LXVI. THE FORMATION OF VITAMIN A IN LIVING PLANT TISSUES.

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An opinion is gradually being formed that the fat-soluble vitamin occurs in the green, actively assimilating parts of plant tissue and that it is generally absent from the localities where chlorophyll is not found. This led us to carry out certain investigations planned to trace, if possible, the origin of the fat-soluble factor in plants.

Experimental Method.

The essential details of the method of testing have already been outlined in a former paper [Drummond and Coward, 1920, 1]. Throughout this work and in fact now generally, we employ a new method of purifying the constituents of our basal diet from residual traces of the vitamin A. As we have pointed out in the paper referred to above, the chief source of impurity is the caseinogen which persistently carries small amounts of milk fat which may serve as sources of the factor A. Since the discovery that this substance is readily destroyed by oxygenation at high temperatures [Hopkins, 1920; Drummond and Coward, 1920, 2] we have employed a process based on this principle to replace the tedious and expensive method of extraction with alcohol and ether formerly adopted. At the present time we use an oven in which the caseinogen is exposed in shallow layers to air at a temperature of 105° for at least 24 hours, the fine powder being frequently raked over to expose a new surface to the air. We have been entirely satisfied with this simplified method of purifying the basal protein, which yields results quite as reliable as the older method. The foodstuffs to be tested are always given as supplements to each rat before the basal diet is given and in nearly all cases it is consumed before the latter is supplied.

The Vitamin A content of seeds.

The work of McCollum and his co-workers summarised in his recent book [1918] has shown us that seeds generally are poor sources of vitamin A. Our first plan was to test a number of seeds and then to compare the vitamin

content of the plants produced from them at various stages of their development under different conditions. Many experimental difficulties were encountered, however, in consequence of which few systematic tests of any one variety were found possible. Nevertheless, the results of these preliminary tests, even if somewhat incomplete, are given because they are all more or less in agreement with those yielded by the more complete studies.

The seeds first studied were carrot, turnip, cabbage, cress, peas, and white and yellow maize. These were all given to the rats in known amounts daily in addition to the basal diet deficient in vitamin A. No resumption of growth followed the administration of turnip, cabbage or white maize, but there was evidence of more or less activity in the case of the peas, yellow maize and carrot. The rats refused to eat the cress seed nor could they be induced to consume the cress shoots. The average results of these experiments are seen in the table p. 538 (Tests 1–7).

Our observations on the two forms of maize confirm the results recorded by Steenbock and his colleagues [1919 and 1920] and in one case we were able to restore the growth of a rat which had been stationary on the white maize supplement by changing it to one of yellow maize.

It is interesting to note also that sycamore seeds, although their cotyledons are distinctly green, are unable to induce growth even when supplied at the rate of 1·5 g. per day without the fruit wall and wing (Test 8).

On the whole our results confirm McCollum's opinion that the majority of these tissues are deficient in vitamin A. Further evidence is also presented in the experiments on sunflower seeds to be described below.

Germination of seeds.

It was originally shown by Fürst [1912] and confirmed by Chick and Hume [1917] that the anti-scorbutic factor is not present in the dry seeds but is produced during germination. Chick and Delf [1919] state, however, that both dry and germinated peas are deficient in the fat-soluble factor. The latter observation was made during a study of scurvy in the guinea-pig, so we decided to make a direct test on rats, with the result that no detectable increase in the amount of the vitamin A appeared to have occurred in these seeds during germination (Tests 9, 10).

Etiolated seedlings.

The first experiments with seedlings were made with sand cultures. The sand in which the seeds were to grow was well washed in running warm water with much stirring for about half-an-hour. It was then treated with strong nitric acid until no further effervescence of carbonates took place, washed with a 2 % solution of mercuric chloride to kill bacteria and finally washed in a Buchner funnel with distilled water. The sand was spread out in large trays in layers about 1½ inches deep, sprinkled with the seed and a very shallow top layer of sand added. Successive sowings on fresh areas were made

every few days to ensure a continuous supply of seedlings of about the same age. The seeds were watered with distilled water for about a week and then with Sachs' solution for a week, after which Sachs' solution reinforced with 0·3 % of sucrose was employed in the hope that by supplying preformed carbohydrate growth beyond the expansion of the cotyledons might be induced. This hope was not realised and in spite of all our care the etiolated seedlings tended to die when about 3–4 weeks old.

