LXXIII. THE NATURE OF THE VITAMIN A CONSTITUENT OF GREEN LEAVES.

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In spite of the fact that green leaves form one of the most potent sources of vitamin A, comparatively little work has been done on the isolation of the active substance from these leaves. The material from an ethereal extract of spinach or other green leaves has been added to a synthetic diet in order to supply vitamin A by various observers, amongst them Osborne and Mendel [1919, 1, 2] and Willimott and Wokes [1927]. In some experiments which were being carried out by E. M. Hume and H. H. Smith, the unsaponifiable matter from which the sterol had been separated was used to supply the vitamin A constituent of the diet, and it seemed desirable to obtain as much knowledge as possible about the nature of the extract used.

Relation of greenness to vitamin A.

The work of Osborne and Mendel [1919, 1, 2] and Steenbock and Boutwell [1919] had indicated an association of the fat-soluble vitamin with the green parts of plants. This view was examined by Coward and Drummond [1921], who reported that vitamin A was not synthesised by etiolated shoots but that green leaves were active in its formation. Wilson [1922], on the other hand, found that etiolated shoots if given in sufficient quantity could supply the fat-soluble vitamin and that this factor was therefore formed in the absence of light. Coward [1923] concluded that, although the presence of chlorophyll was unnecessary, the formation of the fat-soluble vitamins required the action of light though not of ultra-violet radiation, and later [1925] made the interesting observation that green leaves which in the autumn had turned yellow were more active than the original green leaves. Wilson's work has been confirmed by Coward [1927, 1, 2], Moore [1927] and Heller [1928] and there seems now to be conclusive evidence that, although the green shoots show far more vitamin A activity than the etiolated ones, the latter can supply the active factor if they are given in sufficient quantity. The potency of the shoots is much increased under the influence of light and it cannot be considered as quite certain whether the activity of the etiolated shoots is

due to some effect of germination or to the experimental difficulty of ensuring that no light whatever shall reach the seedlings. A similar difference has been described between the green and white leaves of cabbage. Coward and Drummond [1921] noted that green cabbage leaves were more potent than white leaves in promoting the growth of rats on a diet deficient in the fat-soluble vitamins and Hume [1921] made similar observations for guinea-pigs. Steenbock and Sell [1922] reported that the green inside leaves of cabbages which had failed to head properly contained ten times as much pigment as white leaves on the inside of good heads and were far richer in what was then known as vitamin A, but at the same time they thought that more than the minimum demonstrable amounts were present in white cabbage.

Since the very active green leaves, and the inactive or very feebly active white leaves of cabbage may be obtained at the same time from the same plants, the unsaponifiable matter obtained from the lipoid extracted from white and green leaves respectively seemed very interesting material for investigation and we set out therefore to prepare unsaponifiable matter from both sets of leaves, to examine its chemical nature and to test it biologically for vitamin A activity.

In the present investigation a preliminary study of the white and green leaves of the large cattle cabbage led to the conclusion that three to four times as much unsaponifiable matter could be extracted from a given weight of green as from an equal weight of white cabbage. Not only was more lipoid matter extracted by light petroleum from the dried leaves, but the proportion of unsaponifiable matter derived from it was higher. It was just possible that the results of previous workers might be explained by the fact that equal weights of green and white cabbage contained very different amounts of unsaponifiable matter, although the nature of the unsaponifiable matter was identical.

The method of biological experiment.

In an investigation such as the present one, it is clear that the ultimate criterion for the vitamin A value of the materials studied must be the biological test, although many writers have found the latter far from satisfactory. In carrying out the series of tests described which has extended over about a year, a preliminary depletion period was always used and the aim at first was to obtain from the rats, on various doses of the materials to be tested, growth performances which should be comparable. As time went on, however, information accumulated as to the exact size of the minimal dose on which life could be sustained. It was then found that a better comparison could be made by ascertaining the dose which could just sustain life over a measured period and that which failed to do so. In this way it was possible to say, for instance, that the effective dose of one sample of carotene lay between 0·005 and 0·001 mg., while that of another lay between 0·01 and 0·005 mg. In working with doses so far sub-optimal the difficulty is encountered that

the number of animals which fail to recover when dosage begins after the depletion period is increased; for, when the animal is in a precarious state, the dose needed for recovery is often in excess of that just needed for maintenance. Also there is frequently a latent period of several days before the dose begins to act, in the course of which the animal may become very much worse and it is impossible then to tell whether the dose should be increased or not. The dose may be increased without there being a real necessity to do so or failure to increase it may bring about the loss of a specially prepared animal.

