XVIII. THE FORMATION OF VITAMIN A IN PLANT TISSUES. II.

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INTRODUCTION.

INVESTIGATORS who have used plant tissues as a source of vitamin A in research on its influence on metabolism and growth of animals have frequently observed that the green leaf is richer in this factor than the seed of the plant; and in those plants which form "hearts" such as the cabbage and lettuce, they have found that the green outer leaves are richer than the more or less etiolated inner ones [McCollum, Simmonds and Pitz, 1917; Steenbock and Gross, 1919; Hume, 1921]. No one made any exact comparisons between these two types of tissue until Coward and Drummond [1921] examined etiolated and green shoots of various plants, using very small doses for the feeding tests. They found that shoots which had been exposed to light were very much richer in vitamin A than shoots of the same species grown in darkness; in fact the etiolated shoots in most cases were entirely inactive in the doses used in the tests. In one test of the unsaponifiable matter (shown by Steenbock, Sell and Bluell [1921] to contain the vitamin A if present) from the respective shoots, the dosage must have corresponded to a very great many fresh shoots, and as no growth was obtained from the fraction from the etiolated shoots, it would appear to be possible that sunlight had not only increased the concentration of the vitamin in the green shoot but had, perhaps, even initiated its formation in the plant. This suggested a possible association of the vitamin formation with the photosynthetic activity (carbon assimilation) of the plant and a further series of experiments was planned to determine other conditions of its formation. In the meantime, however, a paper appeared by Wilson [1922] in which he contended that "photosynthesis is not necessary for the production of vitamin A in plants." Hence it seemed desirable to repeat one or two of the former experiments, using the same plant that Wilson had used in his work.

METHOD FOR TESTING A TISSUE FOR VITAMIN A.

The general method of procedure was that described by Drummond and Coward [1920], with the modification described later by Coward and Drummond [1921]. The tissue to be tested was cut from the plant, transferred to a box and carried at once to the animal house where it was put inside the nesting box of the cage. As it was given to the rat early in the morning before the daily ration of basal diet, it was practically always eaten at once and so was not subjected to further influence of light than the experiment demanded. Where the fresh tissue appeared to be unpalatable or poisonous, large masses of it were saponified and the unsaponifiable matter was extracted with ether in the usual way, care being taken to prevent oxidation by carrying out the whole process as far as possible in an atmosphere of carbon dioxide. At each stage of the extraction the separating funnels were filled with carbon dioxide, and during filtration a current of the gas was passed over the filter paper the whole time. The unsaponifiable matter was given directly to the rats by dropping from a glass rod into their mouths and thus the dosage was easily determined. Where seedlings of a definite age were to be fed, seeds were set in plant pots each day and the series used daily when the required age had been attained by those first set.

I. THE INFLUENCE OF SUNLIGHT.

Wilson drew the conclusion quoted above from the fact that he obtained just as good growth by including 5 % of dried etiolated wheat shoots in his basal ration as by including 5 % of dried green shoots. Apparently this experiment was performed on rats which had not previously become steady in weight on a diet deficient in vitamin A as no preparatory period is mentioned and the experiment was started when the rats' weights ranged from 40 to 60 g. each. It is becoming a matter of common experience that rats whose mothers have received large supplies of vitamin A during pregnancy and lactation, can grow from this weight to maturity on diets containing only minimal traces of vitamin A. Moreover, Wilson's basal mixture contained 20 % of lard, and Daniels and Loughlin [1920] showed that lard could give growth; and Drummond, Zilva, Golding and Coward [1920] have shown very definitely that lard from pigs whose diet has contained vitamin A may itself contain the vitamin in large enough quantities to restore normal growth to young rats when administered to them as 10 or 15 % of their diet. The fact that two of Wilson's rats died on this basal diet after $8\frac{1}{2}$ and 9 weeks respectively is scarcely sufficient proof of its total deficiency in vitamin A. In a later experiment, three of Wilson's rats became steady in weight on his basal diet at body weights of 130, 120 and 107 g. respectively. These are so high that they indicate a rich reserve of vitamin at the beginning of the preparatory period or the presence of some small amount of vitamin in the basal diet. Also, a resumption of growth (gained in this experiment by adding 8 % of dried etiolated wheat shoots to the diet) is usually obtained by much

