LXXXIX. VITAMIN A AND CAROTENE.

I. THE ASSOCIATION OF VITAMIN A ACTIVITY WITH CAROTENE IN THE CARROT ROOT.

By THOMAS MOORE.

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From the Nutritional Laboratory, Cambridge.

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THE possibility that vitamin A activity may bear some relation to carotenoid pigmentation has attracted the attention of many workers during the past ten years. Of early investigations those of Steenbock and his colleagues [Steenbock, Boutwell and Kent, 1920; Steenbock, 1919; Steenbock and Gross, 1919, 1920; Steenbock and Boutwell, 1920, 1, 2; Steenbock, Sell and Boutwell, 1921; Steenbock and Sell, 1922] were perhaps the most extensive, and furnished a weight of evidence pointing to some connection between yellow pigmentation and vitamin A activity at least in so far as concerned vegetable sources. This connection was confirmed somewhat later by Coward [1923], who found that "some lipochrome (generally carotene) is always associated with the vitamin in plant tissues; and that where carotene is found, particularly carotene exposed to sunlight, there the vitamin can be expected to be present also."

Rosenheim and Drummond [1920], although denying that the vitamin could be either carotene or xanthophyll, were attracted by the possibility of a relationship with some unknown pigment, and suggested a parallelism between yellow coloration and vitamin A activity even in animal sources. In this direction, however, it soon became obvious that no relationship existed [Drummond and Coward, 1920]. It was true that yellow butter fat was an active source whereas colourless lard was inactive, but Stephenson [1920] found that butter fat could be completely decolorised by charcoal without losing its activity. Similarly in the case of cod-liver oil, found by Zilva and Miura [1921] to be an extremely rich source of the vitamin, pigmentation was valueless as a guide to activity; pale cod-liver oils displayed much greater activity than highly pigmented butter fat. Working upon different lines Palmer and his colleagues, who had been the first to prove that the lipochrome pigments of animals are derived from the plant pigments present in their food [Palmer and Eccles, 1914], achieved success in rearing chickens and rats upon diets almost devoid of carotenoids [Palmer and Kempster, 1919; Palmer and Kennedy, 1921].

The above indirect evidence, which seemed to rule out the possibility that vitamin A might be a pigmented body, was supplemented from time to time by direct tests upon isolated pigments. Steenbock, Sell, Nelson and Buell [1921] made a preliminary announcement that carotene, even after several recrystallisations, possessed the activity of vitamin A, but no detailed account of this work seems to have been published. Drummond [1919], on the other hand, found that while impure carotene showed signs of activity in the rat at doses of about 0.3 mg. daily, similar doses of the pure pigment were quite ineffective. Stephenson [1920] tested carotene recrystallised four times and obtained a similar negative result. Subsequently Drummond, Channon and Coward [1925], dissatisfied that the activity of cod-liver oil concentrates might not after all be due to traces of carotenoids, carried out further tests, finding both carotene and lycopin to be inactive. A similar result was obtained in the case of xanthophyll by Willimott and Moore [1927]. Underhill and Mendel [1928], on the other hand, found carotene to possess physiological activity of a kind, since it was found to be highly effective in curing "black tongue" disease in dogs.

In the face of this accumulation of evidence it was not unnatural that the idea of the identity of vitamin A with a carotenoid pigment became generally discredited. Even at an early date Steenbock, Sell and Buell [1921] had been forced to admit that, unless the carotenoids could exist in leuco form, "their coincident occurrence in nature might be due to physiological determination."

The work of Euler, Euler and Hellström [1928], however, has reopened the whole question of the vitamin A activity of the carotenoids. These workers pointed out that in previous biological tests of carotene no provision was made for the presence of vitamin D in the diet, and that the results obtained cannot therefore have been reliable. Attracted by similarities between the colour reactions given by the vitamin and by certain carotenoids with the antimony trichloride reagent [Carr and Price, 1926], they put carotene to further physiological tests, with the result that they found it to be active in doses of 0.005 mg. per rat daily.

It may be said that the general resemblance of the colour reactions of the vitamin to those of the carotenoids had been already familiar [Rosenheim and Drummond, 1920, 1925; Takahashi *et al.*, 1925] and the main interest of the new work obviously rested in the claim that chemically pure carotene could restore growth in rats suffering from vitamin A deficiency. In view of divergent spectroscopic data it was considered improbable by the Swedish workers that the vitamin A of cod-liver oil was carotene itself, and the suggestion was put forward that several substances possessing the physiological activity of the vitamin might exist. The vitamin A of cod-liver oil, for example, might well be derived from the carotenoids of marine algae. In a later communication [Karrer, Euler and Euler, 1928] the suggestion has been made that vitamin A activity is not the property of any single substance, but is due to the presence in various molecules of the "polyene" grouping, a

system of double bonds which gives rise to colour reactions and which is held to possess catalytic powers *in vivo*.