Etiolated seedlings in their prime (generally 13–16 days old) were given to rats in amounts of 0·7 g. per day. Owing to the difficulty of maintaining the supply of sufficient material for testing, the results are not as conclusive as might be desired. No growth was obtained with the carrot seedlings (25–30 days of age) in spite of our having detected some activity in the dry seeds, whilst in the other cases most of the etiolated shoots showed very slight activity (Tests 11–14). Etiolated pea shoots grown from seeds planted in earth and watered with tap water produced some slight increase in weight in the rats (Test 15). As will be shown later, more carefully controlled experiments with sunflower plants indicate that in this species at least, the amount of vitamin A present in the etiolated shoot is not appreciably greater than that in the seed from which it grew.

Green seedlings.

The seeds were treated in exactly the same manner as in the last experiment except that they were exposed to light and only normal Sachs' solution was used for watering. The results of the feeding tests show that green shoots of turnip, maize and peas (soil and sand grown) possess a decidedly higher value as sources of vitamin A than either the seeds from which they sprang or the corresponding etiolated shoots. Abnormal results were obtained in the case of the very young carrot shoots which caused little or no resumption of growth, although fully developed carrot leaves (carrot tops) were more potent (Tests 16–20). Here again we regard these results very much as preliminary in character, and place more reliance on those with sunflower seeds to be described below in which the green shoots were found to be very much more active than the etiolated ones.

Experiments with sunflower plants (*Helianthus annuus*).

The preliminary results briefly outlined above led us to select one species of seed for full investigation and the ease with which the sunflower may be grown under laboratory conditions caused us to choose that plant.

The dry sunflower seeds stripped of the outer husk were found to be relatively inactive as a source of vitamin A when given in daily doses of from one to four seeds per day per rat (Fig. 1). Seeds were germinated in boxes of soil both indoors and in a closely darkened room and after about a fortnight when they had only expanded their cotyledons they were given to the animals as supplements of from one to four shoots daily without inducing

growth (Fig. 2), but vigorous growth followed the administration of a supplement of one or two green shoots daily (Fig. 1). Other seedlings were allowed to produce two further pairs of leaves, and as the cotyledons withered, were tested. The potency of these more mature shoots appeared to be even greater than that of the younger ones (Fig. 2). All attempts to make the etiolated shoots produce further leaves were unsuccessful. It appears reasonable to conclude from these experiments that the formation of the large amounts of vitamin A found in the green leaf of most plants demands the influence of light.

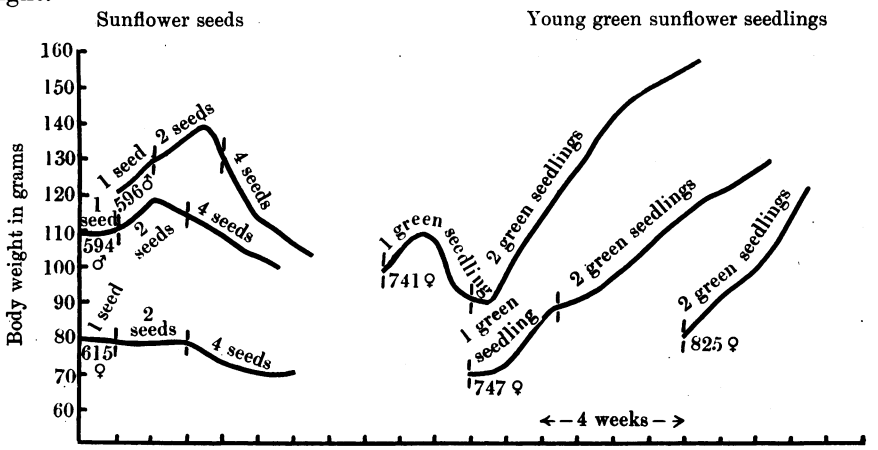


Fig. 1. Curves 594, 596, 615 show failure on sunflower seeds. Curves 741, 747, 825 activity of green seedlings.