In the last series of experiments (spinach-carotene group) about to be described the results were very much impaired by the frequent occurrence in the animals of haematuria and of septic glands in the neck. Several fulminating cases of corneal ulcer also occurred about this time: it might be that the use of minimal doses favoured the development of the conditions; such was found to be the case independently by McCarrison [1927] and Fujimaki [1926], particularly in favouring the development of stone; but it seems more likely that, since in some cases the condition developed before dosage had begun, *i.e.* somewhere about the 7th week of deficient diet, it was also due to the implantation upon the deficient animals of an infection not previously observed among the experimental animals in this laboratory although frequently described by other workers [Macy *et al.*, 1927].

Where possible an attempt was always made to cure a rat which failed during the experimental treatment, by giving cod-liver oil or other material rich in vitamin A; when the attempt succeeded a positive control was thus supplied but when it did not succeed the failure was not significant.

The diet with addition of a source of vitamin B complex was as follows:

Heated caseinogen	300 parts
Wheat starch	750 ,,
Irradiated hardened cotton-seed oil ...	225 ,,
Salt mixture [Hume and Smith, 1928] ...	75 ,,
Lemon juice	75 ,,
Distilled water	900 ,,

From 10 to 25 g. per head per day were eaten, 14 g. being a usual amount. The fractions to be tested for vitamin A were made up in the cotton-seed oil and administered separately with a pipette.

In previous experiments in which estimations of vitamin A have been made by Hume and Smith [1928], marmite has been relied upon as the source of the vitamin B complex, used in such an amount that an average daily consumption for a rat would be about 0·5 g. About the time when the present series of experiments was begun (July 1928) a number of cases was encountered where rats failed to respond by growth to doses of vitamin A believed to be adequate. These cases remained puzzling until one rat also developed skin lesions closely resembling those found in rats on a diet deficient in vitamin B_2 [Goldberger and Lillie, 1926; Chick and Roscoe, 1927]. Dried yeast auto-

claved at 120° for 5 hours, in which the antineuritic factor is almost completely destroyed while vitamin B_2 survives [Chick and Roscoe, 1927], was kindly supplied by Dr Chick and administered in amounts of 0·5 g. daily. Growth was restored and the dermatitis healed; the conclusion was drawn that marmite could not any longer be relied on as a constant source of supply of vitamin B_2. In the present series of experiments, marmite (75 parts in the above diet mixture) was at first retained and supplemented with 0·5 g. of the autoclaved dried yeast to every 10 g. of the wet diet. The animals used in the experiments, the results of which are given in Table I, received the vitamin B in this way. The animals used in the experiments, the results of which are given in subsequent tables, received no marmite in their diet. In those used in Table II 6 % of dried yeast from the Pharmaco Chemical Products Co. Ltd. was added to the wet diet, and in the final experiments on carotene this amount was increased to 10 or 12 %. The higher amounts appeared to give a more satisfactory result and also made it possible to rely more confidently upon the condition of the eyes as an index of vitamin A deficiency.

Preparation of lipoidal material from cabbage.

The method of preparing the material has already been described [Clenshaw and Smedley-MacLean, 1929]. The preliminary dipping of the leaves in boiling water, which is there described as rendering the leaves more brittle and more readily pulverisable, was used in preparing most of the material used for this investigation. It has however the disadvantage of setting free the chlorophyll so that this is also extracted in the light petroleum solution. If the leaves are dried at the ordinary temperature without the preliminary dipping in hot water, the light petroleum extract is yellow and is practically free from chlorophyll [Arnaud, 1885]. We have since found that a preliminary drying *in vacuo* before drying in the hot room also liberates the chlorophyll, but if the leaves are air-dried at a temperature of 37° the petroleum extract is yellow and appears to be free from chlorophyll. In nearly all the material used in this investigation, the leaves were subjected to the preliminary dipping in hot water, the petroleum solutions were deep green in colour and the lipoidal material contained chlorophyll. Treated in this way, 715 g. of dried white cabbage leaves yielded 7·58 g. of light petroleum-soluble material, whilst from 1300 g. of dried green cabbage leaves 25·83 g. of lipoidal material were extracted.