Preliminary experiments by the writer in confirmation of the activity of carotene have been mentioned elsewhere [Moore, 1929]. Two samples of the pigment, kindly made available by Prof. von Euler and Dr R. A. Morton, were both found to restore good growth in rats in doses of 0.01 mg. daily. Dulière, Morton and Drummond [1929] on the other hand, after preparing an exceptionally pure sample of carotene (M.P. 184.9°), have obtained no evidence of growth-promoting power, and have suggested that the specimens used by Euler and his colleagues were probably contaminated with traces of the vitamin carried down as impurity.

In the present communication a study of the vitamin A activity of the carrot root will be described. It will be shown that this material provides a much richer source of vitamin A activity than has hitherto been supposed, and that activity tends to be concentrated in the carotene during its isolation, remaining in the pigment through all stages of purification that have so far been applied. This finding is in agreement with the conclusion of Collison, Hume, Smedley-MacLean and Smith [1929] that carotene isolated from cabbage fat also possesses intense vitamin A activity.

EXPERIMENTAL.

In testing the vitamin A activity of the various materials the following basal diet was used:

Casein (Glaxo "Vitamin Free")	...	...	20 %
Rice starch	...	...	60 %
Arachis oil	...	...	15 %
Salt mixture	...	...	5 %

The vitamin B complex was supplied by the addition of 7.5 % of a standard marmite extract; vitamin D by an allowance of one drop of "Radiostol" (irradiated ergosterol in arachis oil) per animal daily.

Young albino rats receiving this diet at first continued to grow for varying periods, but in the end invariably declined and died. During decline signs of xerophthalmia and of the characteristic lung troubles were frequently in evidence. Growth curves of control rats are shown in Fig. 1¹.

The vitamin A activity of fresh carrot. Calculations based upon the reported activity of carotene led to the obvious implication that if the claim was valid the carrot must be a much richer source of vitamin A activity than hitherto supposed. That this is actually the case has been shown in the following experiments.

¹ It will be noticed that the maximum weights attained by the rats varied within wide limits. Such differences were probably attributable in part to differences in vitamin A reserves between various litters and in part to variations in the initial weights at which the animals first received the experimental diet. The degree of variation in the negative control animals was emphasised by the selection for this purpose of certain rats which attained unduly high maximum weights.

The test rats were restricted to the basal diet until definite signs of vitamin A deficiency had become obvious, whereupon fresh carrot root, cut into pieces of 50, 100 or 200 mg., was presented daily before the basal ration.

Fig. 1. Negative control rats.

x Died

D Discontinued.

Fig. 2. Rats receiving fresh carrot.

The responses obtained are shown in Fig. 2. In all rats receiving 200 or 100 mg. of carrot xerophthalmia was cured and steady growth was resumed

until the discontinuation of the experiment. In one rat receiving 50 mg. of carrot no response was obtained. It would therefore appear that the allowance of fresh carrot necessary to supply the rat's requirement for vitamin A is about 100 mg., although it is unsafe to assume from the evidence of a single animal that a slightly lower dose might not sometimes be effective.

The vitamin A activity of carrot fat. The fresh carrot is said to contain 0.6 % of fat. If we assume that all the vitamin A activity of the root is removed with the fat during the extraction with light petroleum it is obvious that 100 mg. of fresh carrot must be represented by 0.6 mg. of the fatty residues obtained after the evaporation of the light petroleum.

In a trial experiment (prior to the extraction of the pigment on a larger scale) about 75 kg. of carrots were first sliced and dried in a hot room, and were then forwarded to Messrs British Drug Houses, Ltd., who kindly carried out the extraction. After grinding to a fine powder the material (now amounting to 7.5 kg.) was extracted repeatedly with light petroleum, which was finally removed under diminished pressure.

Fig. 3. Rats receiving carrot fat.

On addition of excess of methyl alcohol to the extract so obtained a yield of crude carotene amounting to about 2 g. was precipitated. The unprecipitable fraction, a deep crimson fat of low melting-point, amounted to some 45 g.

The main interest of the present experiments lay in the comparison of the vitamin A activity of carotene with that of the mother-liquor from which it had been separated. The biological test, therefore, was carried out upon the unprecipitable fraction rather than upon the original extract. The growth curves of four rats which received daily doses of 0.4 mg. of this material are shown in Fig. 3. In all four cases a resumption in growth was observed, but one animal died suddenly after receiving the fat for a short period.

Biological experiments at a lower dosage were not carried out. Colorimetric data, however, indicated that the activity of this fat was considerably inferior to that of crystalline carotene. The chromogenic value of the fat,

estimated by means of the antimony trichloride reagent, was found to be only 12 Lovibond blue units per mg. per cm. cube of reagent, as against 165 units per mg. of carotene tested under similar conditions. The blue coloration produced by the fat with the antimony trichloride reagent when inspected by means of a direct-vision spectroscope was found to be characterised by an absorption band at about $590\text{ }\mu\mu$.

Fig. 4. Rats receiving carotene.

Rat No.	Dose of carotene
1, 2	Sample A. 0.02 mg.
3, 4, 5	Sample B. 0.01 mg.
6, 7, 8, 9, 10	Sample C. 0.01 mg.
11, 12	Sample D. 0.01 mg.