smaller doses of vitamin A in rats of this weight than are necessary for rats which have become steady at a lower weight. These considerations all seem to indicate that the amount of vitamin A in etiolated wheat shoots must be very small indeed and without a test of wheat seeds on rats with a similar basal diet, it seems scarcely possible to draw a comparison between the vitamin content of etiolated shoots and that of the seeds themselves. It is much to be regretted that, while making this investigation, Wilson did not proceed to make a comparison between etiolated and green wheat shoots in smaller dosage, for he says that "all physiological activity must be more intense in the sprouts grown in the light than in those grown in the dark."

In view of this criticism it seemed desirable to repeat the light experiment on wheat shoots. Two series of sowings of wheat seeds were made on successive days for a period of six weeks, one series being placed immediately in a large airy dark room and the other in a verandah in sunlight, protected above and on two sides by glass. Twelve days after the first sowing the respective shoots were given to experimental rats whose weights had become stationary on our basal diet, and each day for a period of three weeks or longer, one shoot only was given to each rat. The average weight of a fresh etiolated shoot was 0.14 g., its dry weight being 0.012 g. The average weight of a fresh green shoot was 0.12 g., its dry weight 0.011 g. In case even such a small amount of etiolated shoot should give growth, a test was made of one ungerminated seed per rat per day, though in view of our previous findings on wheat seeds this appeared superfluous. It did indeed prove to be so—no growth being shown by any of the four rats and three of them declining in weight and dying. Six rats were used for testing the etiolated wheat shoots, fair growth being obtained in one case, slight growth in two and none in the other three. In marked contrast to these results are those obtained with the green shoots; in each of three rats normal growth was resumed (Fig. 1).

Confirmation of this result was obtained with extracts of unsaponifiable matter from etiolated and green shoots respectively, though the amounts of such matter were very small and only sufficed for ten days' feeding in a dosage of 5 mg. daily. Three rats on the fraction from the etiolated shoots lost between them, 11 g. in body weight. The three rats on the fraction from the green shoots gained 21 g. Each dosage was equivalent to 15 fresh shoots though it is impossible to say what loss occurred during the process of extraction.

A similar test was carried out on shoots of the yellow maize and also on shoots of the white maize. For the former of these, yellow maize purchased at an ordinary corn dealer's was used and for the latter, Carter's white Horse-tooth maize was used. The seeds were again set on successive days and when 12 days old used for feeding, but only a small part of the oldest leaf (0.15 g.) was used each day. Striking differences in the growth-promoting powers between the etiolated and green shoots in each case were again found. The etiolated leaf of the white maize was entirely inactive in the quantity fed,

while the same amount of the green shoot produced normal growth; the etiolated shoot of the yellow maize produced growth in one rat, but none in two, while the green shoot produced good growth in three different rats (Fig. 2). Those familiar with the biological test for vitamin A will recognize the irregular behaviour sometimes shown by an individual rat, and hence the necessity of using groups of animals for any one test.

Fig. 1.
Preparatory period on A-deficient diet shown in dotted lines.

Thus, while it would be impossible to deny the existence of very small quantities of vitamin A in etiolated seedlings, it would appear to be established that the amount is increased considerably by exposing the seedlings to sunlight.

It was also thought desirable to examine the influence of electric light on the formation of vitamin A. For this purpose a series of sowings of seeds of *Helianthus annuus* (sunflower) was made. One test was made on the etiolated

Fig. 2.