The material obtained from the green leaves was first tested for its activity in vitamin A. The medium chosen to dissolve the given extract was the same hardened cotton-seed oil as was used in the basal diet. In this preliminary experiment one set of doses was also given in paraffin oil in order to see if these were equally well absorbed. On the whole the effect seemed rather better with the hardened cotton-seed oil, especially in the case of the smaller doses and this medium was used in all later experiments. The results

are shown in Table I, which also contains the record of an experiment in which some dried green spinach leaves which had been several times extracted with light petroleum were extracted with acetone. Since xanthophyll is soluble in acetone and insoluble in light petroleum, this extract would contain the xanthophyll present in the leaves. No evidence of any vitamin A activity was found in this fraction, a result in agreement with that observed by Willimott and Moore [1927].

Table I. *Test of green cabbage and acetone-spinach extracts for vitamin A activity.*

Material tested	Dose mg.	No. of litter	No. of rat	Sex	No. of days maintenance	Growth in period of experiment g.	Notes
Green cabbage extract	5·0	1044	516	♀	28+	43	—
	„	1045	518	♀	28+	43	—
(*a*) In cotton-seed oil	„	„	519	♂	28+	66	—
	2·5	1044	517	♀	25+	15	Following larger dose for 15 days
	„	1045	525	♀	25+	15	Following larger dose for 14 days
	0·5	1044	515	♀	21+	25	Following larger dose for 4 days
	„	1045	523	♀	21+	13	Following larger dose for 5 days
(*b*) In paraffin oil	5·0	1044	512	♀	28+	43	—
	„	1045	524	♀	28+	40	—
	„	„	520	♂	28+	61	—
	2·5	1044	510	♀	18	3	Following acetone-spinach extract, died respiratory disease
	„	„	514	♀	25+	10	Following larger dose for 11 days
	0·5	1045	526	♀	21+	9	Following larger dose for 5 days
Acetone-spinach extract	10·0	1044	510	♀	8	2 to −8	Rapidly deteriorating, cured by green cabbage extract
	„	1045	527	♀	14	2 to −5	Deteriorating, cured by other material
	„	„	521	♂	15	−7	Deteriorating, cured by other material

Note. Behaviour of rats when the material tested for vitamin A is supplied after a preliminary depletion period of 5–6 weeks. The growth or maintenance is for a measured period of 28 days though in one or two cases the period was arbitrarily shortened from lack of material or other cause. Where the symbol + fails to follow the days of maintenance it is indicated that a decline set in. Success or failure to maintain should be taken as the criterion and the amount of growth taken as a subsidiary comment.

Preparation of the unsaponifiable material.

This was prepared from the light petroleum-soluble material by adding to its ethereal solution at laboratory temperature an excess over the calculated amount of an alcoholic solution of sodium ethoxide, and allowing it to stand overnight. On diluting and extracting the solution with ether the chlorophyll remains in the aqueous alkaline solution.

From the green cabbage, 10·9 g. of unsaponifiable matter were obtained, a yield of 0·84 % of the dried leaves; the iodine value (Hübl) was 110·2. The white cabbage gave 1·78 g. of unsaponifiable matter, a yield of 0·25 % of the dried leaves; its iodine value was 60·5. The green leaves gave therefore three to four times as much unsaponifiable matter as an equal weight of white cabbage and the substance obtained from the green leaves was much more unsaturated than that prepared from the white cabbage.

Quantities of 1 g. of the unsaponifiable material obtained respectively from the green and white leaves were dissolved in a small quantity of ether and added to 25 g. of hardened cotton-seed oil. The ether was then blown off from the melted fat by a current of CO_2.

