LXXIX. VITAMIN A AND CAROTENE.

V. THE ABSENCE OF THE LIVER OIL VITAMIN A FROM CAROTENE.

VI. THE CONVERSION OF CAROTENE TO VITAMIN A *IN VIVO*.

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V. The absence of the liver oil vitamin A from carotene.

There is now general agreement that carotene of the highest purity so far attained possesses intense vitamin A activity [Euler, Euler and Hellström, 1928; Moore, 1929, 1, 2; Collison, Hume, Smedley-MacLean and Smith, 1929; Kawakami and Kimm, 1929; Hume and Smedley-MacLean, 1930], and the negative results of early workers must be attributed either to failure to supply vitamin D in the test diet, or more probably to insufficient precautions against oxidation of the pigment during the administration of the test doses.

Nevertheless it has been obvious for many years that carotene cannot be identified with the vitamin A of liver oils, since this factor must presumably be an almost colourless substance, differing notably from carotene in its solubility and adsorption properties. Thus vitamin A, present in unknown amounts in the pale orange-coloured liquid concentrates of cod-liver oils, is readily soluble in almost all organic solvents, including methyl alcohol and natural fats. Carotene, on the other hand, although not greatly exceeding the best cod-liver oil concentrates in physiological activity, is so deeply pigmented as to appear almost black in the pure state, and is readily soluble only in such solvents as chloroform, carbon disulphide and hot cyclohexane, being sparingly soluble in natural fats and almost insoluble in methyl alcohol. Similarly carotene is much more readily absorbed from its solutions by charcoal than vitamin A, the difference being so great as to afford an easy means of separation [Stephenson, 1920]. It is necessary, therefore, to account for the possession of a common physiological activity by two very divergent sources, and in the first place to ensure that the activity of carotene is not due to the presence of the liver oil factor in an adsorbed state, or in some other condition that would alter its normal solubility properties.

In supporting the contention that the activity of carotene samples might be due to contamination with the familiar form of vitamin A, Dulière, Morton

and Drummond [1929, 1] described a careful colorimetric and spectroscopic differentiation of carotene from the vitamin A of cod-liver oil. They found, in confirmation of Euler, Euler and Hellström, that the blue colours produced in the $SbCl_3$ reaction were of slightly different shade, that of carotene being characterised by an absorption band at 590 $\mu\mu$, that of vitamin A by a band at 608–612 $\mu\mu$, while in regard to the ultra-violet absorption spectra vitamin A was associated with a band at 320–330 $\mu\mu$, which was absent in the case of carotene. Carotene and the vitamin were therefore certainly different, but since it was also found that less pure specimens of carotene, similar to those found active by other workers, differed but little in their spectroscopic behaviour from highly purified samples, the additional suggestion was made that the presence of effective amounts of vitamin A in carotene might escape spectroscopic detection by reason of more intense superimposed absorption due to the pigment itself [Dulière, Morton and Drummond, 1929, 2].

Although Hume and Smedley-MacLean [1930] have now shown that even the most carefully purified carotene exhibits vitamin A activity, provided that the test doses are made up in a solvent in which decomposition does not take place, the above criticism would still seem to apply, since the persistence of traces of the liver oil vitamin A in carotene of the highest melting point might well be concealed if the absorptions due to the pigment were in fact of relatively overwhelming density. Capper [1930] has already shown that this condition does not hold good in regard to the absorption band at 328 $\mu\mu$. In the experiments described below it will similarly be shown that the blue colour given by carotene in the $SbCl_3$ reaction is of insufficient relative density to conceal the presence of effective amounts of the vitamin. This conclusion is in agreement with the previous qualitative observation that no sign of a maximum at 610–630 $\mu\mu$ could be observed in the colours produced in the $SbCl_3$ reaction by highly active samples of carotene [Moore, 1929, 1].

Experimental.

Method. Specimens of carotene (M.P. 174° and 178°) were compared colorimetrically with specimens of cod-liver oil concentrates (kindly supplied by Messrs Joseph Nathan and Co., Ltd., and Messrs Lever Brothers, Ltd.) using the technique previously described [Moore, 1929, 2]. The same materials were then tested biologically at doses graded to run parallel to the colorimetric values.

