physiological condition of the host plant in defining their symptom. In his opinion the expression of virus activity varied as host physiology changed. The rapid changes in physiology as the plant passes through periods of germination, maturation, flowering, and senescence can affect virus expression

so that there may be evidence of severe injury or no evidence at all. The present work has treated this precaution with respect. The problem of this work has received its genesis from conflicting results on the subject of virus interference in pigment production. The matter of physiological conditions has played an unknown and often undefined part in these results. Frequent reference will therefore be made to Cook whenever judgements of conflicting results are necessary.

3. Transition Between Microscopic and Chemical Study of Symptoms

As we have seen, Cook produced evidence of a microscopic nature to support his argument. The certainty with which he presented his findings regarding inhibition of chloroplast development appears to contradict Sorokin's (30) evidence that chloroplasts are destroyed. Sorokin claims to have demonstrated by direct observation and by microchemical tests a "dissolution of the proteins of the stroma". Compared with Cook, Sorokin has made no attempt to describe the conditions under which chloroplasts are destroyed. Since Sorokin has not been proven wrong, one finds it necessary to propose the conditions which made his results possible. In so doing both his (Sorokin's) and Cook's observations can be accepted as useful in understanding what happens following virus inoculation.

The biologist's approach to finding a link between

virus and the destruction of chloroplasts is often to locate the pathogen at the site of injury. Consequently, Lyons (19) has recently found virus protruding from chloroplasts as though they had been reproducing at that sight. One can only guess at the nature and results of this association. If one supposes that one result may be a destruction of the chloroplast, then evidence may be found to indicate that the chloroplast has something the virus requires and is capable of acquiring by chloroplast destruction.

It is known that virus requires duplicating units to form nucleic acids and nucleoproteins necessary to its multiplication. Using isotopic tracers, virologists have noticed that in multiplying (autoreproducing) a bacterial virus accepts the larger percentage of its duplicating units from its host (5) and very little from the parental virus molecule. This provides some idea of what the virus acquires. It now remains to indicate how and where the virus might go about getting what it requires.

The virus competes with the plant cells for amino nitrogen sources necessary for both normal plant protein metabolism and virus multiplication. When the concentration of virus within the cell increases to a point at which all of the amino nitrogen source is required for virus multiplication, the cell itself is starved for protein duplicating units. Under these conditions normal plant proteins (both cytoplasmic and chloroplastic) may be hydrolized to their

constituent amino acids (10). Starvation appears to initiate this reversal process. Perhaps virus may take advantage of the new nitrogen sources provided by this breakdown for its own requirements.

The observation that chloroplastic protein is hydrolized along with cytoplastic protein in a starving plant (18) is important to the purpose of this section. It began with an attempt to justify Sorokin's finding that chloroplasts were destroyed. In so doing it was pointed out that a high concentration of virus could result in protein starvation of the cell. This in turn might cause hydrolysis of chloroplastic protein. The resulting breakdown in chloroplastic protein would mean what Sorokin termed a "dissolution of the proteins of the stroma." Since the stroma (thread like supporting membranes of the chloroplast) and the grana (pigment bearing grains supported by the stroma) are composed of a protein skeleton (11), protein hydrolysis would mean destruction of chloroplast structure. Wood (35) commenting on this condition noted a rapid and complimentary decrease in all plastid pigments. It would seem that pigments too are stable only as long as the pigment bearing structures are stable.

There now appears to be a solution to the apparent conflict between Cook's and Sorokin's findings. Cook must have been working with a type of virus at a concentration which caused inhibition of development only. Sorokin probably used a virus at a concentration which produced severe injury. The injury may have been caused indirectly through

protein starvation and resulting hydrolysis of chloroplastic protein. Thus he could have observed chloroplast destruction.

The information provided by these two men has contributed to the understanding of what happens to the chloroplasts and their pigments following inoculation. The problem of this work concerns the relationship between virus and plastid pigments under conditions similar to those of Cook's. That is, if a virus does not reach high enough concentrations to cause metabolic starvation, the result will probably be some measure of interference in development rather than destruction. The following section considers published evidence regarding pigment development in diseased and normal plants. The cytological and cytochemical evidence discussed so far will be used in an attempt to find some consistency in the chemical interpretation of the mosaic symptom.

4. Chemical Interpretation of the Mosaic Symptom

A mosaic symptom is recognized by the abnormal variation in leaf coloring. This variation in pigment concentration due to the effects of virus will occupy the following discussion.

Existing evidence published by several investigators (12, 22, 29) working with tobacco mosaic virus indicates a reduction in chlorophyll content of the light green zones of the diseased leaf. McKinney (22) did a study of four different mosaics, reporting on chlorophyll, carotene,

xanthophyll and enzyme chlorophyllase. He concluded that leaves infected with a mosaic virus were consistently lower in all three pigments than in healthy plants. Also, a reduction of 50% in chlorophyll content, compared with healthy tissue, is associated with an approximate doubling of the chlorophyllase activity.

It is appropriate to recall at this point the author's statement from the last section that extreme conditions (such as starvation) might cause chloroplast destruction. This result was expected from Sorokin's observation regarding the "dissolution of the proteins of the stroma". Consequently, it would not be a surprise to find a reduction in plastid pigments in proportion to the loss in chloroplasts. This reasoning seems to support McKinney's work since he used mature leaves that had developed severe symptoms. One would conclude that virus only affected the pigments indirectly through chloroplast destruction.

In contrast to McKinney's observation, Elmer (12) found an approximate doubling in carotene content and a reduction of chlorophyll in diseased areas of a tobacco leaf. This suggests that virus affects chlorophylls and carotenoids (xanthophyll and carotene) separately. If the chloroplasts were destroyed, both chlorophylls and carotenoids would disappear with them (10). It is possible that Cook's experience with virus inhibition of chloroplast development could support

Elmer's findings. If the chloroplasts were not destroyed, as Cook believed, then a measure of pigment concentration under these conditions would decide with more certainty whether or not virus affected each pigment separately. This line of thinking will be developed in the following section where the problem of this work is defined.

5. Definition of Problem

Main Issues. Several questions have been left unanswered by the four men most involved in the past development of this field. It would be interesting to know with some certainty the conditions under which chloroplasts are destroyed as Sorokin observed. The writer only guessed from later evidence that chloroplast structure would break up under severe conditions of protein starvation. When Cook published his work on inhibition of chloroplast development, nothing was known of the ultrastructure of the chloroplast. If chloroplast development is indeed inhibited, it would be interesting to compare an electronmicrograph of the lamellated granum structure (32) in an immature chloroplast with Cook's virus inhibited or retarded chloroplast. There is still little known about the role of granum ultrastructure in its function. A comparison of chloroplast ultrastructure in both normal and diseased tissue might contribute some information to this subject also. Regarding McKinney's work on plastid pigments, the writer again gathered published evidence that might account for his results. It was a guess that chloroplast destruction would account for his results. It was a guess that chloroplast destruction would account for his proportion-

al decrease in all of the plastid pigments. Supporting evidence from Sorokin, Wood and various other men who worked on virus multiplication helped make the writer's guess more reasonable. However, Elmer's observation that carotene concentration increases while chlorophyll decreases required a separate defence. Cook's ecidence that chloroplasts were not destroyed left the possibility that pigments could be affected by virus in some way other than chloroplast destruction. If the virus does not destroy the structure that supports the pigment (granum) then one wonders if it might affect the biosynthesis of the pigment. This appears to be implied by Elmer's and Cook's evidence.

If there were some certainty that Elmer and McKinney were working under identical conditions, there would be no reason to accept their contradictory reports on the same issue. But there is no evidence either that the conditions were identical or in what respect they differed. In the years in which these two men worked there was little awareness of the number of variables that influenced the host-pathogen reaction. Since then the virologist has had to pay extreme care to such factors as variety and age of test plant, strain of virus, description of symptoms over a wide host range, environmental conditions such as temperature, humidity, soil pH, and inoculation techniques. The degree and way in which these factors influence host-pathogen reactions is still not a subject of agreement. But it is recognized that they do affect symptom development. If they are disregarded altogether in the definition of one's experiment, the results may have

an ambiguous meaning. There may be no way of determining how much or what part of the results are due to uncontrolled variables and what part to host-pathogen reaction.

The purpose of this work was designed to reinvestigate the issue on which Elmer and McKinney differed. The present investigation incorporated certain changes in the design and purpose which were considered necessary improvements on the previous work. McKinney and Elmer wanted to know what difference there was in pigment concentration between diseased and normal tissue. For this reason they harvested a mass of plant material; extracted for plastid pigment, separated to carotene from chlorophyll, and did a colorimetric determination. When they had finished they knew the concentration of pigment at the time of harvesting. From the writer's point of view they could have made their evidence more understandable and acceptable if they had made pigment determinations immediately following inoculation and at intervals until the onset of mosaic symptoms. This would have related the evidence that they published as one point in a progression of events. This led directly to the author's organization of investigation which set out to find a series of points, each of which would be related to a direction of development.

The potato was considered a good test plant since it could be sprouted and grown in darkness. The sprout of the potato variety used had only traces of color when grown in the dark. Under these conditions chlorophyll was expected to be absent and the carotenoids (carotene, and xanthophylls) present only in traces. Growing the test plant in darkness amounted to a near zero point in pigment concentration. By

introducing light this concentration could be increased rapidly in normal plants.

This procedure permitted an investigation of the problem of pigment development in two parts. First, inoculation just at light introduction meant that the immediate effects of virus on pigment development could be followed. Second, inoculation with virus several days before light introduction made a complimentary study possible. If the virus had any effect on pigment precursors, it would have an opportunity in the dark grown plant to make a difference in the precursor concentration. A large and significant difference might be recognized as a correspondingly large quantitative difference from the immediate effects as shown in the first part.

With little variation of the above procedure another topic related to pigment development was investigated. That is the topic of virus increase in the plant. If pigment development is affected directly or indirectly by the effects of virus increase, then information of the affect on pigment might suggest some conclusions regarding virus increase. These effects could represent metabolic disturbances which could be the results of virus multiplication. If carotene concentration is sensitive to virus increase (i.e. carotene concentration increases rapidly following inoculation) then the rate of virus increase may be indicated by the rate of change in carotene concentration.

It should be noted that the postulation of the above issue need not make any claim to a quantative indication of virus increase. Owen (25) working on the respiration of diseased tobacco leaves has done a good example of such a qualitative

study of virus multiplication. He noticed a sharp rise in the rate of respiration 9 hours following inoculation. This implied that the effect of virus increase was evident long before any recognized increase in the number of virus particles. The emphasis is placed on the work "recognized". The question is whether methods used by others to recognize or identify virus increase directly were quantatively accurate. A microchemical technique puts the earliest point of measurable virus at 72 hours after inoculation (8). A later method using the electron microscope claims an increase as early as 18 hours following inoculation (6). If it is possible to associate virus increase and metabolic disturbance as cause and effect, then Owen's observation stands as the earliest evidence of virus duplication. Unfortunately there is no way of knowing how directly or by what mechanism the virus acts on respiration. The same criticism could be applied to the writer's attempt to demonstrate a qualitative relationship between virus increase and rapid rate of change in pigment concentration. In spite of this it was considered of some worth to accumulate the data on the pigments, knowing that this evidence of metabolic disturbance and virus duplication were adjacent events. Later developments in this field may or may not justify the author's suggestion that these adjacent events were, in fact, related as cause and effect.

These two closely related issues occupy the major part of the present work. The one concerns the effect of virus on pigment development. The other represents an attempt to find some relationship between virus increase and the rate of change in pigment concentration. In addition to these two

major issues there is a third which was given minor consideration. This latter was considered as secondary since the planning of this part of the work was begun after the completion of the first two issues. It was thought valuable enough to deserve additional time and investigation.

Secondary Issue. Like the major issues which preceeded it the secondary issue was related to the diseased state of the plant. It differed from the others in the emphasis it placed on the function of pigment in photosynthesis, rather than the pigment potential of concentration. This has not been given mention earlier in the Introduction since it would have been premature to discuss it before this point. Before giving a definition of the issue, a short introduction and survey of background literature must be included.

Since 1930 many experiments by Emerson (13), Binks (2), Manning (23) and others have shown that all three types of pigments (chlorophyll, caretenoids and phycobilins) can function in contributing energy to photosynthesis. However in some organisms certain pigments are less effective than others. Such experimentation has been confined to the algae. Thus, the carotenoids of green algae appear to be about one half as effective as chlorophyll (37). In certain species of red algae, light absorbed by chlorophyll is used for photosynthesis less effectively than light absorbed by phycoerythrin.

Algae are particularly adapted to the techniques

used in investigating photosynthetic activity of pigments. Also the fact that red, green, blue green algae and their mutants each have different pigment compliments often assists the investigator in studying the efficiency of one pigment in the absence of some other. For example, the efficiency of phycoerythrin has been studied in the absence of chlorophyll. It would contribute something to our knowledge of physiology if such a study could be made of higher plant pigments as well. The difficulty preventing such a study is the presence in all normal leaves of all three of the following pigment groups: chlorophylls, carotenes, xanthophylls. That is to say it is impossible under normal conditions of growth with normal leaves. However the following possibility developed out of the first two experiments.