The results of the biological tests are given in Table II.

Table II. *Test of unsaponifiable matter from green and white cabbage for vitamin A activity.*

See note under Table I.

Material tested	Dose mg.	No. of litter	No. of rat	Sex	No. of days maintenance	Growth in period of experiment g.	Notes
Unsap. matter from green cabbage	1·5	1142	531	♂	28+	49	— —
	1·0	,,	532	♂♀	28+	31	— —
	,,	,,	535	♀	28+	21	— —
	,,	1160	548	♂♀	—	—	Died at once
	0·5	1142	533	♀	28+	22	Survived a pregnancy, litter lost
	,,	1160	543	♀	28+	41	— —
	,,	,,	547	♂♀	28+	48	— —
	0·25	,,	538	♀	28+	23	Survived a pregnancy, litter lost
	,,	,,	541	♀	28+	26	— —
	,,	,,	544	♀	28+	28	— —
Unsap. matter from white cabbage	10·0	1160	539	♀	28+	16	— —
	5·0	,,	542	♀	11	−14	Cured by green unsap.
	3·0	,,	540	♀	24	21 / −10	Maintained to 28th day by increasing dose to 10 mg.
	,,	,,	545	♂	—	—	Died at once
	,,	,,	546	♂	14	15 / −22	Died
	1·5	1142	530	♂	16	9 / −27	Moribund, killed
	1·0	,,	534	♀	28+	13	— —
	,,	,,	536	♀	15	−25	Very ill, cured by green unsap.
	0·5	,,	537	♀	14	−34	Pregnant, died
Green cabbage unsap.	1·2	1162	553	♂	28+	56	— —
Fraction insol. in hot	0·6	,,	555	♂	28+	35	— —
and cold alcohol. Sol.	0·2	,,	550	♀	28+	15	— —
in light petroleum	,,	,,	551	♀	28+	23	— —
	0·03	,,	554	♂	23+	8	— —

Fractionation of the unsaponifiable material.

Having established that the green leaves yielded an unsaponifiable matter at least ten times as potent in vitamin A activity as the white, the next step

was to fractionate this material and to find out how far this activity could be concentrated. The first method used was by extraction with different solvents.

Cold alcoholic extract. The unsaponifiable fraction was twice extracted with cold alcohol and, from the cold alcoholic extract of 5 g. of the original unsaponifiable matter, crystals of a substance melting at 135° separated on standing; these gave a positive Salkowski reaction and probably consisted of a sterol. A very small amount of red crystals was also separated from this extract; the filtrate from these was evaporated to dryness and yielded 2·3 g. of residue having an iodine value of 130·7.

Hot alcoholic extract. The substance insoluble in cold alcohol was three times extracted with hot 96 % alcohol. From this crystals separated melting from 63° to 68°. By subsequent recrystallisation from alcohol white crystals melting at 75° were obtained, and a substance melting up to 68°. A preliminary investigation of this fraction was described by Clenshaw and Smedley-MacLean [1929], who isolated a hydrocarbon which they regarded as identical with the hentriacontane, $C_{31}H_{64}$, M.P. 68°, which they had already isolated from spinach. Subsequently Channon and Chibnall [1929] published the results of an investigation on cabbage and described the isolation of a hydrocarbon melting at 62·7°–62·8° and of a ketone $C_{29}H_{58}O$ melting at 80·5–81°; these they identified respectively as nonacosane and di-*n*-tetradecyl ketone. The hydrocarbon isolated by Clenshaw and Smedley-MacLean from spinach melted sharply at 68–68·5° and was analysed and satisfactorily identified as the hydrocarbon $C_{31}H_{64}$. The substance which they obtained from cabbage, melting at 68°, was only separated in very small amount: it was not analysed, and for its identification a mixed melting-point with the hydrocarbon obtained from spinach was relied upon. In the present investigation, working with a green Christmas cabbage, we isolated two substances melting at 63° and 80° to 81° respectively and confirm the results published by Channon and Chibnall. We propose to examine again the large white summer cattle cabbage used in their investigation by Clenshaw and Smedley-MacLean in order to determine definitely whether the substance isolated by them was identical with the hentriacontane of spinach or was a mixture of the two substances identified by Channon and Chibnall.