Colorimetric results. The chromogenic activities of the various materials were determined several times in each case and may be approximately represented by the following values[1].

[1] Although the tintometer technique certainly yields results of great value to the individual worker some uncertainty must be felt in expressing the values so obtained on an absolute scale. Apart from avoidable complications arising from variations in temperature and reagent [Wokes and Willimott, 1927; Evers, 1929, 1, 2] or from accelerated fading due to the presence of unsaturated acids [Norris and Danielson, 1929; Norris and Church, 1930] a personal element cannot be excluded in matching the shades produced. The results presented in the test, however, were obtained with the utmost care on the same occasion, and can in any case be regarded as giving satisfactory relative values.

	Blue units per mg. per "1 cm. cube" $SbCl_3$ reagent
Carotene (M.P. 174°)	180 at 590 $\mu\mu$
„ (M.P. 178°)	180 „
Concentrate A	180 at 610–630 $\mu\mu$
„ B	270 „ „

Biological tests. The biological work was carried out in several separate sections, rats nos. 1, 2, 3, 4 (albinos), nos. 5, 6, 7, 8, 9, 10, 11, 12 (albinos), nos. 13, 14, 15 (piebalds), and nos. 16 (piebald), 17, 18, 19 (albinos), being used in successive series of experiments. The usual curative technique was invariably employed, all the animals receiving the vitamin A-free diet previously employed, with the exception of rats nos. 13, 14, 15, which were kept under the care of Miss V. R. Leader at the Sir William Dunn Institute, where they received a slightly different basal diet (caseinogen 23 %, rice starch 40 %, cane sugar 17 %, palm kernel oil 15 %, salt mixture 5 %, + marmite extract 7·5 %).

Since all the materials had been found in earlier experiments to give positive results at 0·01 mg. per rat daily, attention was directed to dosages slightly below this level. The test doses were made up in arachis oil, one of the carotene samples (M.P. 174°) being dissolved by warming in the oil, the other (M.P. 178°) being added to the oil in hot cyclohexane, which was then removed under diminished pressure. During the experiment all the test solutions were kept in cold storage except when actually in use, and to ensure that no serious deterioration had taken place were tested by the $SbCl_3$ reagent at the conclusion of the experiment. The growth curves obtained are shown in Fig. 1.

Discussion.

In the above experiments carotene was found to be effective at levels down to 0·004 mg. per rat per day, while, with the exception of a single rat recovering on 0·002 mg., lower doses were found to be ineffective. The more active of the two concentrates tested was effective in curing a single rat at 0·0033 mg. but negative results were obtained in all other cases. It would thus appear that weight for weight the carotene samples were of slightly greater biological activity than the concentrate.

From the parallel colorimetric determinations 0·004 mg. of carotene may be considered equivalent to 0·7 Lovibond blue unit at 590 $\mu\mu$, while 0·0033 mg. of concentrate A corresponds to 0·9 blue unit at 610–630 $\mu\mu$. The colour value given by a minimal dose of the pigment therefore appears to be slightly smaller than that given by a minimal dose of concentrate. Since the possibility of the concealment of effective amounts of the liver oil vitamin A in carotene would imply that the colour value given by a minimal dose of carotene should be much greater than that given by a minimal dose of the concentrate this result affords strong evidence against this view. When in addition the work of Capper on the absence of the 328 $\mu\mu$ absorption band from carotene is taken into

account the only reasonable conclusion to be reached is that the activity of the pigment certainly cannot be attributed to direct contamination with vitamin A, or at least with the chromogen associated with that factor in liver oils.