Test plants grown in darkness and inoculated in darkness previous to illumination, did not produce chlorophyll until several hours after illumination. During this same period of chlorophyll absence, the plant had a relatively high concentration of carotenoids. This period suggested the possibility of taking advantage of the condition to investigate the role of carotenoids in photosynthesis. A Warburg apparatus was set up to measure gas exchange by Barcroft's differential manometer method. Using this technique any carbon dioxide absorbed from the environment by the plant tissue is taken as an indication that photosynthesis was in progress. Any activity could then be taken due to the carotenoids, rather than chlorophyll which was absent.

Summary. In review, there are three issues arising from this work. The first constitutes a study of pigment development under the influence of virus. The second represents an attempt to find an early indication of virus increase in the rate of change in pigment concentration following inoculation. The third and minor issue investigates the function (or efficiency) of carotenoid pigment in photosynthesis.

II. MATERIALS AND CONDITIONS.

1. Material.

The Potato. The test plant, Solarum tuberogum, used in the present work had to have one important characteristic. It must grow with normal vigour in complete darkness. This suggested the use of a potato tuber since the young plant depends on the tuber food supply for approximately a month after germination. In this case the sprout would not be required to photosynthesize its own food. Its pigment complement could then remain undeveloped. This is a second characteristic required of the test plant. The potato sprouts when grown in darkness remain nearly colorless. In this state the concentration of carotenoids would remain near zero, and chlorophyll would not have developed from its immediate precusor protochlorophyll.

The potato variety, Cherokee, was recommended by Dr. Thomson, Plant Inspection Division, Guelph, Ontario, as a plant which could develop clear virus symptoms of the yellow mosaic type. This variety also had the desirable characteristic mentioned in the preceeding paragraph. Its use facilitated the purpose of the present work, that is, to study the development of pigment in dark-grown and light-grown plants. The advantages of using the potato plant were discussed earlier on page 14 of the Introduction.

Seed stock certified by the Department of Agriculture, Sudbury, Ontario was obtained. According to their methods of inspection the seed was considered 90% free of potato x and

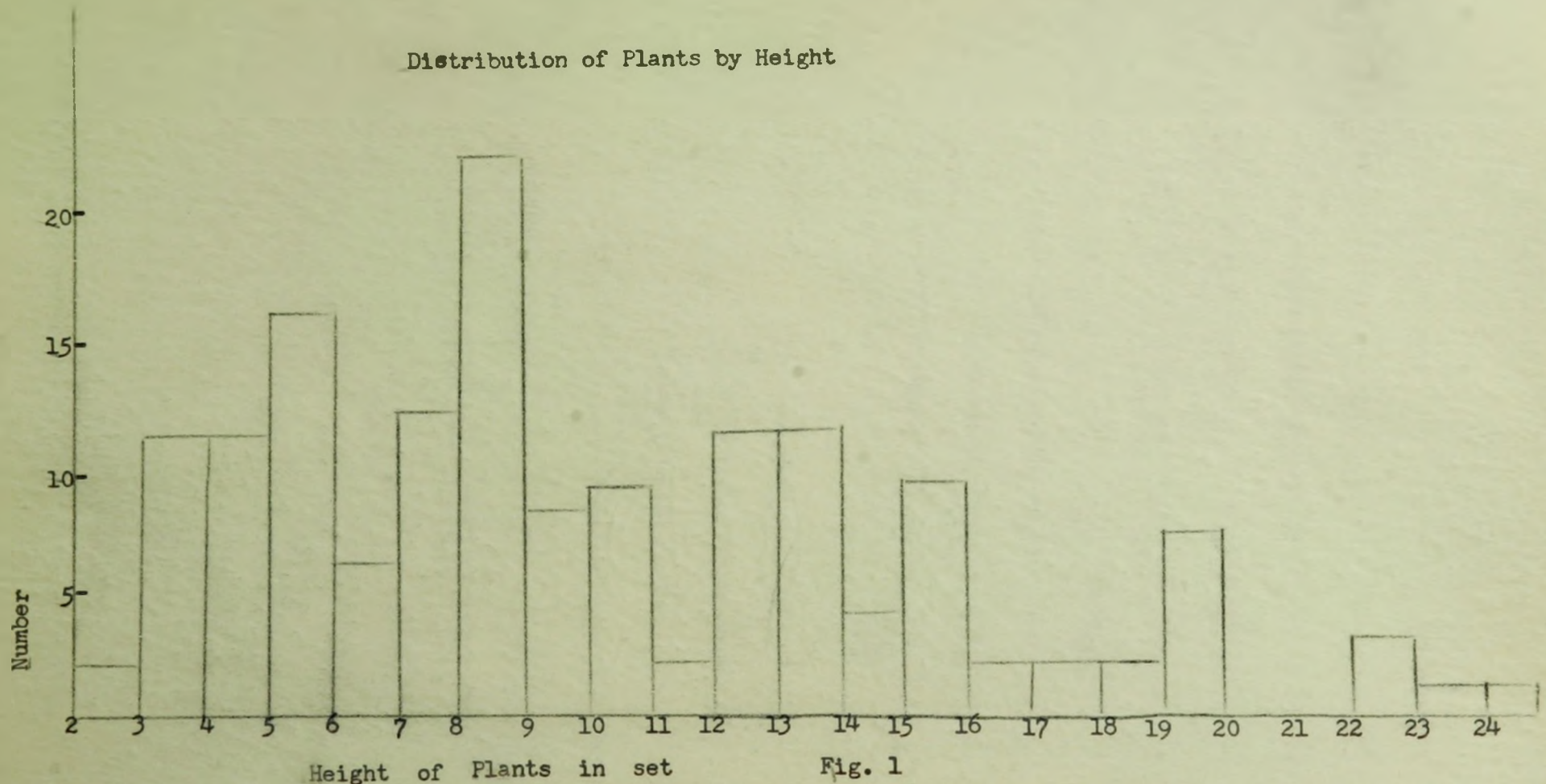
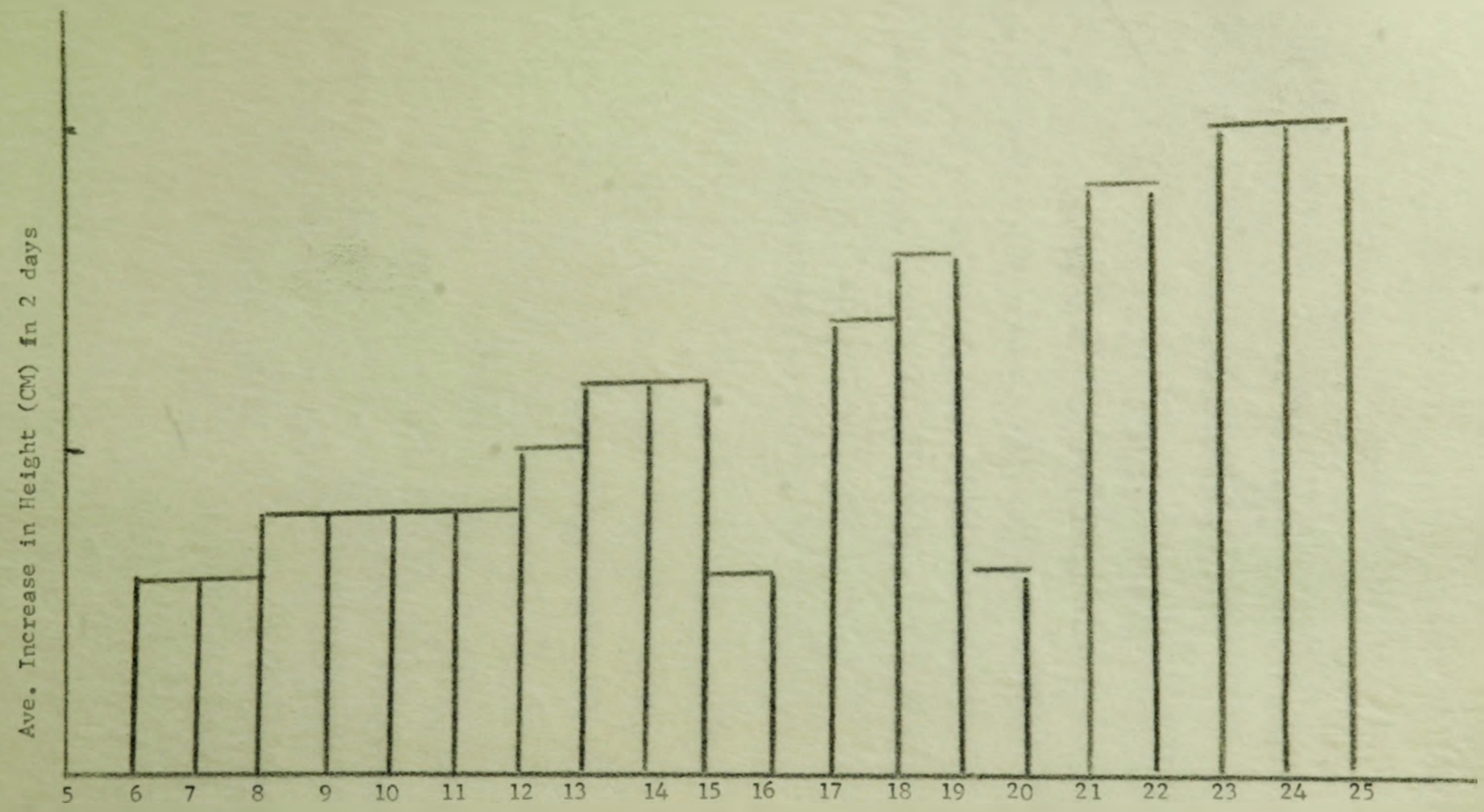

Distribution of Plants by Height
Number
20
15
10
5
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Height of Plants in set

Fig. 1



Weight (CM) of Tagged Plants Fig II

virus. None of the sprouts tested in this experiment showed any evidence of previous virus infection.

Once sprouted, the potato shoots were selected for uniform size. This selection had to be made from flats of sprouts ranging from 2-24 cm height. Numerical distribution over this range is illustrated in Fig. 1. It can be seen from this that 40% of the sprouts were between the heights of 8 to 13 cm. Fig. 11 illustrates growth increases for each size of sprout from 2 to 24 cm. in height. Inspection shows that sprouts from 8-13 c.m. was observed to be nearly constant at 5 to 8 mm. in diameter. On this basis the sprouts from 8-13 cm. in height were selected as the test material. The basis for the claim of uniformity in test material rests on measurements above of growth increases, numerical distribution and stem diameter.

The Virus. The virus source was obtained from the plant Pathology Laboratories, Fredericton, N.B. Dr. D. J. MacLeod provided three cultures, one from a strong potato x, one from medium x and another from weak x. These had been selected from plants grown in their Plant Inspection Division as standard, recognizable sources. On receipt, the virus was cultured in the Cherokee variety used in the experiments.

2. Experimental Conditions

Growth Cubicles. Wooden flats 5' x 2' x ½' were built in controlled temperature cubicles. Temperature remained within 20-24°C, and humidity between 60-70%. A fan helped circulate air in summer and a coil heater helped

maintain temperature in the winter.

A fluorescent light source of 500-600 ft. candles intensity at the level of the growing plants, was used for artificial illumination. Warm white and cold white light bulbs emitted light energy covering both red and blue ends of the plant's active spectrum.

The flats were filled with soil mixed according to John Innes standard greenhouse mixture. It consisted of the following parts in this proportion, 7 sand, 3 loam, 1 peat. The same mixture was used for all experiments.

Conditions. In an earlier statement of the problems under investigation three issues were defined. The first constitutes a study of pigment development in virus diseased tissue. This occupied the larger part of the experimental procedure. The second and third issues required less time since they were considered as smaller issues which had developed out of the first issue. The second issue represents an attempt to find an early indication of virus increase by observing the rate of change in pigment concentration following inoculation. A third issue is an inquiry into the function (or efficiency) of carotene in photosynthesis. The following will continue to develop these issues into a complete procedure.

In preparation for planting, each tuber was cut into three parts, stem end, flowering end and center. Each part was planted separately. Since wounding and soil dampness initiated sprouting there was no need to use artificial or chemical means to force germination. A single potato section

(stem end, flowering end or center) usually contained more than one eye or bud. From each bud a shoot developed. When the shoots had reached the recuired state of maturity, they were separately inoculated. From this point onwerd each shoot was considered a separate plant.

Whenever a virus sample was needed for an experiment, one or more diseased leaves of the potato host plant were pinched off, and ground in a mortar. The cell sap containing the virus was then expressed from the pulp through a gauze pad. This sap was used immediately as the inoculum for a new experiment. The particulars of the inoculation technique used here will be discussed later.