Preparatory period on A-deficient diet shown in dotted lines.

seedlings at the age of nine days, a further one on seedlings that had grown in the dark for nine days and then had been subjected to various periods of illumination from an electric light of 32 candle power at a distance of 16 inches. Various trials were made and most marked contrast was obtained with shoots that had been treated for three days with 10 hours' illumination and 14 hours' darkness (when the lights were turned off at night) alternately. The first test with two etiolated seedlings produced only very slight growth, the second test with two illuminated seedlings produced good growth which was only slightly subnormal. A further test on seedlings which had been illuminated as the last set and then kept in the dark for eight days gave, in two rats out of three, growth at least as good as that from the shoots which had been eaten directly after illumination. Hence it would appear that electric light is also effective in increasing the rate of formation of vitamin A, though no comparison of its power relative to sunlight was attempted; and it may also be concluded that the store is not immediately used up by the plant (Table I).

Table I.

		Body-weight in g.						
		Daily dose	0	7	14	21	28 days	
<i>Helianthus annuus</i> :								
Etiolated seedlings, 9 days old	2 seedlings	58 60	61 63	68 73	75 75	77 69	} Slight	
Green seedlings, after 3 days' electric light	2 seedlings	75 71 63	88 76 74	96 80 78	106 89 80			} Fairly good
Green seedlings, after 3 days' electric light followed by 8 days in the dark	2 seedlings	79 86	96 96	105 108	— —	— —	} Good	
Green seedlings, 14 days old, after 1 day in electric light	2 seedlings	91 90 70	105 80 65	100 91 75	95 96 81	104 102 81		} Slight
<i>Tradescantia</i> :								
Grown in Sachs solution	Fresh Mat. 1.0 g.	95 83 95	112 100 95	115 116 107	119 125 105	122 136 117	} Good	
Grown in solution without calcium	1.0 g.	72 67 66	84 76 66	100 86 68	105 90 70	104 88 70		} Fairly good

By a further test of growing etiolated seedlings for 14 days in the dark and then exposing them to 10 hours' electric light and 14 hours' dark again, it was shown that no better growth-promoting powers were developed than had been in the nine days old seedlings similarly treated. Thus in vitamin formation in *Helianthus annuus* there is no increased activity through delay in starting it, as Briggs [1920] found was the case with the photosynthetic activity (carbon assimilation) of seedlings of *Phaseolus* (Table I).

II. INDEPENDENCE OF CARBON DIOXIDE IN THE AIR.

As the formation of vitamin A has hitherto been generally associated with those tissues showing at some time photosynthetic activity (carbon assimilation) it seemed advisable to investigate the various factors in this process to see whether they were also factors in the process of formation of vitamin A.

Fig. 3.

Preparatory period on A-deficient diet shown in dotted lines.

The influence of carbon dioxide in the air was first tested. This and the following two tests, together with a control, were run concurrently during May 1922 when, fortunately, there was a succession of sunny days practically without an interruption throughout the experiment. The plan adopted was to cut off shoots of etiolated pea seedlings 12 days old, and place their cut ends in a small tube of fresh tap water in the apparatus each day for 24 hours. The apparatus consisted of a large photographic dish with a layer of mercury, and a bell jar of about 5 litres capacity. Within this stood a wide low dish of 6-inch diameter containing strong potash solution—and the tube containing the cut shoots stood in the middle of this. Thus the shoots would be practically free from carbon dioxide, any that was given out by respiration probably being absorbed by the potash. The whole apparatus stood in a large south window.

A daily supplement of one of these shoots produced growth in rats of the type that is described by Zilva and Miura [1921] as resulting from small doses of vitamin A. That is, the rats grew pretty well for a short time (two weeks), then began to slow down in rate, but on administering two shoots further growth was resumed. One rat only failed to respond to the increased dosage (Fig. 3).