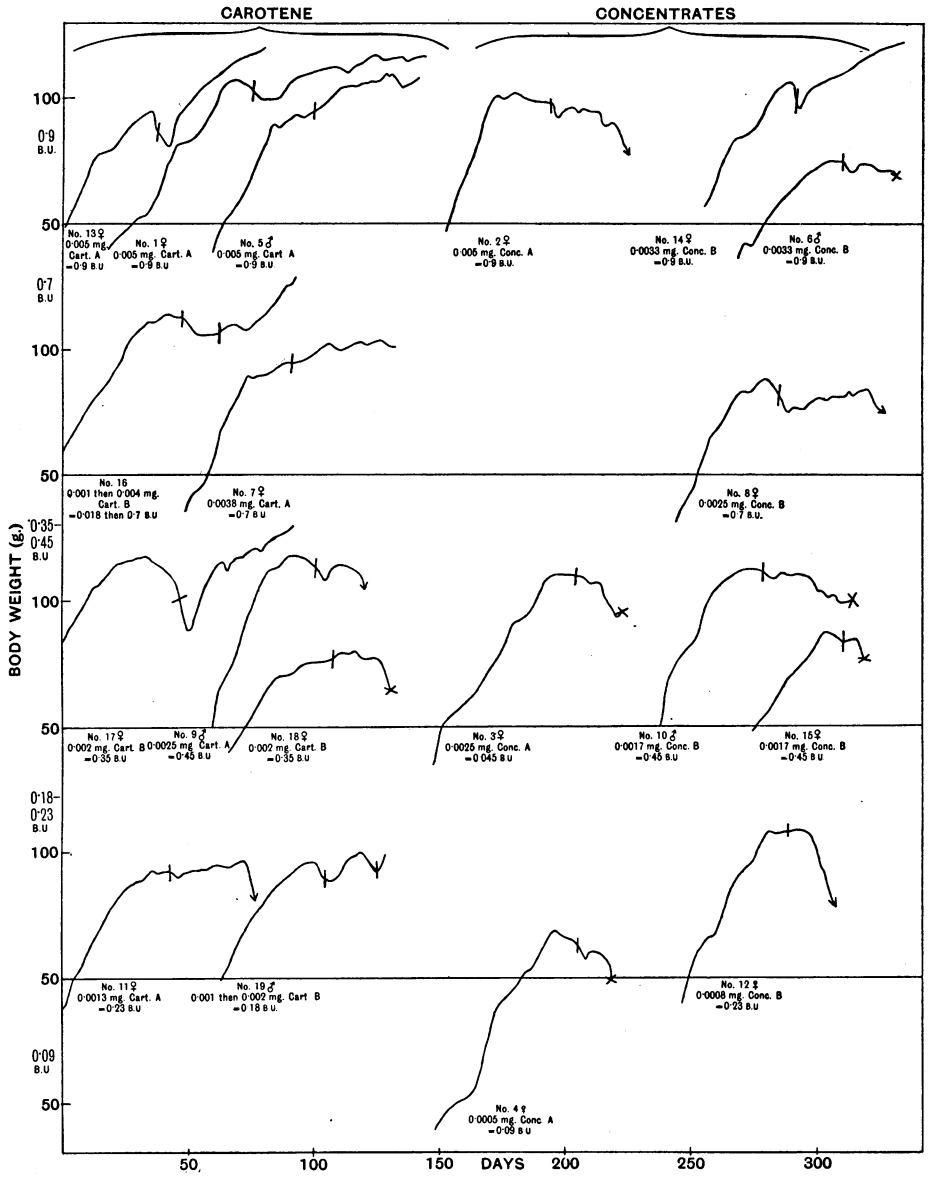

Fig. 1. The relative vitamin A potencies of carotene and cod-liver oil concentrates. B.U. = Lovibond blue ("1 cm. cube") unit. Cart. A = carotene (M.P. 174°). Cart. B = carotene (M.P. 173°). Conc. A, B = concentrate A, B.

VI. The conversion of carotene to vitamin A *in vivo*.

The question was once raised by Palmer [1919] as to whether it was logical to test the physiological activity of carotene in an animal, such as the rat, from which it was invariably absent. Answering the question in the negative Palmer and Kennedy [1920] attacked the question of the alleged identity of carotene with vitamin A by indirect means, and succeeded in proving conclusively that rats could thrive on diets almost completely freed from carotenoids. The assertion was therefore made that the pigment and vitamin were "neither identical nor necessarily associated."

The work of Euler, Euler and Hellström on the activity of carotene therefore seemed in conflict with preconceived ideas of the nature of the vitamin, while the later statement [Euler, Euler and Karrer, 1929, 1, 2] that excess of carotene could be stored in considerable amount in the liver oil of the rat was in direct contradiction to the finding of Palmer and Kennedy[1]. The Swedish workers, however, realised that carotene and the vitamin A of cod-liver oil were not identical, and suggested that several substances of carotenoid or "polyene" type might possess vitamin A activity.