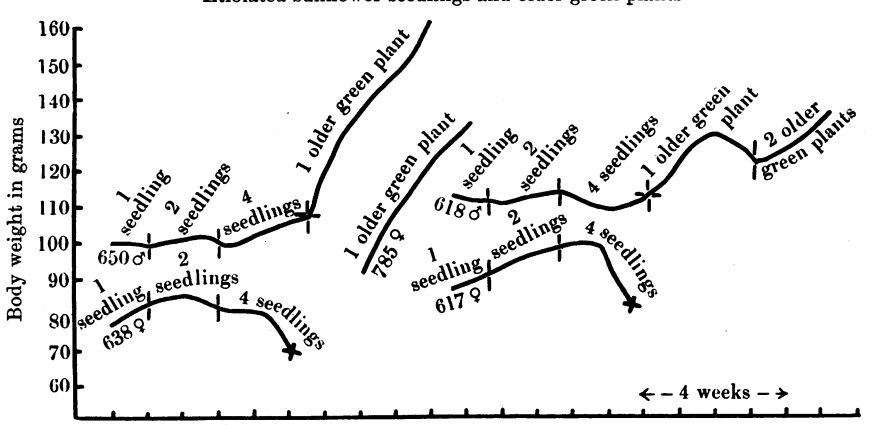


Fig. 2. Curves 650, 638, 617, 618 show failure of growth on etiolated seedlings with subsequent recovery in case of 650, 785 and 618 on older green plants.

WATER-CULTURE EXPERIMENTS WITH *Tradescantia* ("Wandering Jew").

Tradescantia is a plant that lends itself very freely to water-culture experiments and a few tests were made on material obtained in this manner. Preliminary tests showed that shoots of this plant grown in pots could serve as a source of vitamin A for rats (Test 21).

Shoots of about five nodes were taken, cut off sharply immediately below the lowest node, the lowest three leaves stripped off, and the stem placed in a hole in the cork of the culture jar. The stem was padded round with cotton wool and so set that the lowest two nodes were submerged in the culture fluid. Ordinary glass preserving jars of 800 cc. capacity were employed and the cork of each jar carried four cuttings. Sachs' solution was employed as a culture fluid and was renewed every three or four days, the stem and roots of the shoots being at the same time washed and gently brushed in running water. It was found very difficult to keep the cultures free from contamination with moulds, especially in the case of the jars kept in darkness, but the development of green algae in cultures exposed to light was successfully prevented by covering the jars with black cloth up to the level of the fluid.

The shoots kept in the light grew excellently and developed into large sturdy plants in the course of some eight weeks. From the plants, recent shoots were removed and administered to rats in daily doses of 1·5 g. with the result that growth was induced more or less strikingly (Test 22).

That the presence of vitamin A in these shoots did not merely indicate a transference of material from the original cutting is shown by some later experiments in which the same plants were cut down until only half-an-inch of bare stalk remained above the cork. These stumps after a few weeks produced vigorous new shoots with abundant leaves which on testing appeared to possess a value as a source of the vitamin at least equal to that of the original plant.

Trouble was encountered with the cuttings kept in the dark. In the first place they refused to produce new leaves and merely grew by elongation of the internodes, even when the Sachs' solution was reinforced with 0·3 % of sucrose; whilst, secondly, the moulds which frequently appeared in the cultures required constant removal. Testing of these pallid shoots demonstrated that their activity was somewhat less than that of the shoots grown in the light, but it must be remembered that the etiolated shoots were originally green and the loss of chlorophyll may not necessarily have been accompanied by the destruction of the pre-existing vitamin (Test 23).

An attempt was made to grow chlorotic shoots of *Tradescantia* in Sachs' solution devoid of soluble iron salts, but without success. The shoots grown in this fluid appeared to thrive as successfully as those grown in the normal fluid in light and showed no obvious sign of chlorosis. It is possible that the roots were obtaining sufficient iron from the glass jars. These shoots contained at least as much vitamin A as the normal plant (Test 24).