A control of one etiolated pea shoot gave a negative result in three rats. Hence the formation of vitamin A in the plant must be considered as independent of the presence of carbon dioxide in the surrounding atmosphere, if one may ignore the possibility of utilisation of the small quantities admitted to the apparatus in placing the shoot in it, for it might be some time before all the gas became absorbed by the potash (Fig. 3).

III. INDEPENDENCE OF OXYGEN IN THE AIR.

To determine whether oxygen is necessary for the formation of vitamin A in plant tissues, an apparatus was set up similar to that used in the last test but a solution of pyrogallol (10 %) in caustic soda (80 %) was used in place of the potash. Most of the air was blown out each day with CO_2 before the fresh seedlings were put into it, in order to ensure as complete an absorption as possible of the remaining oxygen by the pyrogallate. The apparatus stood in the same window as the last one so that it might receive equal illumination.

The results of the feeding test were very similar to the last, three rats resuming growth for about a fortnight, then becoming stationary. One rat grew again on the addition of two shoots instead of one. Thus it appears that vitamin A can be synthesised by the plant practically in the absence of oxygen in the surrounding atmosphere (Fig. 3).

IV. INFLUENCE OF CHLOROFORM IN THE AIR.

Very small traces of chloroform in the atmosphere are sufficient to inhibit the process of carbon assimilation in a plant. It was determined to find out whether vitamin formation was affected similarly. Cut ends of etiolated pea

shoots 12 days old were placed in a small jar of water for 24 hours under a bell jar in a south window similar to the one used above, and a small flask of chloroform, open, was also placed under it. The base of the bell jar was not sealed with mercury—fresh air was allowed to enter where it could. The shoots remained apparently healthy and developed a decided green tint.

A ration of one shoot per rat per day so treated failed to restore growth in three rats; hence it may be concluded that chloroform inhibits the formation of vitamin A in plant tissues (Fig. 3).

V. INDEPENDENCE OF CHLOROPHYLL.

One difference between the shoots deprived of oxygen in Exp. III and those deprived of carbon dioxide in Exp. II was very noticeable each day. The former invariably remained a pure yellow in colour while the latter developed a decided green colour. It is an accepted fact that etiolated shoots will not develop chlorophyll in the absence of oxygen in the air and thus the feeding test of Exp. III leads to another important conclusion. The formation of vitamin A in the plant must be independent of the presence of chlorophyll in the tissues (Fig. 3).

VI. INDEPENDENCE OF ULTRA-VIOLET LIGHT.

Another important conclusion may also be drawn from the foregoing experiments. The shoots in two cases, II and III, developed vitamin A when the light which they received had passed through two thicknesses of glass, one the ordinary window glass, the other the thick glass of the bell jar. Hence it may be concluded that the formation of vitamin A is independent of those ultra-violet rays of the spectrum which fail to pass through two thicknesses of ordinary glass—a point in which this process is similar to the more generally examined processes carried on in plant tissues.

VII. INFLUENCE OF CALCIUM SALTS.

The close relation between calcium and vitamin A in certain of the latter's activities led the writer to consider whether calcium played any part in the actual formation of the vitamin in the plant, and an attempt was made to cultivate shoots of *Tradescantia* in a medium devoid of calcium. This plant was chosen as it had previously been found very easy to cultivate in water cultures under laboratory conditions. About 200 cuttings were made from normal plants growing in profusion under the staging of a greenhouse. Each shoot, when prepared, consisted of about seven nodes and internodes, the terminal bud and four leaves, the lowest three leaves being cut off and the stem itself cut straight across immediately below the lowest node. The shoots were supported in corks in holes, the stems protected somewhat with a small covering of cotton-wool. Jars of 800 cc. or 1200 cc. capacity were used, four cuttings were put in the former, six in the latter, and the two lowest nodes

of each shoot were submerged in the solution. Half the cuttings were set in normal Sachs solution, half in a solution consisting of 1 g. KNO_3 , 0.5 g. NaCl , 0.5 g. NaH_2PO_4 , 0.5 g. MgSO_4 , and two or three drops of FeCl_3 sol. to a litre of distilled water. This last was obtained from an ordinary copper still, and was not distilled again. The jars were covered with brown paper, and placed on a window sill with a north aspect.