××××× Rat receiving inactive substance.

The vitamin A activity of carotene. Four samples of carotene in all have been tested. Of these the samples A and B, both specimens of a fair degree of purity, were made available through the kindness of Prof. von Euler and of Dr R. A. Morton. Sample C (M.P. 174°) was prepared in Karrer's laboratory by submitting a sample already tolerably pure (M.P. 169°) to nine further crystallisations from light petroleum. Sample D (M.P. 174° in air¹) was prepared by the writer from a specimen (M.P. 174° in air¹) already purified to a

¹ The melting-points of carotene samples vary considerably according to the technique employed. The melting-point of this specimen, for example, was found by Prof. Drummond, to whom my best thanks are due, to be about 180° when heating was carried out rapidly in an atmosphere of nitrogen.

high degree by many crystallisations from methyl-alcohol-chloroform mixtures, by submitting it to a further five crystallisations from cyclohexane.

In order to facilitate administration the samples to be tested were dissolved in warm arachis oil in such concentration that standardised drops of the oil (20 mg.) contained 0.01 mg. of carotene. The growth responses obtained by administering the pigment daily to rats deficient in vitamin A are shown in Fig. 4. The activity of all the samples at this low level was unquestionable. Xerophthalmia was invariably cured and good growth restored over prolonged periods¹.

Spectroscopic examination of the blue colour produced by samples of carotene when treated with antimony trichloride showed a marked band at about 590 $\mu\mu$, *i.e.* the position described by Euler, Euler and Hellström, and confirmed by Dulière, Morton and Drummond. No trace of absorption was visible at 610 $\mu\mu$, the position considered characteristic of the cod-liver oil vitamin A.

DISCUSSION.

The above results confirm the claim of Euler, Euler and Hellström that carotene possesses the physiological action of vitamin A. The suggestion of Dulière, Morton and Drummond that pure carotene is inactive, and that the activity reported by the Swedish workers may have been due merely to contaminating traces of vitamin A, must be considered improbable for the following reasons. (1) Carotene samples of a high degree of purity were found invariably to be active. (2) The activity of the carrot fat from which the carotene was crystallised was inferior to that of the isolated pigment. The activity of the pigment therefore could not have been due to the presence of fat carried down as impurity. (3) Both carotene and carrot fat produce with antimony trichloride a blue coloration which is characterised by an absorption band at 590 $\mu\mu$. If the physiological activity of these sources were attributable to the same vitamin A as contained in cod-liver oil, intense absorption would be anticipated at 610 $\mu\mu$, but no band was visible in this position.

The possibility of the presence of an uncharacterised active impurity, even in the purest samples of carotene so far obtained, cannot of course be ruled out. Neither can we be sure that carotene samples, even if pure, do not tend to change with the formation of an active product during the processes involved in the administration of test doses to rats. It seems highly probable, however, that the samples found active in the present experiments differed but little in regard to purity from those found inactive by Drummond and

¹ In order to decide whether the addition of vitamin D to the test diet was really essential in maintaining growth, two rats (Sample A) were deprived of the customary allowance of "Radiostol," but were given carotene (0.02 mg.) almost from the start of the experiment. Under these conditions no check in growth was observed. Whether growth may continue in the absence of vitamin D (as suggested by Euler, Rydbom and Hellström, 1929) or whether the present test diet was not free from traces of this vitamin cannot yet be decided. A direct test of the vitamin D content of carotene is in progress.

his colleagues, and that the main cause of such completely divergent results may well be found in some unsuspected difference in biological technique. It may be pointed out that, whereas those workers who have employed basal diets containing fat have obtained positive results, Drummond and his colleagues, presumably using the standardised "fat-free" diet of their laboratory, have obtained a negative result.

While there can be no doubt as to the activity of carotene under the experimental conditions described, it remains to be explained how molecules, differing so obviously as do carotene and the vitamin A of cod-liver oil, can be interchangeable in function. The "polyene" theory of Euler and his colleagues is open to the criticism that a very low order of specificity, quite unlike that of vitamin D, must be implied. Preliminary experiments by the writer, on the other hand, have indicated that a chemical relationship between carotene and vitamin A may exist. It is hoped to make this question the subject of a later communication.

The high vitamin A activity of the carrot may be of interest from an economic standpoint. The minimum dosage of 100 mg. found effective in the present experiments contained only 10 mg. of dry matter, and represented only one-tenth of the dosage previously considered necessary [Osborne and Mendel, 1920].

SUMMARY.

1. Under the experimental conditions specified, fresh carrot root was found to be a much richer source of vitamin A activity than hitherto supposed. Daily doses of 100 mg. of the fresh root sufficed to cure xerophthalmia and restore good growth in rats deprived of vitamin A.
2. Under similar conditions a sample of carrot fat, from which much of the carotene had been removed, was found to be active in a dose of 0.4 mg. daily.
3. Carotene (m.p. 174° in air) was found, even after many recrystallisations, to be active in doses of 0.01 mg.
4. A possible explanation of the divergent results of other workers is put forward.

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