Light petroleum extract. A small amount of blackish oily residue (iodine value, 173) remained which was practically insoluble in hot and cold alcohol: this was extracted with warm light petroleum (B.P. 40° to 60°) and from the solution red crystals of carotene separated. After several recrystallisations from light petroleum 0·018 g. crystals, which softened at 174° and melted at 178°, were obtained; they had an iodine value of 324. Very much less of the material from the white leaves was available than from the green: the whole of it was found to be soluble in alcohol and there was no blackish residue, corresponding to that obtained in working up green cabbage, insoluble in alcohol and soluble in light petroleum and rich in carotene. The quantity of

carotene present must have been so small as to have been completely dissolved in the alcoholic solutions.

Colour test with antimony trichloride. These various fractions were tested by the method of Carr and Price [1926] with the following results.

Green cabbage:

(1) Unsaponifiable matter	Blue-green colour
(2) Cold alcohol extract, 0·016 g. per cc.	Blue-green colour
(3) White substance from hot alcohol extract, 0·020 g. per cc.	Colourless
(4) Ether extract, insoluble in alcohol, 0·003 g. per cc.	Green colour
(5) Carotene crystals, 0·001 g. per cc. ...	Permanent blue colour
(6) Carotene crystals, 0·0001 g. per cc.	Permanent blue colour

White cabbage:

(7) Unsaponifiable matter	Blue-green colour, much less intense than (1)

The fact that solutions of carotene give a blue and not a green colour may be interpreted as showing that the yellow colour of carotene disappears and that it is therefore the yellow coloured carotene itself that enters into combination with the antimony trichloride solution. The green colour produced by the yellow unsaponifiable fractions which contain carotene must therefore be ascribed to a mixture of the blue compound formed from carotene and antimony trichloride with a yellow substance which remains yellow and is not changed by the antimony trichloride solution.

Preparation of carotene.

It appeared from the above results that the activity was associated with the carotene-containing fractions, and was certainly greatest in the most unsaturated fractions. It seemed desirable therefore to isolate carotene from the unsaponifiable matter and to determine whether the vitamin A active fraction was still associated with the carotene crystals. Three materials were selected, cabbage, spinach and carrots, and from each of these a small specimen of carotene was prepared.

From cabbage. 10·9 g. of the unsaponifiable matter from green cabbage were fractionated as described above. A very small amount of carotene was obtained from the fraction soluble in hot alcohol: the fraction insoluble in alcohol but soluble in ether deposited dark reddish crystals which were several times recrystallised and finally obtained softening at 174° and being completely melted at 178°. Examination of a dilute solution of this carotene in light petroleum solution showed the presence of two bands extending from 450 to 455 $\mu\mu$ and from 478 to 486 $\mu\mu$ respectively.

From spinach. When similarly treated 11·05 g. of the lipoidal matter from spinach yielded 5·09 g. of unsaponifiable matter (I.V. 108). In this case the carotene was separated by dissolving the unsaponifiable fraction in carbon disulphide and fractionally precipitating it with alcohol. The earlier precipitates contained most of the impurities; the later (I.V. 183) were rich in carotene and were redissolved in carbon disulphide, again fractionally precipitated with alcohol, and then several times recrystallised from ether. The iodine value was 352. After further recrystallisation, the material melted at 163–164° and had an iodine value of 338. This fall in value cannot be regarded as significant since very small quantities of material were used for the determinations.

Part of the material (I.V. 183) was recrystallised from ether, dissolved in light petroleum, shaken up with chalk and filtered through a layer of chalk. The solvent was then evaporated from the filtrate and the residue used for a biological test on one rat.

From carrots. Slices of carrots were dried at 37° and extracted with light petroleum. The residue from the petroleum extract (I.V. 142·3) was worked up similarly to the unsaponifiable matter from spinach. The reddish yellow crystals first obtained had an iodine value of 181°. By fractionally precipitating with alcohol from their carbon disulphide solution, crystals were obtained melting from 164° to 170° (I.V. 220). By further purification 22 mg. recrystallised carotene of iodine value 300 were obtained. Examined microscopically it consisted of characteristic red diamond-shaped crystals; at this stage it was used for the biological tests, and was compared with the cruder specimen of iodine value, 220.