III. EXPERIMENTAL PROCEDURE.

Experiment I. Preparation for an experiment involved the following sequence. Tubers were sprouted and grown in total darkness. The shoots of uniform size were selected three weeks after planting. The procedure of the first experiment necessitated inoculation and immediate exposure to a ten hour light day. The length of light day is not critical in this experiment although 10 hours is the optimal period for growth. Following inoculation, samples were collected, ground to a pulp and extracted as described later. The choice of the appropriate intervals between collection of the samples were determined after a preliminary trial experiment. The results of this indicated a rapid change in carotene concentration immediately following inoculation, as compared with chlorophyll's more gradual rate of change. It was, therefore, not considered

necessary to sample for chlorophyll concentration as often as for carotene. For this reason samples for carotene determination were collected every four hours for two days. Samples for chlorophyll determinations were collected every day for six days.

The purpose of the above discussed experiment was to determine whether or not chlorophyll and carotene were affected similarly or dissimilarly by virus inoculation. This point was discussed at length in the Introduction. It was suggested then that any evidence received, that indicated a dissimilar effect (i.e. reduction in concentration of one pigment compared with an increase of another) would support Elmer's point of view. Any evidence to the contrary meant support for McKinney. This point of dissimilar effect is pursued in a second experiment described as follows.

Experiment II. The investigation of the affect of inoculation is the object of this second experiment. In contrast to the first experiment, the second provides for the inoculation of some of the plants previous to illumination, others at the beginning of the illumination. Of the 150 sprouts grown in the dark, 50 were inoculated three days before light introduction, 50 three days later at the time of illumination, and the remaining 50 were left uninoculated as healthy controls.

The reasons for inoculating at two different times may be clarified in the following comment. The first experiment was designed to provide an answer to the issue on

which McKinney and Elmer differed. By inoculating 50 plants three days before the others, it was hoped that the effects of the earlier inoculation would produce a relatively larger change in pigment concentration than the set inoculated three days later. Thus, by exaggerating the effects in one inoculated set in comparison with the other, the relationship between virus and pigment might be more evident. The second experiment was a repetition of the first in this sense. The change in the design of the experiment was the means of making the repetition effective.

Samples from each of these three sets of 50 plants were collected once every three days for nine days. These samples were then weighed and extracted separately. Separate pigment determinations were done for each set. Sampling at these longer intervals was considered adequate for the purpose of the experiment. Since the pigment concentration determinations were to be plotted graphically against time after light introduction, three points were suitable. For example, the concentration of carotene in the set of 50 plants inoculated earlier was compared with the concentration of the same pigment in plants inoculated later, as well as with the healthy controls. This comparison was made on the basis that all three sets had been collected on the same day, and had been growing under the same light, temperature, humidity and soil conditions.

It was arranged to take additional data for the sprouts in this same experiment. Data were collected on increase in height, weight and leaf size for separate plants in each of these three sets of 50. Since measurements on all

three sets were taken at the same time each day, an indication of the effect of virus on increase in height, weight and leaf size could be had by comparing these three sets together. That is, if the healthy plants were taller, heavier and had larger leaves than the inoculated plants, then one would conclude that virus had inhibited growth in the inoculated plants. If the reverse were true, then virus could be considered to stimulate growth. If the differences in height, weight and leaf size between the three sets were of approximately the same size, some proportional relationship would be suspected. If these differences increased or decreased from the earliest inoculated, to the latest, to the healthy control, the difference might be considered a function of the time inoculated. These comparative measurements were based on a total sampling of 878.5 gm. wet weight and 19,327 cm. of potato shoots. This data was taken in anticipation of the need to support pigment determinations and comparisons made in the second experiment with some general information of the effect of virus on growth rates as well as pigment concentrations. When the results of the second experiment will have been discussed later, the reason for this data will be clearer.

Experiment III. Experiment III differs from Experiment II in two respects. First, in the second experiment, the plants were inoculated in the dark, and the results studied only in the light period, while the third experiment gathered data on pigment concentration in both dark and light periods. Second, inoculation in Experiment III was six days before illumination as compared with three days in Experiment II. The organization of the experiment was as follows.

Plants were grown in the dark, selected for the appropriate size, and inoculated in this same dark period. Sampling began at the moment of inoculation, and was continued in darkness at 24 hour intervals for six days. Following this six day dark period, the plants were illuminated. Three samplings were taken at four hour intervals, the first day after illumination, and once, each day, for the next four days.

Inoculations in Experiment III were of two kinds. For this reason a flat of 150 plants was divided into three sets of 50 each. Set I was inoculated with virus and Set II with water. The inoculation technique (described later) was identified in both sets. These two inoculations differed only in the content of the inoculum. Set III was left uninoculated as a control.

The results of Set II were later compared with Set I (virus inoculated) and Set III (uninoculated) to determine whether water inoculation would have effects similar to virus, or no effects at all. The reason why this was necessary will be considered later in the discussion.

Samplings of all three sets were made regularly in the dark grown period at the intervals indicated. However, only Set I (virus inoculated) was sampled at regular interval in the light period for the following reason. By the end of the dark period, Set II (water inoculated) showed no evidence of causing changes which might be confused with effects due to virus. In addition, since there was no significant difference between the water inoculated and the uninoculated in Experiment III, and between these and the uninoculated in previous experiments, the experiment

was modified to reduce the number of determinations in Sets II and III. Thus, only three determinations of uninoculated and two of water inoculated were made during the light period. This permitted a concentration of time and effort on the more significant virus inoculated plants.

Experiment IV. The second issue which received attention in the Introduction forms the center of a fourth experiment. The discovery from a preliminary experiment that carotene concentration appeared to change rapidly following virus inoculation suggested the possibility of using this pigment as an indication of the progress of the virus. For example, the effects of virus are assumed, as indicated in the Introduction, to be due to the virus demand for duplication units. The stages through which the virus passes in duplicating and the levels of demand on the host physiology are not as yet known. Any rapid change in any one of the cell's components (e.g. carotene) following inoculation could be an indication of the effects of one stage of virus duplication. If the change is connected only with a final stage, then a change in carotene concentration may indicate the point at which increase in the final virus unit takes place.

The procedure in the fourth experiment was identical with the first part of the third. Inoculated dark grown plants were sampled for carotene concentration. The plants were kept in darkness for the duration of the experiment to ensure that any immediate changes in pigment concentration would be the result of virus and not light energy. Sampling began immediate-

ly after inoculation and was repeated every two hours for 8 hours. The resulting data on carotene concentrations was plotted on graph paper against time following inoculation.

Experiment V. The third and secondary issue which the present work considers is the function of the carotenoid pigments. The experiment makes no claim to be a broad study. Rather it represents an attempt to obtain evidence of photosynthesis in the absence of chlorophyll. The conditions that presented the opportunity to study this process in the absence of chlorophyll can be seen in the results of the third experiment. When it was realized that the sprouts inoculated six days before light introduction did not yield any detectable chlorophyll until some time following light introduction, this seemed to offer an interesting possibility. At the beginning of this period there was a higher than normal concentration of carotene due to the influence of virus. This state of chlorophyll absence and high carotene concentration provided a convenient condition under which to study the possibility that carotene may function as an active photosynthetic pigment. By using Warburg apparatus and Barcroft's differential manometer method, any photosynthetic activity in the diseased leaf could be recognized. Earlier in the Introduction a statement was given of the reasoning behind this experiment.

IV. TECHNIQUES.

1. Inoculation Technique

In each of the five experiments an inoculation technique has been used here similar to that used by Rawlins and Tompkins (38). The use of their carborundum powder to pierce the cuticle and epidermal cell wall is standard, as is phosphate buffer to maintain the virility of the virus inoculum. The inoculum was obtained by crushing diseased tissue in a mortar and expressing the sap through a fine cheese cloth.

The one difference taken from the commoner finger rub method was the use of a camel's hair brush charged with carborundum to produce the abrasion. Manual rubbing was considered possibly too drastic in view of the danger of inoculation damage. The gentler technique using a camel's hair brush reduced the visible wounding. The possibility that any damage in inoculation might cause effects indistinguishable from effects due to virus was tested in Experiment III. An inoculation with water was done beside a normal control. By comparing the water inoculated with the healthy control a satisfactory measure of the extent of any effects due to damage was found.

2. Techniques of Pigment Assays

Original Technique. The first pigment determinations were always made of young sprouts not over 13 cm. in height. Each sprout was cut at a level one third the distance from its tip toward the base of the plant. The leaf buds and stem portions constituted the same for pigment analysis.

The plants were watered the night before sampling to ensure uniform turgidity when the samples were taken. Collections of samples were made in the morning. For any one sample, five shoots were selected, cut as described above, and 10 grams of this weighed out immediately.

The extraction and separation technique used on the above samples was similar in most respects to that of Seybold and Egle (28). However, Willstatter's original method and the modifications of Schertz formed the background in the author's evaluation of an adequate technique for his purpose. Willstatter had developed an elaborate and time-consuming method for separating plant pigments between solvent phases. This method depended entirely on the physical properties of solvent and soluable pigment. Schertz, instead of attempting to separate chlorophyll from carotene and xanthophyll by solvent partition, saponified the chlorophyll with dilute sodium hydroxide. This left two fractions, carotene and xanthophyll, to be separated on an adsorption column. The difficulty here is that one can never completely saponify all the chlorophyll in solution, since a small but significant percentage of the total still remains unchanged. Further, since it was necessary in the present work to estimate chlorophyll concentration as well as carotene and xanthophyll, chlorophyll had to be maintained in its original form.

Seybold and Egle (29) have worked on separation procedures and arrived at a satisfactory method for chlorophyll, carotene and xanthophyll. They recommend the use of petroleum ether and 95% methanol in a 9.1 mixture for

pigment extraction. The total extract is allowed to separate in a separatory funnel into two phases. The upper phase (petroleum ether) contains chlorophyll and carotene. The lower phase (95% methanol) contains xanthophyll and traces of carotene and chlorophyll. The lower 95% methanol is diluted to 85% with water. At this point traces of chlorophyll and carotene are transferred from the methanol phase to the petroleum ether. Carotene and chlorophyll are not soluable in methanol dilutions less than 88%. Precautions are now taken to ensure that the methanol phase contains only xanthophyll and the petroleum ether only chlorophyll and carotene. First the two phases are separated. The ether phase is washed with 85% methanol to ensure the removal of xanthophyll. The methanol phase is washed repeatedly with petroleum ether to remove any remaining carotene or chlorophyll. When the washings cease to show any traces of pigment, those washings which had contained color were combined with the parent phase. Seybold and Egle reduce the petroleum ether extract in vacuo and run this through a sugar column. The chlorophyll absorbs strongly to finely powdered sugar while the carotene passes through into the percolate.

It is only in the use of solvents to develop the chromatogram and to wash the absorbing chlorophyll from the sugar that the author's procedure differs from Seybold and Egle's. The author found that ethyl ether moved the front of carotene through the column into the percolate more rapidly and more completely than petroleum ether. The absorbed chlorophyll was best removed from the sugar absorbent with a 1:1 mixture of ethyl ether and acetone. Seybold and Egle used

petroleum ether in place of the author's use of ethyl ether and ethyl ether-acetone mixture. Other than this the two procedures were similar in theory. In practice smaller details such as type of glassware, mixture of sugar absorbent and drying column differed. The author varied such details as they appeared to increase the efficiency and convenience of the procedure. Since the concentration of each of these pigment extracts was eventually determined colormetrically, the determinations had to be based on a measure of weight or area. McKinney (22) found that wet weight coincided closely with area. For the same reason the author accepted wet weight as the basis for comparison.

Author's application of Original Technique. Details of pigment assay used in the present work are given as follows. Samples for extraction were immersed in hot (70-75°) water for three minutes, and ground in a mortar containing sand and 15 ml. of 95% methanol. Heating and treatment with methyl alcohol prevents decolorization through induced enzymatic oxidation.

The resulting mash was extracted with 140 ml. petroleum ether and 70 ml. 95% methanol. The mixture was kept in the refrigerator for 10 hours. Frequent shaking during this period kept the mash suspended in the solvents. This mash was then filtered through a Buchner funnel into a 50 ml. suction flask. The filtrate was transferred to a separatory funnel and enough water added to dilute the methanol to 85%. On dilution any chlorophyll in the alcohol layer was transferred to the petroleum ether fraction. The methanol layer, contain-

ing xanthophyll, cleared after setting, was drained into a second separatory funnel and extracted with four 20 ml. portions of petroleum ether to remove any traces of chlorophyll. These washes were combined with the parent extract, the whole of which was now washed with 85% methanol to remove xanthophyll traces, and with water to carry away any remaining methanol. These two solvents (petroleum ether and methanol) containing the pigment (chlorophyll and carotene in petroleum ether, and xanthophyll in methanol) were independently reduced in volume for the procedure to follow.

The methanol solvent with xanthophyll was reduced to 50 cc. in partial vacuum. It was found that concentrating the methanol fraction beyond 50 ml. caused pigments and various colored resins to separate out of solution. The resulting turbidity made it undesirable to concentrate the methanol fraction below 50 cc. since the colormetric reading of concentration would represent the total light absorption due to turbidity as well as pigment.