In the first paper of this series [Moore, 1929, 1] it was pointed out that the "polyene" theory would imply an unexpectedly low order of specificity in vitamin A action, and the alternative suggestion was made that a chemical relationship between carotene and the vitamin might exist. Preliminary experiments supporting this view have already been described elsewhere [Moore, 1929, 3]. In the present paper more extensive evidence on the same point is presented.

Experimental method.

The method adopted in proving the conversion of carotene to the vitamin was essentially as follows. Young albino rats were "run out" upon a basal diet deficient in vitamin A [Moore, 1929, 1] until the "plateau" condition indicative of exhaustion of reserves of the vitamin was attained. Certain of the rats were then killed in this condition. To the remaining rats, varying amounts of carotene, either in the purified state or as red palm oil or carrots, were administered until complete cures and considerable increases in body weight had been effected. These rats were then killed.

The livers of all the rats were dissected out, finely minced, and mixed with twice their bulk of 5 % aqueous KOH, after which they were placed in cold storage until a suitable opportunity for examination arose. Even pro-

[1] (*Note added in proof.*) In a recent communication accessible to the present author only after the above paper went to press, Euler and Rydbom [*Svensk. Kemisk. Tidskrift*, 1929, **41**, 223] have shown that when rabbits are fed carrots or carrot extracts a "pale yellow coloured carotenoid," and not carotene, is stored in the liver. Although no attempt has been made to identify this "carotenoid" with the classical vitamin A, it is obvious that this result is in complete agreement with the experimental findings described in the present paper in the case of the rat.

onged storage did not lead to deterioration, although in most cases examinations were carried out in the course of a few days. On removal from cold storage the gelatinous masses were gently warmed until almost complete solution of the liver tissues was effected. The solutions were then extracted four times with about twice their volume of ether. The combined extracts in each case were washed thoroughly three times with water, and if necessary dried by shaking with anhydrous Na_2SO_4. The ether was then removed by evaporation under diminished pressure in a current of nitrogen, and the residual liver oils tested immediately for carotene and vitamin A by colorimetric methods.

The colorimetric differentiation of carotene from vitamin A.

It should be noted that, since carotene and the liver oil vitamin A both give positive results when tested biologically, it is unavoidable that resource to colorimetric data should be made in studying the conversion of one to the other. The validity of the colour reaction of the vitamin has indeed been questioned from time to time by several workers, but Drummond and his colleagues [see Ahmad and Drummond, 1930] have generally shown that such criticisms have been quite unjustified.

Suppose we compare the natural yellow colour of carotene itself in chloroform solution with the blue colours which it produces when it is dissolved in the same volume of $SbCl_3$ reagent. A yellow to blue ratio of about 11 to 1 is then found. (The yellow colour of the sample tested is replaced on treatment with the $SbCl_3$ reagent by a much less intense blue colour.) On the other hand, when we compare the faintly yellow colours of cod-liver oil concentrates with the corresponding $SbCl_3$ blue colours a ratio yellow to blue of about 1 to 100 is found. (The concentrate when so dilute as to be almost colourless will produce on treatment with the $SbCl_3$ reagent an intense blue colour.) Thus when one constituent is present in a given material in predominant amount the yellow to blue ratio suggests at once whether this constituent is a carotenoid or the almost colourless vitamin, while in the case of equal mixtures a rough working approximation as to the probable proportion is afforded.

In the present work the yellow to blue ratio was used as a first indication of the nature of the chromogen present in the liver oils. Results were then confirmed by the position of the absorption band produced in the $SbCl_3$ reaction (carotene 590 $\mu\mu$; vitamin A 610–630 $\mu\mu$). Certain samples were then despatched to Mr N. S. Capper for examination in regard to the ultra-violet absorption, while others were employed in biological tests.

Results.