Solutions (0·14 %) of all three specimens of carotene in hardened cotton-seed oil gave a blue colour with the antimony trichloride reagent. A 0·07 % solution of the specimen from cabbage was also tested and gave a marked blue colour.

The evidence at present available as to the constitution of carotene, $C_{40}H_{56}$, indicates that it possesses 11 double bonds of which probably 8 are conjugated [Zechmeister and v. Cholnoky, 1928]. The iodine values for the specimens of carotene now examined varied between 300 and 350, corresponding to about 7 ethylenic linkages in the molecule. It seems unlikely that 22 halogen atoms would be added by interaction with the iodine reagent. It is known for instance that ethylenic linkages in the α–β position to an OH or COOH group do not react with the iodine reagent [Smedley-MacLean and Thomas, 1921], and the addition of so many halogen atoms would probably modify the reaction. The variations from 300 to 352 cannot be regarded as significant in view of the very small quantities of material which were used for these determinations; the experimental error was large, since only 2–3 mg. were available for the test, and might well be 10 % of the observed value.

The highest melting point of the various specimens of carotene was that from cabbage which softened at 174° and finally melted at 178°.

Table III. *Test of carotene for vitamin A activity.*

See note under Table I.

Material tested	Dose mg.	No. of litter	No. of rat	Sex	No. of days maintenance	Growth during period of experiment g.	Notes
Cabbage carotene:							
I.V. 330	0·01	1228	572	♀	28+	40	— —
M.P. 174–178°	,,	1227	577	♂♀	28+	76	— —
	0·005	,,	579	♀	28	7	Moribund at end of experiment with septic bladder condition
	,,	,,	578	♂	28+	60	— —
	0·003	1223	563	♂♀	28+	25	— —
	,,	1225	601	♂	28+	10	Following smaller dose, septic glands in neck
	0·002	1223	564	♂	28+	16	— —
	,,	1275	601	♂	6	− 5	Following smaller dose, changed to larger dose
	0·001	1223	561	♀	24	6 −15	Maintained to end of period on 0·002 mg.
	,,	1228	574	♂♀	4	− 2	Cured by cod-liver oil
	,,	1275	604	♂♀	3	− 5	Died—septic uterus
	,,	,,	605	♀	14	5 − 4	Haematuria
	,,	,,	601	♂	6	− 5	Changed to larger dose
Carrot carotene:							
(*a*) I.V. 220	0·04	1272	596	♀	28+	29	— —
M.P. 169°	0·02	1223	567	♂	28+	7	Following dose mother-liquor
	,,	1272	600	♀	28+	23	— —
	,,	,,	597	♂♀	28+	29	— —
(*b*) Recryst.	0·01	1223	560	♀	28+	31	— —
I.V. 300	,,	1228	573	♂	7	− 6	Fulminating corneal sepsis. Treated perhaps prematurely with other material—cured
	,,	1227	576	♂	28+	36	— —
	0·005	1275	607	♀♂	10	− 4	Cured by cod-liver oil
	,,	,,	602	♂	16	—	Changed to 0·01 mg. with temporary improvement
	0·004	,,	608	♀	13	− 8	Cured by cod-liver oil
	0·002	1275	603	♂♀	12	− 8	Cured by cod-liver oil
	0·001	1228	575	♂♀	5	− 4	Cured by other material
	,,	1275	608	♀♂	13	− 8	Changed to 0·004 mg.
	,,	,,	607	♀♂	12	−10	Changed to 0·005 mg.
	,,	,,	602	♂	7	− 8	Changed to 0·005 mg.
Spinach carotene:							
(*a*) I.V. 338	0·012	1300	611	♂	8	—	Haematuria, died, severe stone
M.P. 164°	,,	,,	616	♂	28+	36	Foetid urine
	0·008	,,	609	♀	28+	15	Haematuria before beginning dosage
	,,	,,	610	♀	18	4 −13	Haematuria—died
(*b*) I.V. 352	0·010	,,	613	♂	28+	51	Foetid urine — enlarged gland in neck
	,,	,,	614	♂	8	−10	Haematuria before beginning dosage—died
	,,	,,	615	♂	25	25 −14	Haematuria—recovered with cod-liver oil
(*c*) After shaking solution with, and filtering through, chalk	0·006	1300	612	♂	28+	27	— —