The resulting porridge was extracted with 140 ml. petroleum ether and 70 ml. 95% methanol. This was allowed to stand with frequent shaking for 10 hours in a refrigerator ate 10°C. This brew was then filtered through a Buchner funnel into a 500 ml. suction flask. The filtrate was transferred to a separatory funnel and enough water added to dilute the methanol to 85%. On dilution any chlorophyll in the alcohol layer is transferred to the petroleum ether fraction. The methanol layer containing xanthophyll cleared after settling was drained in a second separatory funnel and extracted with four 20 ml. portions of petroleum ether to remove any traces

of chlorophyll. These washes were combined with the parent extract, the whole of which was now washed with 85% methanol.

For the purpose of clarity the two techniques employed in partition chromotography are discussed together in this paragraph. Since carotene and chlorophyll were both taken up in the ether fraction, a separation was necessary before their concentration could be read independently.

For this reason the ether fraction was reduced to 5-10 ml. in experiments 1 and 2, and 2 ml. in experiment 3. The 5-10 ml. concentration in experiments 1 and 2 was found to give the most complete absorption of chlorophyll in a recognizable ring at the top of the absorption column. The use of filter paper, instead of an absorption column, in experiment 3 necessitated the reduction of the petroleum ether fraction to 2 ml. or less. To get clear separation by this last method the spot of solvent mixture on filter paper must be kept close to 1 cm. in diameter. Furthermore a 1 cm. spot would take no more than 2 ml. of solvent mixture before becoming saturated.

The two paragraphs to follow treat the partition techniques used separately since the details of procedure are quite different in each. The sugar adsorbent for the adsorption column in experiments 1 and 2 was prepared by mixing granular and powdered sugar in a 2:3 proportion by volume. The granular sugar increased the rate of flow to a speed sufficient to have the chromatogram completed in two hours. The adsorbent was packed evenly with a rubber plunger into a 2 cm. column 10 cm. long. This column was then set into a suction flask with a rubber

stopper. A slight negative pressure was produced at the bottom of the column by hooking up the suction flask to the water aspirator with rubber tubing. The column was first prepared by washing with petroleum ether to remove impurities before the reduced petroleum ether extract (5-10 ml.) was pipetted onto the adsorbent. When this had adsorbed, the chromatogram was developed with ethyl ether, carotene being carried into the percolate while the chlorophylls separated into a and b zones on the column. The percolate, containing the carotene was reduced almost to dryness; the residue was dissolved in ethyl ether and the concentration determined colorimetrically. The column, on which the chlorophyll was absorbed, was sucked dry and the chlorophyll bands removed with a spatula. The chlorophyll was eluted from the adsorbent with a 1:1 ethyl ether acetone mixture, and transferred to the ether by addition of water to the acetone layer. The ether extract was made up to a 50 ml. volume. A 10 ml. portion taken from this was pipetted into the colorimeter's curvette for reading.

In preparation for paper partition chromatography, potato sprouts were sampled and extracted with methanol and petroleum ether as described earlier. On standing, the extract separated into methanol and petroleum ether fractions. The methanol was diluted to 85% with water. Since chlorophyll is insoluable in 88% methanol, any traces were then transferred to the petroleum ether fraction. As stated earlier, the fractions were independently reduced in volume. A 10 ml. portion of the 50 ml. methanol extract was pipetted into the colorimeter's curvette for reading. The 2 ml. extract of petroleum ether, which contained any chlorophyll and

carotene was now spotted on the center of a sheet of Waterman No. 1 filter paper, dried and chromatographed using the following apparatus.

The sheet of filter paper on which the petroleum ether extract had been spotted was placed between two glass plates. The upper plate had been pierced in the center with a hole 7 mm. in diameter. The paper was adjusted so that the spot of extract lay directly under this opening in the upper plate. The 7 mm. opening was just large enough to receive the nozzle of a 5 ml. pipette through which the developing solvent (carbon disulfiede) was run onto the paper.

The solvent spread slowly outward in an increasing circle from the center to a large circle 20 cm. in diameter. The circumference of the spot, or solvent front, carried with it the carotene, while the chlorophyll remained behind in the center. By this method it was possible to separate small quantities of pigment quickly and accurately. The chromatogram completed its run in a half hour. The carotene ring was then cut from the outer circle and any chlorophyll present cut away from the center. Each of these paper separations was independently washed of its pigment with 10 ml. of ethyl ether. Each sample was then pippetted into the colorimeter's curvette for reading.

The Colorimeter. The separated pigment fractions were measured quantatively in the photoelectric colorimeter, a Bausch and Lomb Spectronic 20 model. The instrument operated under the following principle. Light emitted from a tungsten lamp was focused on a crystal diffraction grating. Light striking this crystal emerged

from it at an angle proportional to its wave length. A small slit opening was aligned with the crystal in such a position that a narrow band of light (20 mm.) emitted from the crystal could pass through. A photomultiplier tube set up in line with the slit opening responded to the intensity of the light band. This tube released a positive potential to a sensitive indicating instrument, which in turn registered the impression on a dial scale. This scale is marked both as optical density and % transmittance.

In practice, placing any absorbing substance between the photomultiplier tube and the slit opening reduced the intensity of light striking the tube by an amount corresponding to the number of absorbing particles in solution. When a 10 ml. sample of pure solvent (ethyl ether or 85% methanol) was placed in the path of the light beam, the intensity of light passing through the solvent was adjusted at the tungsten lamp so that the dial scale read 100% transmittance of 0 optical density. This reference adjustment was always necessary before placing a sample of the extract containing pigment of unknown concentration in the colorimeter. Replacing the pure solvent with a 10 ml. sample of the extract changed the scale reading from 0 optical density to some positive number proportional to the concentration of absorbing particles at that wave length. This reading was recorded for reference to the standard plot (described later) from which the proportional concentration (mg/ml) was taken.

The manner in which light of a specified wave length is selected has been discussed earlier. By rotating the

diffraction crystal any wave length within the limits of 350-650 mm. could be obtained. However, due to the limitation of slit opening the incident light band included light quants over a range of 20 mm. wave lengths. Since the pigments in any extract consisted of more than one component, each of which absorbed at a similar, but not identical, wave length. This last mentioned limitation of the equipment was an advantage in the present experiment since the wave length at which the absorption of the pigment was measured was broad enough to include all of a pigment's components. Xanthophyll, for example, has three major components, lutein, vialoxanthim, fluoxanthim with absorbtiontion maxima at 442, 446 and 450 mm. respectively. An average wave length at 445 mm. was then selected, which ensured that any particles absorbing in the range of 435 to 455 mm. would affect the reading of optical density in proportion to their number. In the same manner average wave lengths for carotene at 435 mm. and chlorophyll at 430 mm. were chosen. Each time a reading was taken for an extract containing one of those pigments, its average wave length was selected by rotating the diffraction crystal.

Standard Plot. Since the data recorded from the colorimeter are only functions of the concentration, the relationship between the reading and the concentration was first established in the following manner. A 10 mg. sample of chlorophyll was dissolved in 10 ml. of ethyl ether. This solution was called standard concentrations of 0.1 and 0.01 standard were also made. Readings of the optical density of these three solutions of known concentration were taken

from the colorimeter. On ordinary graph paper the optical density was plotted for each concentration. A straight line plot was obtained indicating a linear relationship between optical density and concentration. The same procedure for making standard concentrations was followed using a purified carotene sample. Readings of optical density for each concentration were recorded, and the data plotted in the same manner as described for chlorophyll. A straight line plot was again obtained, indicating the linear relationship true of solutions which follow Beer's Law.

These standard plots (Fig. III, V) were used in the following manner. When an extract contained a pigment of unknown concentration, a 10 ml. sample was pipetted into a curvette, and a reading of its optical density taken from the colorimeter. Referring this reading to the standard plot, every unit of optical density on the vertical axis has a corresponding concentration on the horizontal axis; thus, the corresponding concentration for that optical density is read directly from the plot. A separate plot was not constructed for xanthophyll. The use of carotene as a standard for xanthophyll was recommended by Snell and Snell. Since xanthophyll (hydroxy-carotene) is structurally similar to carotene, it was expected that a molecule os xanthophyll would absorb (for all practical purposes) the same amount of light as a molecule of carotene. The fact that the 20 mm. band width of the incident light spanned the absorption