The results obtained are shown in Table I. It will be seen that the liver oils of rats nos. 1 to 10, which had either died or been killed in a condition of acute avitaminosis A, invariably gave negative results when tested with the $SbCl_3$ reagent. The liver oils of rats nos. 11 to 14, in which growth had been restored by small doses of carotene, similarly gave negative results, as indeed would be

expected at levels sufficient to meet immediate needs but insufficiently liberal to permit storage of the vitamin. On the other hand, in the case of the liver oils of rats nos. 15 to 22, which received either purified carotene, red palm oil, or fresh carrots in considerable excess of the minimal dosage, strongly positive reactions were given, which in all cases could be attributed to the appearance of large amounts of vitamin A, and not to the storage of unchanged carotene.

Since the strongest evidence in favour of the conversion must be derived from those cases in which carotene of the highest purity was used, special attention may be first directed to rats nos. 15 to 18 which received carotene (M.P. 178° uncor.) which had been obtained from carrots and recrystallised twelve times from cyclohexane, operations being conducted for the most part in an atmosphere of nitrogen. The pigment was added in cyclohexane to the arachis oil used in compounding the basal diet, the cyclohexane being removed under diminished pressure. After having attained the "plateau" condition each rat received the carotene during from 16 to 26 days at the rate of 0·75 mg. per 10 g. of diet eaten, which was the average amount consumed daily. At autopsy, despite very liberal amounts of pigment ingested, the body fat was always found to be quite colourless, apart from occasional small loci in regions where the intra-peritoneal fat lay in contact with the gut wall. The liver oils were certainly yellower than the liver oils of the control rats which had received no carotene, but these increases in yellow values were trivial when contrasted with the enormous concentration of yellow pigmentation that would have been expected if the intense blue colours now given in the $SbCl_3$ reaction had been due to unchanged carotene. The following observations confirm the view that conversion had been effected. (1) These blue colours were now characterised in all cases by a well-defined band at 610–630 $\mu\mu$, the position characteristic of vitamin A, and not at 590 $\mu\mu$, the position characteristic of the ingested carotene. (2) In preliminary work by Mr N. S. Capper (private communication) it was found that the untreated liver oil now displayed the absorption band at 320–330 $\mu\mu$ characteristic of vitamin A. (3) As a final precaution against the possibility that the chromogen should represent an inactive degeneration product of the pigment the vitamin A activity of specimen liver oils was confirmed by biological tests (Fig. 2).

In regard to rats nos. 18 to 22 which received even more liberal supplies of carotene as red palm oil or carrots, similar results were obtained, in which the yellow and blue values of the liver oils attained somewhat higher levels. For example the liver of rat no. 21, which had received during several weeks a diet consisting largely of carrots, was bright orange in colour. Such obvious pigmentation might at first sight be taken to support the claim of Euler, Euler and Karrer that the carotene had been stored in an unchanged condition, but it may be pointed out that the blue value of the liver oil would have corresponded to the presence of about 80 mg. of the pigment, *i.e.* the liver oil would have appeared deep crimson and would have deposited crystalline carotene freely on standing.

Expression of results.

The colorimetric examination of the liver oils was carried out according to the technique previously described [Moore, 1929, 2]. The method of expressing results may be explained most simply by an actual example.

Rat no. 18. Liver oil dissolved in 10 cc. chloroform.

Yellow units. The colour of this solution was matched by Lovibond glasses 4 yellow, 0·7 red. Therefore total number of yellow units in liver = 4 × 10 (volume of solution in cc.) = 40 units.

Blue units. 0·05 cc. of the above solution was made up with washings of chloroform to 0·5 cc. $SbCl_3$ reagent (2 cc.) was then added. The resulting blue colour was matched by Lovibond glasses 4·0 blue, 1·0 yellow (average of several readings). Therefore total number of blue units

$$= 4{\cdot}0 \times \frac{2{\cdot}5 \text{ (volume of reaction mixture)}}{0{\cdot}05 \text{ (volume of solution used in test)}} \times 10 \text{ (volume of the original solution)}$$

= 2000 units.