At first the shoots appeared to grow equally well in the two solutions, practically everyone of them taking root. But a striking difference between the roots was noticed on removing the paper to search for possible moulds. The roots in the Sachs solution had produced masses of root hairs throughout their length, while those in the solution without calcium were devoid of root hairs except for a short distance about half-an-inch long beginning about a quarter-of-an-inch behind each root tip. The root hairs never developed as far as those on the normal roots and apparently died away quite early. This confirms an observation made by Maquenne and Demoussy [1917, 1] in their work on the influence of calcium salts on the growth of seedlings (Plate I, Fig. 2).

When two or three more leaves had expanded on each shoot, the older ones were cut off, and later, when the root systems were well established, the plants were cut down so that only two nodes with undeveloped axillary buds were left. Practically all the plants survived this drastic treatment but the next shoots from the Sachs solution were distinctly more vigorous than those from the calcium-free solution. By this time, three months after the setting of the original cuttings, it was decided to begin the feeding test. 1 g. of the fresh shoot was given to each rat daily and although the shoots grown in the calcium-free solution did not give quite such good growth as those grown in the Sachs solution, yet the growth was very definite in two cases out of three. Maquenne and Demoussy [1917, 2] state that distilled water will dissolve out enough calcium from the glass of the culture vessels to supply the need of seedlings for some time. It would seem probable, however, that after three months with two changes of culture fluid, there would be only traces of calcium left, and it may be concluded that vitamin A can be produced in the presence of only the merest traces of calcium salts in the nutrient solution (Table I).

The question of examining the influence of other elements such as phosphorus, potassium, sulphur, etc., on the formation of the vitamin was also considered, but the difficulty generally experienced in getting cuttings to develop more than one or two more leaves (and no side shoots) in solutions free from these elements, individually, made the matter impossible. Briggs [1922] has just shown that the "photosynthetic activity of plants of *Phaseolus vulgaris* grown in culture solutions devoid of potassium, magnesium, iron or phosphorus is less than that of plants grown in full culture solution." Possibly a lack of calcium also retards photosynthetic activity which would account for the retarded growth of the *Tradescantia* cuttings in the calcium-free solu-

tion. It is impossible to contend that retarded photosynthetic activity in a leaf was the cause of the retarded formation of vitamin A as it has been shown that vitamin A can be formed under conditions ((a) lack of chlorophyll, (b) lack of CO₂ in atmosphere) in which photosynthesis (carbon assimilation) does not take place to any appreciable extent. And in fact, it is even open to question whether the observed difference in the response of the animals to the two sets of shoots (those grown with calcium and those grown without) was really significant or whether it lay within the limits of experimental error often observed in the only available and decidedly crude test at present used for the vitamin. The main point is that the vitamin can be formed in the presence of only very small traces of calcium in the nutrient solution.

SUMMARY.

1. Light is necessary for the formation of vitamin A in plant tissues.
2. This process can be carried on in the absence of carbon dioxide and of oxygen in the surrounding atmosphere.
3. It is independent of the presence of chlorophyll in the plant.
4. It is also independent of the ultra-violet rays of the spectrum (or such of those as are held back by two plates of window glass) and can be carried on under the influence of electric light in the absence of sunlight.
5. The presence of chloroform in the atmosphere prevents the formation of the vitamin.
6. The almost complete absence of calcium salts from the nutrient solution of a water culture of *Tradescantia* does not prevent the formation of vitamin A in the leaves of the plant.

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TRADESCANTIA CUTTINGS

Fig. 1
Full Sachs Sol. Calcium lacking
Difference in Vegetative Growth

Fig. 2
Full Sachs Sol. Calcium lacking
Difference in Roots