Standard Dilution Curve

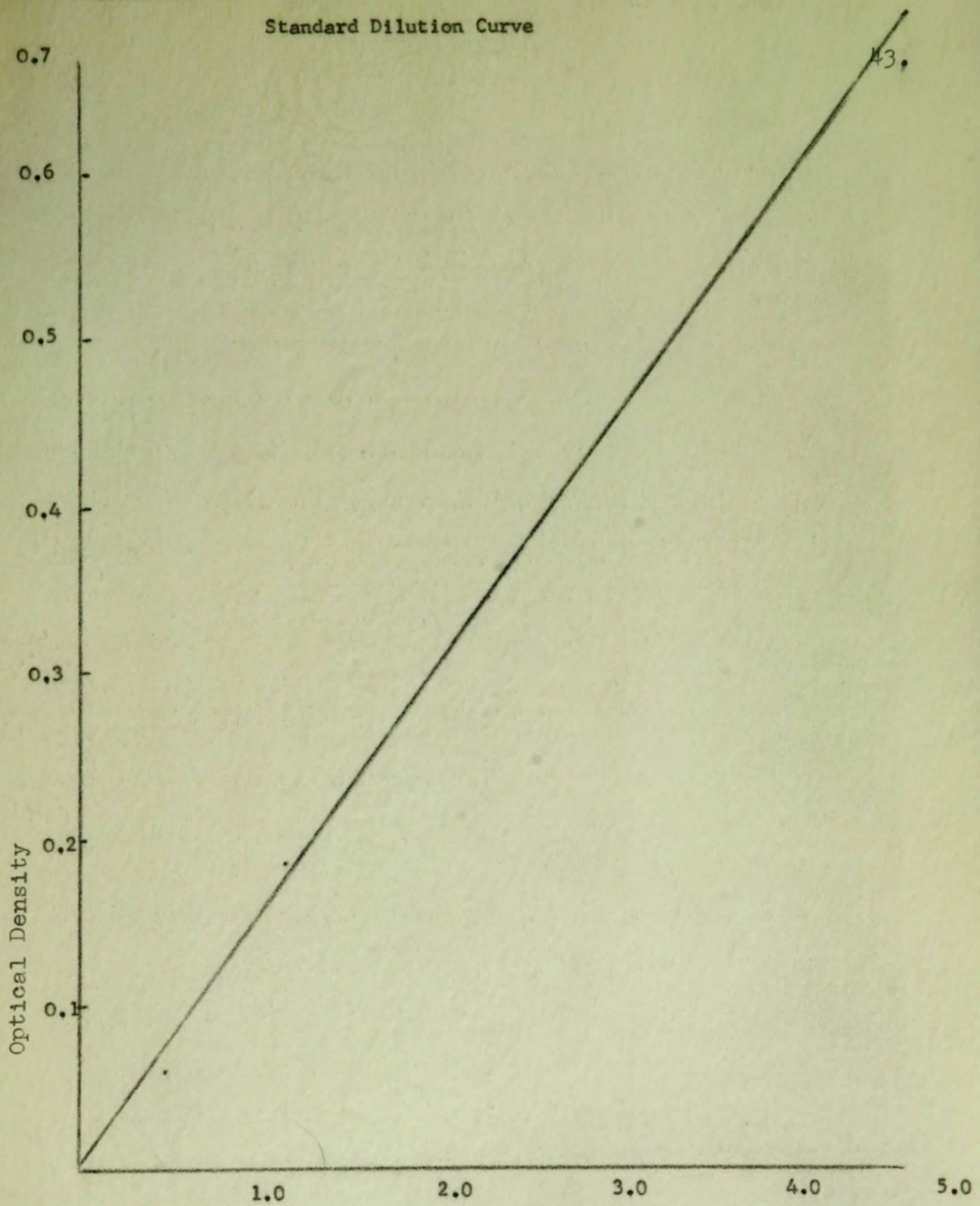


Figure III

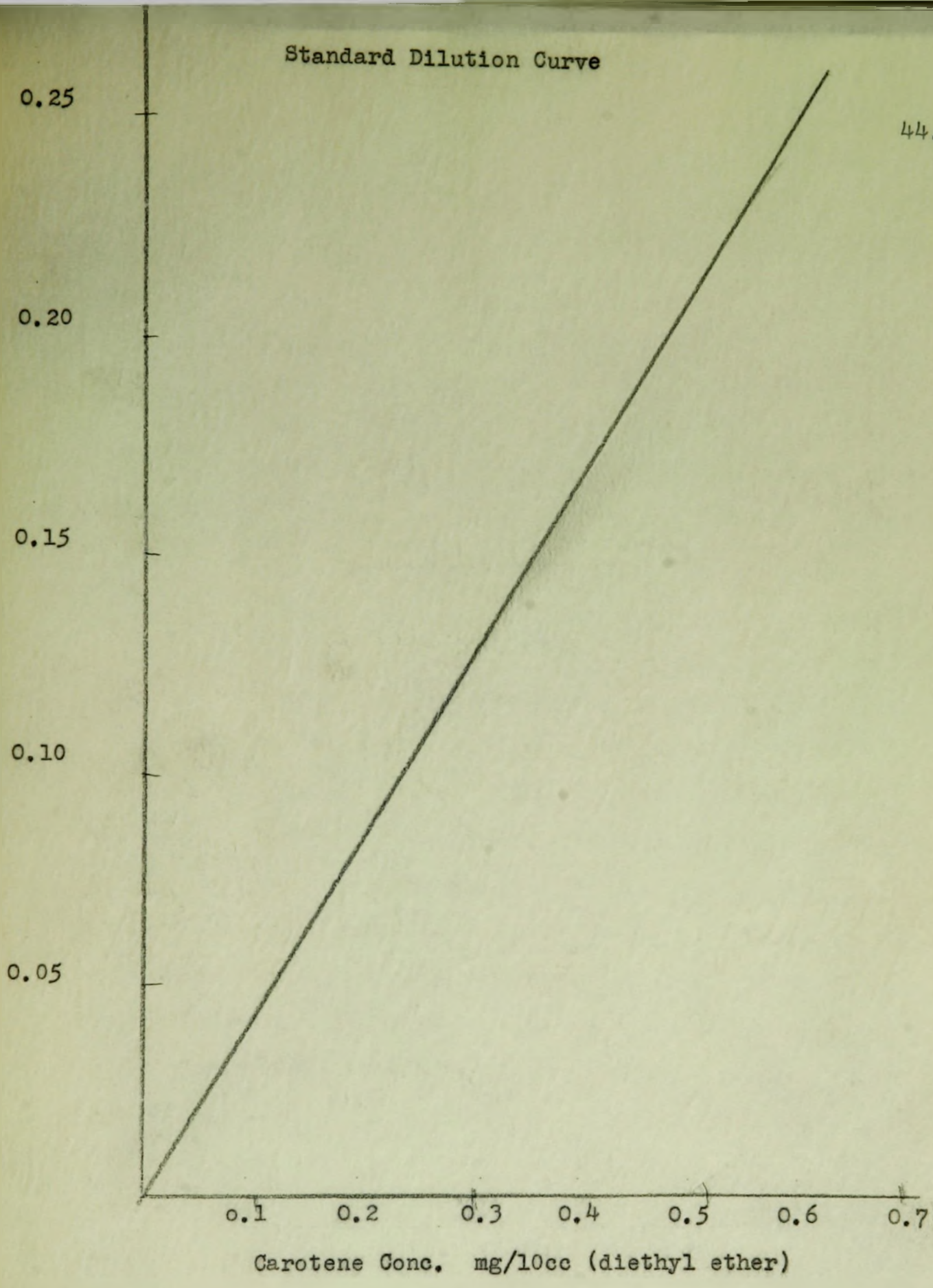

Standard Dilution Curve
0.25
0.20
0.15
0.10
0.05
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Carotene Conc. mg/10cc (diethyl ether)

Figure IV

maxima of both carotene and xanthophyll favored the acceptance of carotene's standard plot for use in determining concentrations of xanthophyll as well.

The use of these quantitative techniques discussed above produce emperical data from which conclusions can be drawn regarding the possibility of photosynthesis. The manometric technique discussed in the following section is related to the above in so far as it also studies some part of photosynthesis, but from an experimental point of view.

3. Manometric Techniques Used in Studies of Photosynthesis

In view of the complexity and number of variations of this technique, no claim is made to any completeness of familiarity with it or the processes which it studies. It has been noted that the use of this technique was considered after the results of the present work indicated that the development of a single point (the function of carotenoids in the absence of chlorophyll) might be worth while. It is therefore only with limited assurance that this study, using this technique, was undertaken. The major argument in this thesis will rest with the results of the quantitative pigment studies.

As noted in the Introduction, page 17, this work attempts to gather information from gas exchange experiments which may indicate the presence or absence of photosynthesis activity under the following conditions. First, virus will

have prevented the development of chlorophyll in potato sprouts up to a period of 6 hours following illumination. Second, virus will have caused the accumulation of carotenoid in the same plant previous to illumination. The investigation of this photosynthetic activity is complicated by the fact that photosynthesis and respiration cannot be measured separately during a period of illumination. Warburg apparatus can only record gas exchange between cells and their environment. The difficulty lies in determining how much of this gas exchange is due to photosynthesis, and how much to respiration. In effect the observed rate of photosynthesis is less than the true rate because of the evolution of carbon dioxide by respiration. For this reason it is necessary to measure photosynthesis by a differential method involving three separate determinations. These are made in the following order: (1) the determination of respiration during a dark period; (2) a determination of gas exchange during the period the cells are illuminated; (3) a determination of respiration following the light period. In other words, respiration is measured immediately before and after the illumination period, and the average values of these two added to the observed rate of photosynthesis to give the true rate (37). These three determinations are represented later in a formula by the symbols h_{r1}, $h_{p\&r}$, h_{r2}. Symbol h_{r1} indicated the change in height of fluid in the manometer before the illumination period, $h_{p\&r}$ during illumination, and h_{r2} after illumination.

The procedure in this experiment follows this order. First, the leaves from the diseased plant (inoculated six days previously) were cut away at the petiole. The clean wound was dipped in lanolin to reduce effects due to wound respiration. These leaves were collected in a match box, weighted indirectly, and transferred in darkness to a 21 ml. reaction flask. The flasks were now joined directly to the manometer through a ground glass joint. Since active leaf tissue within the flask resulted in gas exchange, gas pressure changed as the rate of uptake exceeded liberation or vice versa. This changing gas pressure caused the level of fluid in the manometer to rise or fall accordingly. The readings of the fluid levels are the "h" values referred to in the following formula given by Bancroft (37). The symbol "hphoto" is the change in fluid height due to photosynthesis. The other symbols (h_{r1}, h_{r2}, h_{par}) have been described in the preceeding paragraph. Each reading is expressed as change (+ or -) per minute. Substitution of readings for "h" values in the formula permits a solution for hphoto, the change in height of fluid of the manometer due to photosynthesis.

$$\text{hphoto} = \left[-\frac{1}{2}\left(\frac{h_p}{\min r_1} \; \frac{h_{r2}}{\min r_2}\right) - \frac{h_{p\,r}}{\min_{p\,r}} \right]$$

Manometric apparatus recording gas exchange has two variables, internal temperature and external barometric pressure. The first of these is maintained constant at $27 \pm 0.5^{\circ}C$ with thermostatically controlled water bath. Thus it does not

affect the calculation. But barometric pressure cannot be controlled as easily. Experimental "h" readings include the effects of external barometric as well as internal gas pressure. It is therefore necessary to record the effects of barometric pressure on the system at 27°C from a separate manometer. In practice a manometer was hooked up to a flask identical with that used in the experiment. This control manometer was placed beside its identical piece of apparatus containing active leaf tissue. Any change in the height of fluid in the control manometer was recorded and subtracted from the reading made from the reaction manometer. For example, if the manometer attached to the reaction flask registered a change of 22 mm. and the barometric control 2 mm. in the same direction, then the real change in internal pressure would be found by 22 mm. - 2 mm., giving 20 mm.

In the present work, three experiments were done, each over different time intervals. In the first readings were taken at 10 minute intervals; in the second, 30 minutes; in the third, 60 minutes. The purpose in doing three experiments is supported in the following reasoning. As noted earlier, "h" readings were expressed as mm. change per minute. In practice the observed change at the end of a time interval was divided by the number of minutes intervening. An "h" reading per minute therefore represents a mean change for that interval. The shorter the interval,

the smaller the number of individual changes. The supposition to this point has been that photosynthesis and respiration are independent variables acting singly or in combination in dark and light periods respectively. However, the availability of carbon dioxide and oxygen, carbon dioxide fixation, possible enzyme-substrate interference by virus, and many other factors may influence photosynthesis and respiration to a greater or lesser degree. Choosing a larger interval such as 30 minutes or 60 minutes therefore accounts for a larger number of individual changes (caused by a larger number of variables). If the mean value is more inclusive in longer intervals, it can be taken to represent only a qualitative and directional change in photosynthesis or (and) respiration. Differences in the calculated values for hphoto found in the three experiments may be significant, but there is no attempt here to indicate a direct connection between any single factor, such as those mentioned above, and a difference in calculated hphoto. It was hoped in designing the experiments that the longer intervals by contrast with the smaller might yield data open to further speculation and investigation. Finally, if an experiment had been done over a short interval only, there would have been some question whether similar results would have been obtained in longer intervals. If there are more variables possible in a longer interval than a shorter, then results indicate a photosynthesis rate in both are more reliable

than a single one. It is reasonable to conclude that the rate is positive in spite of, rather than due to, any single variable not under investigation.

V. RESULTS

1. Pigment Assays in Dark and Light-Grown Plants

Experiment 1. The experimental results given in the following section will follow in the order of the experiments' description in the preceeding section. The purpose of Experiment 1 as stated was to determine whether chlorophyll and carotene concentrations were affected similarly or dissimilarly by virus inoculation. In preparation for these determinations of pigment concentrations, the plants were grown in darkness, and selected for uniform size as described in Experimental conditions, page 33 . These were inoculated with cultured potato x virus and immediately exposed to light in the constant temperature room. Following the collection of 5 gm. samples of plant material at the intervals stated below, each sample was separately extracted. The pigments were separated as described in the Techniques of Pigment Assay. Their concentration was separately determined in the Bausch and Lomb photoelectric colorimeter.

An earlier discussion (14) stressed the importance of studying alterations in normal pigment development due to virus. In order to investigate what is happening in the virus-pigment relationship, it was reasoned that pigment concentrations had to be studied in a series of

events following inoculation. Since carotene concentratiion in diseased plants changed rapidly following inoculation, determinations were made at short intervals. The first and second days following inoculation determinations for carotene concentration were made at three, four hour intervals each day. Since chlorophyll concentration developed more slowly in diseased plants, one determination per day over a six day period for chlorophyll yielded sufficient information to follow chlorophyll development in inoculated plants. The data showing the results of these determinations for carotene and chlorophyll are found in Tables 1 and 11 respectively. These two Tables illustrate the results of Experiment 1. They are found on pages 55, 56.

Table 1 demonstrates the affect of virus on carotene development in inoculated plants growing in the light. By comparing data on carotene concentration in the healthy control with pigment concentration in the inoculated samples, one may draw three conclusions. First, both healthy and inoculated show an increase in carotene concentration over a two day period. Second, there is a significantly more rapid increase in carotene concentration in the inoculated as compared with the healthy control. What is meant here by a "significant" change will be taken up later in the Discussion, which follows the results. Third, an approximate four fold increase of 8.60 mg. of carotene in the inoculated over 1.89 mg. in the healthy

strongly suggests the influence of some introduced variable, namely virus.

Table 11 demonstrates the affect of virus on chlorophyll development in inoculated plants growing in the light. In the same way, by comparing data on chlorophyll concentration in the healthy control with pigment concentration in the inoculated samples, one may draw three conclusions. First, both inoculated and healthy control show an increase in concentration over a six day period. Second, the inoculated plants appear to show a significantly lower concentration of chlorophyll than the healthy control. Third, the final determinations made on the sixth day found a two fold reduction of 21.0 mg. of chlorophyll in the inoculated compared with 54.0 mg. in the healthy plants. Again this suggested the influence of some introduced variable, namely virus.

From the data given in the two preceeding paragraphs one can make the following comparisons between carotene and chlorophyll development in inoculated plants. Carotene concentration in inoculated plants increases at a rate greater than that in the healthy plant. By contrast, chlorophyll concentration in inoculated plants increases at a rate less than that in the healthy plant. By way of comparison, Elmer and McKinney expressed their results as a state of pigment concentration at a given time. It was understood

that this state of pigment concentration remained continuous throughout the life of the diseased plant. However, the present work has taken this concept in question. In collecting a series of data on concentration, and plotting this series against time, the development of pigments can be traced. This experiment has attempted to find some of the general characteristics of pigment development in diseased plants. Perhaps the most important characteristic the evidence suggested was that carotene and chlorophyll were not similarly affected by virus. Rather carotene concentration increased and chlorophyoll decreased in inoculated plants compared with healthy.

This characteristic was the issue on which Elmer and McKinney differed. Since Elmer believed that pigments were not similarly affected by virus, the evidence to this point supported Elmer.

Table I. Carotene Concentration in Light-Grown Plants

Time in hours from inoculation	Carotene Concentration	
	Inoculated	Healthy
0 hour	0.03 mg.	0.05 mg.
Δ 4	2.05 mg.	...
Δ 8	2.45	0.12 mg.
	Overnight	
Δ 18	3.24 mg.	...
Δ 24	5.40 mg.	...
Δ 28	8.60 mg.	1.89 mg.