If one is permitted to exclude the complications introduced by the absence of complete sterility in these experiments (and the later results indicate that lower organisms devoid of chlorophyll do not synthesise vitamin A), the production of this substance by the green plant from inorganic sources appears to have been demonstrated from these experiments.

Vitamin A in white and green cabbage leaves.

For a long time the value of green cabbage as a source of the fat-soluble factor has been recognised but a definite comparison of the white and green leaves has not been made. Recently, however, Hume [1921] has reported that growth in guinea-pigs was more satisfactory when green leaves were given than when the white inner leaves were used. In our experiments white and green cabbage leaves respectively were given to rats in quantities of 1·5 g. of the fresh material daily. Practically no growth was obtained with the white leaves but very fair growth was obtained with the green leaves. The quantity corresponded to about 0·24 g. of dry matter per day which appears to be somewhere near the minimum for obtaining growth (Tests 25, 26). Rats which had for some time been stationary on dried white leaves resumed growth when transferred to dried green leaves.

Fat-soluble vitamin in some lower plants.

Mushrooms (*Agaricus campestris*) when fed to rats in rations of 1·5 g. per day induced very slight growth (Test 27). Microscopical examination indicated that the spores tended to pass through the intestinal tract unchanged but that the remainder of the fructification was digested.

Seaweeds. The examination of the marine algae is of very great importance since it would appear that the ultimate origin of the vast supplies of vitamin A which are available in the form of fish oils and liver oils may be represented by these organisms. On two occasions we have tested samples of common green seaweeds found on the south coast of England (*Ulva* and *Cladophora* sp.?) and have found them to be at least as potent as the green land plants such as cabbage (Tests 28, 29).

The examination of a typical brown seaweed, *Fucus vesiculosus*, was impossible since the animals refused to eat it. It is significant that no animal is known to eat this weed.

A red seaweed, *Polysiphonia* sp., induced a slow rate of growth at first, but this was not maintained (Test 30).

The difference in the food value of these weeds may be significant in spite of the small amount of experimental evidence advanced here, since we may recall the relationship between the coloration of these weeds and the activity of the photosynthetic processes at the different depths at which they flourish [see Palladin, 1917; Engelmann, 1883].

Another sample of a red weed, *Chondrus crispus* (Carrageen moss) which had been sun-dried showed no growth-stimulating powers when given in an amount of 0·5 g. per day (Test 31).

The condition of the vitamin A in plant tissues.

Certain observations having led us to suspect that the fat-soluble vitamin might occur in the leaf of green plants in the form of a complex with protein we undertook the following experiments. Preparations of the proteins of

green and white leaves were made by the method described by Chibnall and Schryver [1921]. The fresh leaves were minced and, together with the juice pressed out in the process, allowed to stand in water saturated with ether overnight in the cold. The solid matter was then removed by filtering through a cotton cloth and the residue pressed. Carbon dioxide was passed through the filtrate for 2–3 hours to remove the ether and the solution was heated on the water-bath to 40–50° to precipitate the proteins. The flocculent precipitate was filtered through a folded filter and washed only once with water at which point our method deviated from that employed by Chibnall and Schryver in that further washing to remove substances of a fatty nature was avoided. The residue was spread out on porous tiles in a vacuum desiccator over calcium chloride until practically dry when it was tested on rats in amounts of about 0·1 g. daily, which represented about 40 g. of fresh spinach. Unfortunately, green cabbage leaves were not available at the time in sufficient quantity for a similar test, and tests were made only with protein matter prepared from fresh green spinach and white leaves of cabbage. The results showed a slight activity on the part of the green protein precipitate from the spinach whereas that from the white cabbage was completely inactive (Tests 32 and 33).