The separation of carotene from cabbage is certainly very incomplete, especially when, as in our separation, no stringent precautions were taken to guard against oxidation. The actual amount of crystals separated must represent only a small proportion of the carotene present in the original cabbage.

Figures are not available as to the minimum weight of fresh or dried cabbage which will maintain a rat on a vitamin-A deficient diet in a good state of health under the conditions of the experiment.

From the figures now given 11·17 kg. cabbage leaf gave 1300 g. dry material from which 25 g. lipoidal substance and 10·9 g. unsaponifiable matter were extracted. In our experiments 0·25 mg. of unsaponifiable matter was more than sufficient for the minimum dose to supply the vitamin A factor and must therefore contain the minimum dose of carotene, here determined as 0·003 to 0·005 mg. This would correspond to rather more than 2 % of carotene in the unsaponifiable matter. Channon and Chibnall [1929] estimate the amount of carotene in the acetone-ethereal extract of cabbage with which they worked as 0·86 % and as the unsaponifiable fraction would be only a portion of their total extract, these figures seem to be of a comparable order.

The biological results are given in Table III.

The specimen of carotene which melted at the highest temperature and was therefore presumably the most pure was that from cabbage which was active in a dose of 0·003 mg. *per diem*: in this group the division between the active doses which maintained life for 28 days with fair growth and those of 0·001 mg. which failed to do so was well brought out. The specimens of carotene from spinach and from carrots respectively were less pure, the melting point was lower and the positive dose required was appreciably higher, being in the neighbourhood of 0·01 mg. The animals were not in such good condition in the later groups used for these experiments and it is not therefore permissible to make the deduction that the purity of the carotene specimen is in inverse ratio to the dose required, but we can say that we found no indication that the activity of the dose diminished with increasing purity of the carotene. The residue from the mother-liquor from which we had separated the carotene crystals from cabbage was tested on three rats and gave negative results with doses of 0·005 and 0·02 mg., doses which were active in the case of the carotene crystals, so that there was a definite concentration of activity associated with the carotene crystals. The residue from which the carotene had been extracted with ether was fed to 5 rats and was inactive in doses up to 0·1 mg.

The material obtained by shaking up an ethereal solution of the spinach carotene in light petroleum with finely precipitated chalk and filtering through a layer of chalk, and evaporating the solvent, showed no diminution in activity; a dose of 0·006 mg. gave a good result but only one rat was available for this test (Table III, no. 612). It seems that the active substance, like the carotene, is not absorbed by chalk.

Discussion.

The results obtained with carotene are closely in agreement with those recently published by v. Euler *et al.* [1928, 1929] and by Moore [1929]. The association of vitamin A activity with the carotenoid pigments has been frequently referred to by different workers. The idea has been supported in a series of papers by Steenbock and Sell [1922], who however do not definitely identify the active factor as carotene: the association of the carotenoids with the active vitamin A substance was discussed by Rosenheim and Drummond [1920] but the identification of the vitamin A as carotene was definitely rejected by them and by Drummond, Channon and Coward [1925]. The latter observers isolated from carrots a specimen of carotene which after being recrystallised four times melted at 167·5°, and which they regarded as free from vitamin A activity, although the dosage given is not stated. More recently Dulière, Morton and Drummond [1929] have separated from carrots a specimen of carotene melting at 184·9° after recrystallising it several times from hexane and working under anaerobic conditions. This preparation was found to be almost inactive even in doses of 0·5 mg.

The fact that both specimens of carotene isolated by Drummond and his colleagues, melting respectively at 167° and 183·9°, were found to be inactive suggests that the inactivity of the purer specimen described in the more recent work is not due to the elimination of an active impurity although the possibility that Dulière, Morton and Drummond have isolated an inactive constituent from the crude carotene cannot be entirely excluded.