Plants in this experiment were inoculated at illumination. The symbol "Δ" represents time elapsed between inoculation and sampling. Concentrations are expressed as mg. of carotene per 5 gm. wet weight of tissue.

Note the increase in carotene concentration in the inoculated plants compared with the healthy.

Table II. Chlorophyll Concentration in Light-Grown Plants

Time in hours from inoculation	Chlorophyll Concentration	
	Inoculated	Healthy
0 hour	0.0 mg.	0.0 mg.
Δ 24	1.4 mg.	3.3 mg.
Δ 48	3.8 mg.	5.5 mg.
Δ 72	5.7 mg.	11.2 mg.
Δ 96	8.8 mg.	40.3 mg.
Δ 120	24.0 mg.	54.0 mg.

In this experiment plants were inoculated at illumination. The symbol "Δ" represents time elapsed between inoculation and sampling. Concentrations are expressed as mg. of Chlorophyll per 5 gm. wet weight of tissue.

Note the decrease in chlorophyll concentration in the inoculated plants compared with healthy.

Experiment 11. The second experiment was designed partly to repeat the results of the first. The purpose of Experiment 11 was to produce evidence which might further clarify the relationships found in Experiment 1 between virus and plant pigments. In preparation for this experiment, a flat of 150 plants were divided arbitrarily into three sets of 50. Each of these three sets was then treated separately. Set 1 received an inoculation three days after illumination, and Set 11 three days later when all plants were illuminated.

There are two important differences between the two inoculated sets above. First, Set 1 was inoculated three days earlier than Set 11. Second, Set 1 was inoculated in darkness while Set 11 was inoculated three days later in the light. What affect light has on the development of pigment is uncertain. This point is left for the Discussion later. The present experiment is most concerned with the effect on pigment concentration of exposing plants to virus for a relatively longer period of time than Set 11, for example. By comparing Set 1 (earliest inoculated) with Set 11 (inoculated later) four conclusions were drawn regarding the relationship between time of inoculation and pigment concentration.

First, at the beginning of day 1 of illumination (0 hour in Table 111, xanthophyll and carotene in Set 1 were found in higher concentrations than in either Sets 11 or 111. Sets 11 and 111 showed only traces (0.14 and 0.12 mg.) of carotene and xanthophyll. Since up to this

point all three sets had been grown in the same dark conditions, the difference in concentrations between pigments in Set 1, and the other two sets was assumed to be caused by the earlier inoculation of Set. 1.

A second conclusion arising from the results in Table 111 concerns chlorophyll concentration. At the beginning of day 1 chlorophyll shows no previous accumulation (0.0 concentration). However, 72 hours later the concentration in Set 1 has increased to 36.0 mg. This compares with 60.2 mg. in Set 11 which had been inoculated later. The healthy Set 111 yielded a still higher concentration of chlorophyll, 98.7 mg. One could then observe that the degree of effect of virus on chlorophyll concentration is related to the length of time theplant has been exposed to the virus. One might also observe that by 148 hours following illumination, chlorophyll concentration in Set 1 was still relatively lower than Set 11, and Set 11 correspondingly lower than the healthy Set 111.

As pointed out earlier, Set 1 contained a higher concentration of both carotene and xanthophyll than Sets 11 and 111 at 0 hour. Data collected at intervals of 72 and 148 hours later again indicated a higher content of these pigments in Set 1 than in either of the others. In addition, a progressive increase in concentration was observed when one compared the concentrations of each of

the pigments from Set 111 to Set 11 to Set 1. In Set 111, carotene concentration at 148 hours amounted to 1.82 mg. in Set 11 to 5.70 mg. and in Set 1 to 6.35 mg. Thus one is led to the fourth conclusion that pigment accumulation in Set 1 compared with Set 11, and Set 11 compared with Set 111, is related to the length of time the plant has been exposed to virus.

The carotenoid pigments (carotene and xanthophyll) are affected as well as chlorophyll. As with chlorophyll, the carotenoids showed the greatest change in concentration in the set inoculated earliest (Set1). It can be said that during the early stages of pigment development, the pigments are sensitive to the influence of virus. Furthermore, the earlier the inoculation the greater the change in pigment concentration from normal.

It was understood at the beginning of Experiment 11 that its main purpose was to repeat Experiment 1, but with some changes in the design of the experimental conditions. The results of Experiment 11 led one of the same conclusions arrived at in Experiment 1, namely that the carotenoids and chlorophyll are not affected similarly by virus. In addition Experiment 11 produced a new piece of evidence indicating a relationship between tiie of inoculation and pigment development.

Growth Rate. While using the three sets in Experiment 11, it was thought useful to collect some information

on the rate of growth in each set. Since growth rate is the result of a large number of metabolic processes, a measure of growth rate might provide a general indication of metabolic conditions in the diseased plants. It was reasoned that this data could be related to pigment concentration, and therefore should be collected for comparison.

Previous to taking measurements of increase in weight, height, leaf length, each plant in all three sets was labelled according to height. As explained earlier (page 13), only those plants distributed within the height range of 8-13 cm. were kept for an experiment. Any plants not within this range were harvested, and thrown out. At this point, any plant growing in the flat was labelled as belonging to a group of plants 8 cm. high, or 9, or 10, etc. These were approximate groupings within 0.5 cm. Each height then represented a group of plants. Measurements of growth increase were listed as an average increase in that plant group over a period of time.

Table IV shows the record of increases in weight in each of the three sets over a six day period. All plants at the beginning of the six days belonged to one of the group of plants between 8 and 13 cm. These plants were labelled according to groups just previous to illumination. Although Set 1 had been inoculated three days earlier,

and Set 11 was inoculated at illumination, the 0 hour of Experiment 111 was considered to have begun with illumination. Results in the table indicate an increase in weight for each set over the six day period. However, Set 1 has increased relatively more in weight than Set 11. Set 11 is also relatively heavier than Set 1 at the end of six days.

Table V shows a similar type of increase as TableIV. Plants in Set 1 have increased in height more rapidly than Set 11. The results in Table V do not show as sharp a contrast between the three sets as Table IV. This would seem to indicate that virus plays a more active role in increasing weight than height of a plant.

In the experiments preceeding, an increase in leaf length was noticed as a characteristic of diseased plants. Table VI contains data on leaf length in Experiment 111 which indicates that the earlier observation was correct. Leaves of plants in Set 1 were relatively longer than leaves in either Set 11 or 111. But the inoculated Sets 1 and 11 show a marked increase in leaf size over the 6 days compared with the healthy Set 111.

Generally speaking, these results appear to place the results on pigment concentration in Experiment 111 on firmer ground. This work indicates that the length of period the plant is exposed to virus is related to an increase in growth rate in diseased plants relative to

normal. Pigment concentrations in Experiment 111 also indicated a similar relationship between the time of inoculation and pigment concentration in diseased plants.

Table III. Pigment Concentration in Light-Grown Plants

Time in hours following illumination	Pigment Concentrations in mg.			Pigments
	Set I	Set II	Set III	
0 hour	0.0	0.0	0.0	Chlorophyll
	0.87	0.14	0.12	Carotene
	1.56	0.68	0.69	Xanthophyll
72 hr.	86.0	60.2	98.7	Chlorophyll
	6.40	5.90	1.89	Carotene
	4.40	2.38	1.62	Xanthophyll
184 hr.	52.4	68.9	108.0	Chlorophyll
	6.35	5.70	1.82	Carotene
	8.80	8.40	4.73	Xanthophyll

Table III.

Plants in this experiment were inoculated at two different times. Set II was inoculated at illumination, and Set I three days before in the dark period. The 0 hour of the experimental was the beginning of illumination.

Concentrations are expressed as mg. of pigment per 10 gm. wet weight of tissue. Note the increase in concentration of the carotenoids in inoculated Sets I and II, and decrease in chlorophyll in Sets I and II relative to Sets III, the healthy plants.

Table IV . Increase in Weight over 6 Day Period

Height in cm of each Plant Group at beginning of Experiment II	Ave. Weight in gm. of plants in each group at beginning of experiment	Ave. Wt. in gm. 6 days later	Ave. Wt. in gm 6 days later	Ave. Wt. in Gm 6 days later
		Set I	Set II	Set III
8	o.9	5.3	3.7	1,9
9	1.0	5.9	5.8	1.7
10	1.2	6.6	5.8	2.8
11	1.8	6.5	6.4	4.2
12	2.4	9.5	8.2	5.2
13	3.3	10.6	11.6	6.3

A comparison is made between weight of plants in three sets. Set I has been inoculated three days before illumination; Set II has been inoculated immediately after illumination (point at which experiment began); and Set III was a healthy control.

Notice that each set has increased in weight over the six day period. Set I has increased relatively more than Set II, and Set I[1] more than Set III.

Table V Increase in Height Over 6 Day Period.

Height in cm of each plant group at beginning of Experiment II	Ave Height in cm. of plants in each group at beginning of Experiment	Average increase in cm. 6 da later	Average Increase in cm. 6 da later	Average increase in cm. 6 da. later
		Set I	Set II	Set III
8	8	22	17	14
9	9	20	20	12
10	10	17	15	13
11	11	14	10	10
12	12	15	11	8
13	13	15	14	10

A comparison is made between height increase of plants in three sets. Set I has been inoculated three days before illumination. Set II inoculated immediately after illumination and Set III was a healthy control.

Although each set has increased in height, Set I has increased relatively more than the other two.

Table VI. Increase in leaf length over 6 day period.

Height in cm. of each plant group at beginning of Experiment II	Average increase in cm. 6 da. later	Average increase in cm. 6 da. later	Average increase in cm. 6 da. later
	Set I	Set II	Set III
8	3.0	2.5	1.0
9	4.0	3.0	2.5
10	4.0	2.5	1.5
11	3.5	3.0	1.5

A comparison is made between increases in leaf length of plants in three sets. Set I has been inoculated three days before illumination, Set II immediately after, and Set III was left a healthy control.

Although each set has increased in length, Set I has sown a relatively greater increase than Set II, and Set II was relatively longer than Set III.

Experiment III. By the time Experiment III had begun, the dissimilar effect of virus on chlorophyll and carotenoids was accepted. The investigation then turned to another aspect of the problem, namely, the affect of virus on pigments (carotene and xanthophyll) in dark-grown plants. Following the study of these pigments over a six day period in darkness, the plants were illuminated. A second period of study was begun which covered a period of 5 days after illumination. The results are found in Table VII at the end of this experiment.

As in Experiment II, a flat of 150 plants was divided into three equal parts, Sets 1, II, III. Set 1 was inoculated with virus, Set II with water, and Set III was left uninoculated as a control. More detailed reference to these three sets may be found on page 21 of Experimental Procedure. Both Sets 1 and II were inoculated 6 days before illumination. Pigment determinations were recorded at the intervals indicated in Table VII. The symbol "Δ" represents time elapsed between inoculation and sampling.

Results at Δ8 hours show an increase in carotene concentration in the inoculated Set 1 of 0.41 mg. compared with 0.17 mg. in the uninoculated Set III. Set II, which was water inoculated, yielded 0.11 mg. of carotene. This evidence suggests that water inoculation did not have any effect on carotene similar to virus inoculation in Set 1.

Carotene accumulation at Δ120 hours in Set 1 had levelled off at 0.96 mg. This again shows an increase over

the uninoculated Set 111 which had not changed significantly from the concentration shown at 8 hours. The water inoculated Set 11 again did not show any evidence of difference in carotene content from Set 111.

Xanthophyll in determinations as recorded in the table follows the same development as carotene in all three sets. These two pigments appear to be closely related to one another since a change in concentration of one is accompanied by a similar change in the other.

It may be noted that values are not recorded for any pigments at 0 hour in Table V11 since the concentration of carotenoids in Experiments 1, 11, 111 remained consistantly within the limits of 0.12-0.18 mg. in dark-grown plants (0 hour), it was not considered necessary to repeat the zero point in this table.

As noted earlier on page 29, Experiment 111 was not terminated at the end of the 6 day period in darkness. The plants on which the first part of the experiment had been done were illuminated, and the sequence of determinations continued into the light period. The objective in continuing these determinations was to link the earlier information in this experiment on pigments in dark-grown plants with data recorded for changes in pigment concentration in light-grown plants from Experiments 1 and 11.

During the light period, determinations were made for carotene, xanthophyll, and chlorophyll. The data and the intervals at which they were taken are recorded in Table V111.

Chlorophyll concentration at Δ^4 hours was 0. At Δ^8 hours there was still only a trace, 0.2 mg., of chlorophyll. At 32 hours the extract yielded 0.6 mg., an unusually low concentration for this length of time following illumination. A determination of chlorophyll concentration in the uninoculated Set 111 (not recorded in Table V111) yielded 3.8 mg. at Δ32 hours. It is interesting to compare the results of Table 11, page 56 with the data given for chlorophyll in Table V111. Table 11 shows a contrast between an uninoculated set and a set inoculated at illumination.