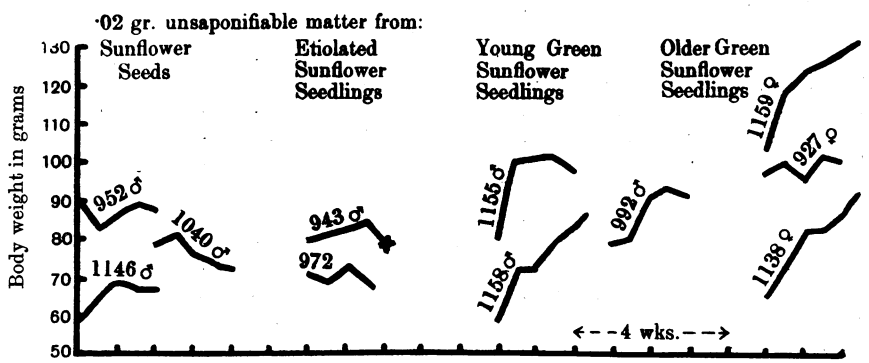
The results of these experiments indicate that vitamin A is not present in the form of a complex with protein in green leaves unless the method of preparation was responsible for the resolution of such a complex. The small activity of the preparation from green leaves when given in doses representing so large an amount of the fresh vegetable would suggest that a small amount only of the vitamin had been carried down by adsorption. Chibnall and Schryver mention that substances of a fatty nature can be removed from the crude protein precipitate by extraction with solvents.

Vitamin A in the unsaponifiable matter in green leaves.

It has been shown by Osborne and Mendel [1919] and others that vitamin A can be extracted from green leaves by fat solvents. That it may be obtained in the unsaponifiable fraction of the fatty substances extracted from a typical green plant, alfalfa, by alcohol, was first shown by Steenbock and Boutwell [1920]. This would appear to confirm the original observation of McCollum and Davis [1914] who stated that the vitamin in butter-fat withstood saponification at room temperature in a non-aqueous solution. This view was at first opposed by Drummond [1919] but we have since found that, if care is taken to prevent oxidation, a highly concentrated fraction may be obtained by the cold or the hot saponification of certain animal oils or plant tissues. The previous failure to confirm McCollum's finding is attributed to destruction by oxidation during the process. We have applied this work to a study of certain plant tissues.

Dried Peas. 200 g. of dried peas ("gradus" variety sold for germination) were warmed on a water-bath with a litre of 50 % aqueous potassium hy-

droxide for 14 hours and then allowed to stand for two weeks at room temperature, after which about two volumes of alcohol were added and the saponification completed by boiling under a reflux for 24 hours. The alcohol was removed by distillation *in vacuo*, and the unsaponifiable matter removed by extraction with ether. The subsequent treatment of the ether fraction, washing, drying and concentration by removal of the solvent were conducted in an atmosphere of carbon dioxide. The residue was semi-liquid and was stored in a tube filled with carbon dioxide, and refilled with the gas after each sample had been removed for feeding tests. This fraction was given to rats in a dose of about 0·02 g. corresponding to about 4 g. or 15 peas, daily, and produced definite growth (Test 34). In view of the results previously obtained with the small ration of dried peas themselves, it was decided to ascertain whether a diet containing the amount of dried peas equivalent to the dose of unsaponifiable matter would induce growth. Accordingly a diet consisting of 65 % of dried peas (ground up), 15 % caseinogen, 5 % salt mixture, 5 % yeast extract, and 5 % of lemon juice was tested and found to produce growth.



Etiolated and green pea shoots were saponified by essentially the same process after having grown for about 20 days in boxes of soil. The shoots only were used, the remains of the cotyledons and the roots being discarded. The crop of etiolated pea shoots, about 425 plants weighing 285 g. yielded very little material but the unsaponifiable fraction when given to rats in daily doses of 0·02 g. appeared to be slightly active. This dose corresponds to 20 g. of fresh shoots. The unsaponifiable matter from the green pea shoots was very small in amount and apparently inactive. For several reasons this test was unsatisfactory, chiefly because of the very small amount of material available (much of it was lost through an accident) and the possibility that in the case of the extract from the green shoot, loss by oxidation might have occurred. Better results however were obtained when the unsaponifiable fractions were prepared from larger quantities of etiolated

and green sunflower shoots. These fractions, prepared by the method we have described, were tested by giving 0·02 g. per day to the rats. As may be seen from Fig. 3, the difference in the growth-promoting power of the material from the two types of shoots is very marked, there being little evidence of the presence of appreciable quantities of the vitamin A in that from the etiolated seedlings.