All the workers who find that carotene shows vitamin activity include fat in their basal diet, whereas Drummond and his co-workers give a fat-free diet. It is possible that in the latter case the pigment is not so well absorbed and it is interesting to note that Hart, Steenbock, Kletzien and Scott [1927] found that the antirachitic unsaponifiable constituent of cod-liver oil did not establish the calcium balance in a goat unless it was given in solution in a liquid fat such as corn oil, although the corn oil itself had no effect on assimilation[1]. On the other hand, Hume and Smith (unpublished experiment) have found that the unsaponifiable fraction from a light petroleum extract of spinach, freed from sterols, is an adequate source of vitamin A, when dissolved in liquid paraffin and added to a fat-free diet. The latter observation suggests that it may be some element of the unsaponifiable fraction of the fat in the diet, rather than the fat as fat, which is needed in conjunction with carotene, to produce the biological effect of vitamin A.

Evidence appears to be accumulating tending to show that more than one substance can function as vitamin A. Differences in the stability of the vitamin A derived from plant and animal sources have been described [Sherman, Quinn, Day and Miller, 1928], and it seems difficult to accept as

[1] Since writing the above, attention has been drawn to the influence of fat in the diet by Burr and Burr [1929] and McAmis, Anderson and Mendel [1929].

identical the highly coloured crystals which are associated with the vitamin A activity in plants with the much paler substances derived from animal oils. The recent publication of v. Euler, v. Euler and Karrer [1929], which records the inactivity of α-crocetin and the activity of its dihydro-derivative, furnishes evidence in support of the view that the property of vitamin A activity is to be regarded rather as belonging to a special grouping of atoms which may be common to several individual molecules than to a definite molecule.

Dulière, Morton and Drummond have laid stress on the appearance of an absorption band 608–612 $\mu\mu$ in the reaction product of antimony trichloride and specimens of the unsaponifiable matter from cod-liver oil which are very active in vitamin A and have drawn the inference that this band is associated with the actual vitamin A. The absorption spectrum of the blue compound produced when carotene reacts with the same reagent does not show this particular band [v. Euler *et al.*, 1928], though it is suggested that the difference may be due to the presence or absence of an oily medium. If the same vitamin A which produces this band be indeed a contamination in the carotene crystals, as Dulière *et al.* suggest, it is difficult to see why the characteristic 608–612 $\mu\mu$ band does not appear in the absorption spectrum of the carotene compound. Since the carotene crystals are as potent in vitamin A activity as any unsaponifiable fraction from animal oils yet investigated, one would expect the 608–612 $\mu\mu$ band to be as strongly marked in the spectrum of the carotene antimony trichloride compound. If the vitamin A of animal oils be indeed responsible for the production of the band, its absence when the very active carotene crystals are used would furnish additional evidence that the active substance in the cod-liver oil is not identical with that in the active carotene crystals.

Summary.

(1) The vitamin A activity of the unsaponifiable fraction from white cabbage is very small compared with that of the corresponding fraction derived from green leaves; this fraction, however, is active if given in sufficient amount. The respective minimal doses are about 10 and 0·25 mg.

(2) The vitamin A substance of green spinach and cabbage leaves and of carrots is contained in the most highly unsaturated fraction of the unsaponifiable matter and, as far as the process of purification here employed extends, remains associated with the carotene crystals.

(3) The carotene crystals obtained from cabbage softened at 174° and melted at 178°, those from spinach at 163–4° and from carrots at 164–9°. The vitamin A activity is certainly not diminished in the specimen of higher melting point and of therefore presumably greater purity: in this specimen the active dose lies between 0·002 and 0·005 mg. No claim to a great degree of purity in the specimens of carotene separated is made, since the work carried out was on too small a scale to admit of sufficient recrystallisations to ensure the separation of all impurities.

(4) It is possible that the crystals of carotene may themselves be homogeneous and active or that they may consist of two or more closely related substances only one of which possesses vitamin A activity.

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