Earlier, a claim was made that the test plant chosen might produce some evidence on the affect of virus on chlorophyll's precursors. The second part of Experiment 111 may have justified that claim. The observation that chlorophyll does not develop immediately following inoculation suggests that some type of block prevented the formation of chlorophyll's immediate precursor, protochlorophyll.

The concentration of carotene and xanthophyll in these light grown plants has produced no information not already known from the preceeding four experiments. The results are similar to those recorded in Tables 111, 1V, V in which an increase in concentration of carotenoids in diseased relative to healthy plants was recorded.

Table VII. Pigment Concentration in Dark-Grown Plants.

Time in hours after inoculation	Virus inoculated Set I	Water inoculated Set II	Healthy control Set III	Pigments in dark-grown plants
Δ^{8}	0.41 mg. 0.38 mg.	0.11 mg. 0.25 mg.	0.17 mg. 0.29 mg.	Carotene Xanthophyll
Δ^{24}	0.74 mg. 0.86 mg.	0.18 mg. 0.30 mg.	0.15 mg. 0.28 mg.	Carotene Xanthophyll
Δ^{48}	0.85 mg. 0.80 mg.	0.25 mg. 0.23 mg.	0.18 mg. 0.31 mg.	Carotene Xanthophyll
Δ^{72}	1.30 mg. 0.82 mg.	0.39 mg. 0.28 mg.	... ...	Carotene Xanthophyll
Δ^{96}	1.32 mg. 0.98 mg.	0.30 mg. 0.31 mg.	0.23 mg. 0.34 mg.	Carotene Xanthophyll
Δ^{124}	1.26 mg. 0.96 mg.	0.28 mg. 0.34 mg.	0.21 mg. 0.32 mg.	Carotene Xanthophyll

Table VII.

The results are expressed as mg. of pigment per 11 gm. wet weight of tissue extracted.

These plants were sprouted, grown and inoculated in darkness. All pigment determinations made were on the dark-grown plants.

Set II shows no significant difference from Set III in pigment concentration over the 124 hour period. Concentration of pigments in Set I increases rapidly until 72 hours following inoculation.

The symbol Δ represents the time interval between time of inoculation (0 hour) and time determinations were made (e.g. Δ 8 hours).

Table VIII. Pigment Concentration in Light-Grown Plants

Time in hours following illumination	Concentration in mg.		
	Chlorophyll	Carotene	Xanthophyll
0 hour	...	...	...
Δ 4 hr.	0.0	1.07	0.70
Δ 8 hr.	0.2	1.10	1.44
Δ32 hr.	0.6	2.44	4.84
Δ56 hr.	3.0	4.72	8.30
Δ80 hr.	7.2	5.21	8.10

In this experiment plants were inoculated six days before illumination. Concentrations are expressed as mg. of pigment per 10 gm. wet weight of tissue.

Note the absence of chlorophyll until Δ 8 hours following illumination.

Increase in Carotene Concentration Within First Eight Hours After Inoculation

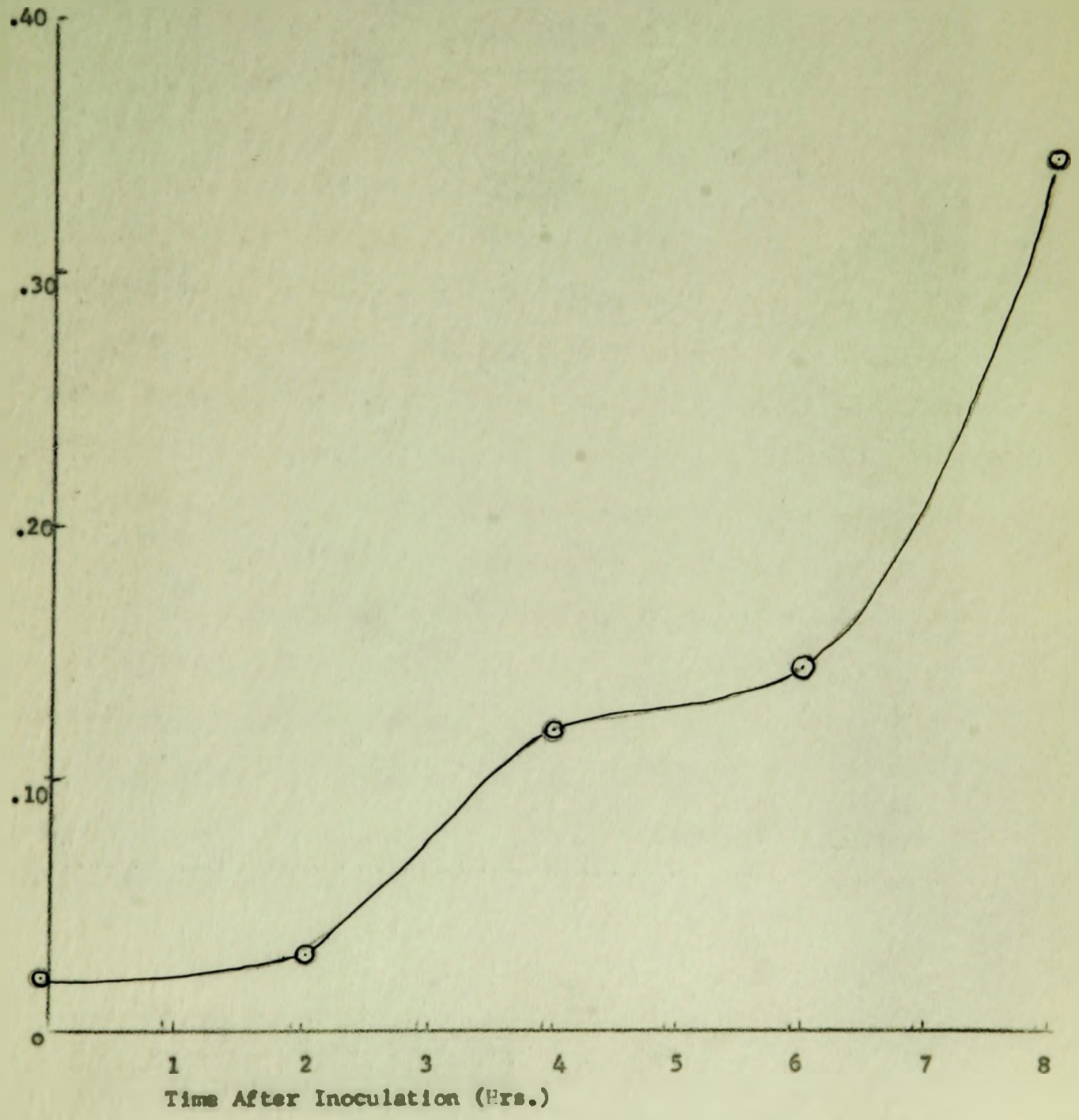


FIGURE V

Experiment 1V. As mentioned earlier, a preliminary experiment indicated a sharp rise in carotene concentration following inoculation. This suggested some immediate relationship between virus and pigment concentration. On this basis a fourth experiment was designed to follow the change in carotene concentration at 2 hour intervals after inoculation. This issue was discussed on page 15 of the Introduction.

Data from Table 1X is illustrated in Figure V. Figure V expresses the relationship between concentration of carotene accumulated from 0 hours to Δ 8 hours. There appears to be no significant increase in concentration between 0 hour and Δ 2 hours. However, between Δ 2 and Δ 4 hours a relatively large increase in concentration was recorded. Again between Δ 4 and Δ 6 hours there was no significant change in concentration. However, concentration recorded in the period Δ 6 and Δ 8 hours again shows a sharp and relatively significant increase in concentration. From this data one would conclude that the indication of greatest change in concentration can be found in two periods; (a) between Δ 2 and Δ 4 hours, (b) between Δ 6 and Δ 8 hours. These periods may indicate stages of greatest activity following inoculation.

Table IX. Carotene Concentration at Short Intervals following Inoculation in Dark-Grown Plants

Time in hours following inoculation	Concentration in mg. Carotene
0 hour	0.040
Δ 2	0.060
Δ 4	0.120
Δ 6	0.145
Δ 8	0.450

Plants in this experiment were grown and inoculated in the dark. Plants were kept in darkness for the duration of the experiment. Concentrations are expressed as mg. of carotene per 10 gm. wet weight of tissue.

Note the apparent rapid change in concentration in the intervals between Δ 2 - Δ 4 hours, and Δ 6 - Δ 8 hours.

Experiment V. Table VIII demonstrates that no chlorophyll could be found in plants before Δ 4 hours. Another determination at Δ6 hours (not recorded in Table VIII) yielded only traces of chlorophyll, less than 0.1 mg. As stated earlier in Experimental Procedure, this period provided an opportunity to study the efficiency of carotenoid pigments in photosynthesis. Although chlorophyll was absent for a short period, a relatively high concentration of carotenoids was present in diseased tissue.

The use of the differential manometer method (described under Manometric Techniques) to measure carbon dioxide uptake in photosynthesis produced the data recorded in Table X. Table XI represents the calculations of the photosynthetic rate found by substituting these data in the formula found on page 42. The three runs were each carried out over a different period length: Run 1, alternating periods of 10 minutes each in dark, light, and dark conditions; Run 2, periods of 60 minutes each; and Run 3, alternating periods of 30 minutes each. This fact may explain the variation in photo value recorded in Table XI. The largest value recorded is 0.10 mm. change in gas pressure due presumably to photosynthesis. The signifigance of this value will be considered in the Discussion.

Table X. Manometer Readings.

Conditions	Dark h r1	Light h r+p	Dark h r2	Run No.
Time intervals 10 minutes	- 1.30 mm	- 1.10 mm	- 1.40 mm	I
Time intervals 60 minutes	- 7.04 mm	- 2.40 mm	- 7.33 mm	II
Time intervals 30 minutes	- 2.62 mm	+ 0.33	- 2.75 mm	III

The "h" readings refer to change in height of fluid in manometer as a result of gas uptake or liberation in the active tissue. The time intervals state the length of alternating dark and light periods.

Table XI. Calculated Values for Photosynthetic Rate.

Run No.	hphoto
I	0.025 mm
II	0.080 mm
III	0.10 mm

VI. DISCUSSION

1. Relation of Results in Experiments I, II, III

The first three experiments were preoccupied with the issue on which Elmer and McKinney differed. For this reason they are basically united in a single purpose. Reference has been made in the Introduction and on several occasions later regarding the relation of the present work to this issue. The results of these experiments strongly support Elmer's conclusion. But it is only the conclusion that can be supported. Since the conditions under which they were working were unspecified, it was difficult to know exactly what their results implied. McKinney's evidence, for example, that all pigments in diseased tissue showed a decrease in concentration relative to healthy, did not necessarily imply that Elmer's contradictory evidence was incorrect. The reasoning to support this last statement will be clarified in the following discussion.

The choice in the present work of a plant that grew well in the dark, allowed the author to escape some of the uncertainty that qualified Elmer's and McKinney's wark. The virus was introduced into the plant either while growing in darkness or at the time of illumination. Since the potato contained only traces of carotenoids and no chlorophyll under dark-grown conditions, any introduced variable (e.g. virus) at this point produced a change from zero concentration. If the virus were introduced at illumination, then there would be two variables, light and virus. By making a mental subtraction of the concentration of pigment in a diseased plant from a healthy plant the difference was taken to be that change in pigment concentration due to virus effects.

This was the case in Experiment I as indicated in Tables I and II. In a set of plants inoculated at illumination, chlorophyll and carotene concentrations were traced from 0 concentration in both diseased and healthy tissue. If the concentration in the diseased plants were subtracted from the healthy, the result would be a + or - quantity depending on whether the healthy had shown an increase or decrease over the diseased. This operation was similar to the continuous comparison drawn in Results between pigment concentrations in inoculated relative to uninoculated plants.

A statement of Cook's evidence in the Introduction was made partly to justify the use of a young plant such as the potato in the present experiments. Cook noticed a difficulty in producing symptoms in a mature plant. In contrast to this he found that a young plant easily developed a mosaic symptom. He explained that he thought this was due to the effect of virus as an inhibitor of histogenesis.

For these reasons the present work chose the young potato plant as an appropriate test plant. Growing the plant in the dark and inoculating at a zero or near zero concentration produced a condition in which the uncertainty of what was being measured did not arise. The choice of a young potato plant insured that symptoms would develop when inoculated with the right virus of adequate concentration. Had Elmer and McKinney defined the history of their plant and virus with care, their results could have been of greater value.

The second experiment attached the same problem as the

first. Once it was evident from the results of the first experiment that the pigments were not similarly affected, a second experiment was designed to find out what effect inoculation time had on pigment development. For this reason two sets of plants were inoculated at two different times. Set II was inoculated at illumination and Set I three days previous in the dark. The effect of the earlier inoculation produced a change in the pigment concentration in diseased plants almost proportional to the time the plant was exposed to virus. This again is another condition Elmer and McKinney did not allow for in their experiments. If pigment concentration were affected quantitatively by time of inoculation, then the quanitative aspect might also be altered through time. The combination of this variable and the effect of age of the plant on the development of a symptom, such as pigment alteration, may have been great enough to account for the contradictory results published by Elmer and McKinney.