Typical results of feeding substances as test for fat-soluble vitamin.

Test No.	Substance tested	Daily supplement per rat to basal diet devoid of A	Average weight in g. of rats during test period[1] 0	7	14	21	28 days	Remarks
1	Cabbage seed	0·2 g.	85	88	91	94	88	Very slightly active
2	Turnip seed	0·1 g.	80	78	72	80	77	Inactive
3	Carrot seed	0·4 g.	97	110	115	130	—	Active
4	Dried peas	1 pea seed	93	95	107	100	110	Slightly active
5	„	5 pea seeds	124	129	130	140	—	Slightly active
6	Maize seed (yellow)	1 g.	100	106	109	108	119	Active
7	„ (white)	1 g.	88	87	87	79	80	Inactive
8	Sycamore seed	1·5 g.	112	109	104	105	—	Inactive
9	Germinated peas	1 plant	95	98	105	102	100	Inactive
10	„	4 plants	79	80	86	—	—	Inactive
11	Etiolated cabbage shoots	0·7 g.	60	61	70	72	79	Slightly active
12	„ carrot „	0·7 g.	84	90	87	—	—	Inactive
13	„ turnip „	0·7 g.	68	67	78	85	—	Slightly active
14	„ maize „	1·0 g.	86	90	96	96	—	Slightly active
15	„ pea „	0·6 g. av.	81	92	91	91	96	Slightly active
16	Green turnip shoots	0·7 g.	59	65	75	81	97	Active
17	„ carrot „	0·7 g.	67	70	70	—	—	Inactive
18	„ „ tops	0·6 g.	135	144	150	—	—	Active
19	„ maize (white) shoots	1·0 g.	61	65	70	75	83	Active
20	„ pea	2 shoots	79	90	102	113	119	Active
21	*Tradescantia* in soil	1·5 g.	101	104	112	112	—	Active
22	„ water culture	1·5 g.	105	116	123	—	—	Active
23	„ etiolated	1·5 g.	65	72	75	—	—	Active
24	„ iron free culture	1·5 g.	88	92	100	103	113	Active
25	Green cabbage leaves	1·5 g.	56	61	66	71	80	Active
26	White „ „	1·5 g.	56	53	55	57	60	Inactive
27	Mushrooms	1·5 g.	65	68	74	77	78	Very slightly active
28	*Ulva*	1·5 g.	91	106	106	108	110	Active
29	*Cladophora*	1·0 g.	100	105	118	123	137	Active
30	*Polysiphonia*	1·0 g.	82	92	91	97	90	Slightly active
31	*Chondrus crispus*	0·5 g.	120	125	129	128	—	Inactive
32	Protein ext. spinach	0·1 g.	82	89	95	100	104	Active
33	„ white cabbage	0·5 g.	95	90	91	91	—	Inactive
34	Unsap. matter from dried peas	0·02 g.	62	80	78	81	93	Active

[1] Weights during preliminary periods on basal diet are omitted for simplicity.

SUMMARY.

1. Dried seeds vary in their content of vitamin A, but are in general deficient in this substance.

2. The amount does not appear to be increased by germination.

3. Etiolated seedlings and leaves deficient in chlorophyll (white cabbage) do not apparently synthesise the vitamin.

4. Green leaves form large amounts of vitamin A, and from evidence obtained from water-culture experiments this synthesis may be effected from inorganic salts.

5. Lower plants (marine algae) containing chlorophyll synthesise this dietary factor; others which are differently adapted for photosynthesis (red weeds) are not so active in this respect, whilst those devoid of pigments which play a role in carbon assimilation (mushroom) are almost completely deficient.

6. The vitamin A in green leaves does not appear to be associated with proteins. It may be extracted in the fat removed by solvents and appears in that fraction of the fat which is resistant to saponification.

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