Owen (25) found that if he did not define the age of the plant he was using, he could find a respiration rate following inoculation which was either greater or less than normal. Furthermore, he again noticed that after 15 days had elapsed between inoculation and respiration measurements, the rates began to drop from above to below normal. One can only speculate what caused such a change. His work indicated that one must be careful when interpreting results as an increase or decrease relative to normal. If variables such as age of plant and time

of inoculation are not accounted for, the results may be ambiguous.

The third experiment can be said to have an interest both in the problem of the preceeding two experiments as well as an independent interest of its own. The development of pigment in dark-grown plants was of interest in itself, but also because the results of this period substantiated the effect in the light-grown plants. In both light and dark period the carotenoids were caused to accumulate more rapidly in diseased than in normal plants.

There were two kinds of inoculations in Experiment III, as mentioned in Experimental Procedure. One set was inoculated with virus (Set I) and the other with water (Set II). The necessity of this was suggested by work being done in virus transfer problem in St. Catharines, Ontario. They found that lesions could be produced in healthy tissue by rubbing a leaf with carborundum powder and water (i.e. water inoculation). The author wondered if the same result could be produced on the tissue used in this experiment. Observation indicated that no visible effects could be found from inoculation with a brush. Also, no change in pigment concentration in the water inoculated compared with the uninoculated was indicated. This represented a precaution taken to establish what part, if any, inoculation damage alone played in the change in pigment concentration. Since it was found to alter the concentration from the uninoculated, the effect was concluded to be negligible.

The determinations which had been made at intervals over six days on the dark-grown plants were continued after illumination. The results in Table VIII, generally speaking, supported the observation in the two preceeding experiments that carotene and xanthophyll concentrations were higher in diseased plants compared to uninoculated. Chlorophyll contents were lower in inoculated than in uninoculated. In addition chlorophyll accumulation was observed to have been blocked until at least 4 hours after inoculation. This block, if it may be called that, appeared to influence the concentration of chlorophyll for two days following illumination. During this time the diseased plants held a consistantly low level of chlorophyll concentration. Later in this section the topic of genetic and biosynthetic blocks will be discussed. It is intended that some parallel be drawn between these possible mechanisms of inhibition and these findings in Experiment III.

The purpose of Experiment IV was to examine at short intervals the effect of virus on carotene concentration. A suggestion was made in Experimental Procedure that this change in concentration might indicate a stage by virus duplication. Since the number and nature of the stages are not as yet known, there is no certainty that virus is in any way directly connected with one of these. However, the evidence that there were two active periods (2-4 hr. and 6-8 hr.) and two non-active periods (0-2 hr. and 4-6 hr.) implies that there is some level of direct relationship between this pigment and virus.

This suggestion of a direct relationship between carotene and virus leads one to consider the possibility of a practical application. To date no simple and effective means have been found of estimating in a quantitative manner the progress of virus infection in plants. Further work might find carotene to be a useful means of providing a quantitative index to the stage or progress of infection. This might be tested by comparing a qualitative symptom, such as protection, with the change in carotene concentration following inoculation.

2. Pigment Accumulation and Inhibition

The first four experiments dealt in each case with some aspect of the influence of virus on chlorophyll and carotenoid concentration. In each case evidence obtained in the experiment indicated that the carotenoids (carotene and xanthophyll) in diseased plants increased more rapidly than in the healthy plants. The reverse was true of chlorophyll. Chlorophyll could be said to have decreased in diseased plants relative to healthy plants.

This increase and decrease could better be termed accumulation and inhibition. Terms such as these can be used to introduce a biochemical discussion of the results. If an accumulation was observed, then under what conditions or by what mechanism is this possible? Similarly, if any inhibition has been observed, what mechanism or condition may lead to its understanding? The present discussion makes no claims to have any special information available to solve these questions. Rather, the arguments are based on existing evidence from related

research. Before entering into an analysis it is taken for granted that the problem is related to the biosynthetic chains of carotene, xanthophyll and chlorophyll. If it were not related to the biosynthetic chain, then the only other alternative would be the chloroplast structure, for, as discussed earlier in the Introduction, the chloroplast might be destroyed. In this case the pigments would be dispersed and disappear chemically. This disappearance would apply equally well to chlorophyll as well as the carotenoids. However, such was not the case. All pigments in diseased tissue did not disappear or show a uniform decrease in concentration following inoculation. The removal of this latter possibility leaves the first suggestion as the most probable of the two. To discuss this aspect, some general idea of the normal biosynthetic chain is needed. A general idea of the chain is all that is available since very little is known for certain about the biosynthetic steps in this chain. There is some evidence to support a belief that chlorophyll begins with the small building blocks, glycial and acetate molecules. These are condensed in a series of steps to form the complex molecule protoporphyrins and the magnesium porphyrins which gives rise eventually to chlorophyll. Less is known about the formation of the carotenoids. Carotene is represented by the formula C_{40} H_{56} and xanthophyll, being a hydroxy carotene, by the formula C_{40} H_{56} O_2. They are thought to be formed from one of the hydrocarbons, a tetraterpene of the formula $(C_5H_8)_4$. In theory four of these molecules would condense to form the two ring structures and joining side chain of caro-

and xanthophyll. Some oxidative enzyme may be connected with the formation of hydroxy carotenes (xanthophyll).

Opinions differ on the pathways and mechanisms of biochemical construction. The course of carotenoid and chlorophyll synthesis could proceed on the following hypothetical pattern. First, it is axiomatic that a biological compound is formed in discrete steps, converting a compound 1→2 and 2→3 etc. The second assumption is that each of these stepwise changes is brought about by a specific enzyme. A third requirement is that a gene should give rise directly or indirectly to an enzyme, catalyzing the conversion of compound 1→2.

From the point of view of the experiments conducted here, the virus might effect one or more of the three steps given above. It is probable that the most likely place for the virus to intrude is the enzyme system. Bawden and Pirie (39) refer to many cases in example of interference. However, there appears to be no common factor in all cases which would lead to an understanding of the mechanism. Perhaps McKinney's (22) report of high chlorophyllase activity in diseased plants might account for the low concentration of chlorophyll in those plants. Although no comparable enzyme is known for the carotenoids, there is the possibility that virus might not inhibit an enzyme system governing the synthesis of carotene from its hydrocarbon building block. However, there is not even a shread of evidence to support this latter conclusion. Therefore a more

favourable solution may be sought in the following evidence.

Recent work of Kay and Phinney (17) can be taken to suggest a possible explanation of carotene and xanthophyll accumulation. These workers have been particularly interested in the extent to which a gene, pale yellow-1, of maise governs the synthesis of carotenoid and chlorophyll pigments. In the course of their work it is apparent that a single gene does not control the biogenesis of all pigments. Rather, a mutant gene may block chlorophyll synthesis, but only affect carotene or xanthophyll by the lack of photosynthetic products (18).

Part of the evidence from the present work might be related to such an interference in genetic controls that determine pigment development. One might suspect some kind of genetic regulation of the above type in causing an accumulation of carotenoids and a block of chlorophyll synthesis. For this to be reasonable, the assumption that a virus may act as a gene, or be able to form some complex with a gene should have to be defended. Evidence that molecular structure and weight of virus and gene are similar, suggests a structural relationship between the two. In the case of a tomato plant inoculated with a tomato virus, leaf shape had been observed to be altered in a manner suggesting a known inheritable factor. Study of this type of virus action could lead to an understanding of the mechanism of the interference indicated in the present work.

Considering the uncertainty of available evidence on these topics, it is not surprising that the interpretation or explanation for the present work, remains indefinite. Of the

possible conclusions suggested, the operation of enzymatic destruction of chlorophyll, and a stimulation of genetic control of carotenoids appear the most reasonable.

3. Limits of Error

This matter has been a topic of considerable precaution in the present work. In order to be assured that the error could be defined within exact quantitative limits one would need to feel assured that all the variables including environmental conditions such as temperature, humidity, light, soil etc., and all factors concerning the variation in host-pathogen reactions had been located. One would then need some estimate of the degree of variation caused by each of these variables. The mathematical sum of all of these positive and negative quantities might be used to define limits in practice. However, since in biological research this is impossible, a compromise must be found.

The results of the present work do not require such exact definition of limits. As stated earlier, the results are expressed as a development of pigment change in diseased plants relative to healthy. For this purpose a qualitative indication of direction of change is appropriate. Since the interest is in a decrease or increase relative to something else, quantitative data must be translated into qualitative information.

If such questions as the following were asked, only a

partial answer could be given. First, how closely identical are samples of equal weight and size from two plants? If a pigment determination was made on each of these (assuming them to be healthy plants), the results might show only slight variation. Since no two plants are identical there is no reason why the results should be truly identical. The only reply to such a question if the type of answer found in practice. In Experiments I and II column chromatography increased the possible error in pigment determinations somewhat more than the use of paper chromotography in Experiments III and IV. For this reason when a difference between two concentrations is said to be "significant" in Experiments I and II it means the difference must be greater than 0.1 mg. of pigment per 10 gm. wet weight of tissue. In Experiments III and IV "significant" is taken to mean a difference greater than 0.05 mg. of pigment per 10 gm. wet weight of tissue. To answer the question asked, if two samples equal in weight and size yield pigment of concentration within 0.1 mg. difference for column separation they are considered (for the purposes of the experiment) "identical".

Emphasis should be again placed on the qualitative nature of these "significant" differences. They were established in practice without first attempting to analyse all of the variables concerned. The conditions of the experiments were held as constant as possible in order to reduce the effect of these variables. If the same variables were affecting the experimental results to a constant degree each time the experiment was repeated, then these variables would not be evident in the results. Since

the results are used as a basis for comparison of diseased and healthy plants, it is the affect of virus on these controlled conditions that makes the difference in the results.

4. Function of Carotenoids

Some evidence from literature on this topic was given in the Introduction, page 18, to indicate that carotenoids often do play an important role in photosynthesis of lower plants. There is a complete absence of information suggesting the possibility of a similar role in higher plants. It was reasoned that part of the explanation for this lack was the difficulty of finding a plant with a high concentration of carotenoids and a complete concentration of chlorophyll.

When evidence in the present work was found to indicate that virus had caused some sort of block preventing the formation of chlorophyll for a short period in the light, the opportunity to investigate photosynthesis in a higher plant arose. Since only the carotenoids (carotene and xanthophyll) are present in the leaf under these conditions any photosynthesis would probably be related to these rather than chlorophyll. It was observed that virus had apparently increased the concentration of the carotenoids of these plants in darkness. When introduced to light these plants were high in carotene concentration and deficient in chlorophyll. Kay and Phinney (17) observed a period comparable to this in which a genetic mutation caused a block to the formation of chlorophyll precursors. It was

not until 44 hours following exposure to light that their plants were able to produce any measureable quantity of chlorophyll.

At this point the topic of a chlorophyll block can be left and the main issue discussed. Although pigments other than chlorophyll may be suppliers of energy for the photosynthetic process, they do not replace chlorophyll in this later process. The fact that chlorophyll absorbs far into the red makes it an ideal pigment for trapping excitation quanta. Other pigments in the leaf might absorb light quanta but these must be transferred to a chemical substance with the most stable excitation level. Chlorophyll's efficiency depends upon its ability to maintain energy for a longer period of time in a more stable state than any other pigment. The second most stable pigment in the leaf is carotene. It is stable for only 10^{-5} of a second whereas chlorophyll is stable for 10^{-2} of a second (40). If carotene had the opportunity to act as a photosynthetic pigment it would probably only do so with a limited efficiency.

The results in Table XI indicated a low photosynthetic rate. If the manometric apparatus used to measure the gas pressures was as sensitive to small changes as it is described to be by those who make constant use of it, then the data recorded in Table X might well be significant. However, without further evidence no definite claim should be made to a positive photosynthetic rate. It can be said that date were found which led one to suspect a significant photosynthetic rate.

SUMMARY

The purpose behind the definition of the issues under investigation in this work has been stated with reference to virus research in two aspects, cytology and physiological chemistry. The program of research was designed to improve the understanding of the cytology and physiology of mosaic diseased plants.

The carotenes and xanthophylls were found to accumulate (in both dark-grown and light-grown plants) faster in diseased than in normal plants. A suggested interpretation of this evidence proposed either a genetic or a metabolic control of precursors, or a combination of these.

Carotene concentration was found to change rapidly immediately following inoculation. This change was related to some stage of increase in virus. A possible application of this evidence was suggested.

Dark-grown plants when later exposed to light developed chlorophyll more slowly in diseased than in healthy plants. Furthermore, this inhibition was found to be related to the length of time the plant had been subject to infection.

On finding a short period of 0 chlorophyll concentration following the exposure of dark-grown infected plants to light an experiment was conducted to study photosynthesis during this period. The weak photosynthetic rates calculated from the results suggested an inefficient photosynthetic function of the carotenoid pigments present. Evidence was not considered strong

enough to be conclusive.

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