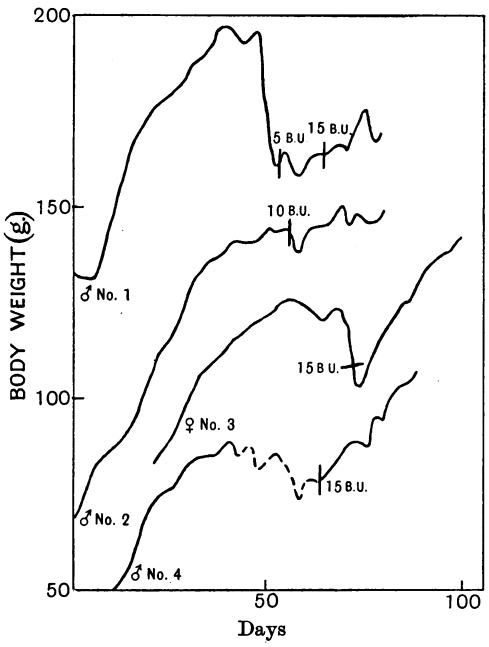

Fig. 2. The vitamin A activity of the liver oils of rats (no. 15 and 18 of Fig. 1) which had received excess of carotene.

Doses are expressed in Lovibond blue ("1 cm. cube") units. Rat no. 3 was completely cured of severe xerophthalmia and a septic cheek. N.B. Rat no. 4 received an ineffective material in a separate experiment during the period indicated by the broken curve.

It should be noted that the yellow and blue values so expressed are directly comparable. The method of calculation will be seen to be exactly the same in each case when it is remembered that the addition of the $SbCl_3$ reagent in obtaining the blue value necessarily involves a further dilution of the chromogen.

Table I. *Analyses of liver oils for vitamin A and carotene.*

Rat no. and sex	Body wts.: initial; plateau; final (g.)	No. of days on vitamin A-free diet	Dosage of carotene (mg.)	No. of days of carotene feeding	Total Lovibond units ingested by rat: Blue units in $SbCl_3$ reagent at 590$\mu\mu$	Total Lovibond units ingested by rat: Yellow units in $CHCl_3$	Total Lovibond units found in liver: Blue units in $SbCl_3$ reagent at 610–630$\mu\mu$	Total Lovibond units found in liver: Yellow units in $CHCl_3$	Total yellow units to be expected in liver if blue chromogen were carotene
			Rats receiving vitamin A-free diet. No treatment.						
1 ♂	54, 171, 124 (D)	77	—	—	—	—	0	4	—
2 ♂	41, 121, 105 (K)	67	—	—	—	—	0	9	—
3 ♂	45, 105, 89 (K)	67	—	—	—	—	0	5	—
4 ♀	42, 119, 109 (K)	51	—	—	—	—	0	6	—
5 ♀	54, 99, 92 (D)	33	—	—	—	—	0	2	—
6 ♀	31, 58, 58 (D)	26	—	—	—	—	0	2	—
7 ♀	33, 60, 45 (D)	39	—	—	—	—	0	1	—
8 ♀	85, 127, 105 (K)	54	—	—	—	—	0	5	—
9 ♂	140, 167, 145 (D)	50	—	—	—	—	0	10	—
10 ♀	62, 100, 77 (D)	54	—	—	—	—	0	3	—
			Rats cured by small doses of carotene (M.P. 174°).						
11 ♀	45, 111, 133 (K)	54	0·02	20	70	770	0	10	0
12 ♀	51, 128, 178 (K)	47	0·01	43	80	880	0	16	0
13 ♀	52, 114, 133 (K)	53	0·05	45	400	4,400	0	7	0
14 ♀	—, 89, 89 (K)	—	0·5	3	270	3,000	0	23	0
			Rats cured by large doses of carotene (M.P. 178°).						
15* ♂	38, 95, 159 (K)	41	0·75	16	2,200	24,000	2,000	90	22,000
16* ♀	33, 95, 139 (K)	41	0·75	22	3,000	33,000	3,300	110	36,000
17 ♀	36, 66, 106 (K)	29	0·75	26	3,500	39,000	3,700	100	41,000
18 ♂	41, 76, 137 (K)	28	0·75	26	3,500	39,000	2,000	40	22,000
			Rats cured by red palm oil.						
19 ♂	33, 73, 152 (K)	29	R.P.O. 1·5 g.	26	12,000	130,000	5,000	280	55,000
20* ♀	38, 94, 127 (K)	41	R.P.O. 1·5 g.	16	7,400	83,000	4,500	400	50,000
			Rats cured by fresh carrots.						
21 ♀	35, 86, 147 (K)	56	Carrots *ad lib.*	55	?	?	16,000	600	180,000
22 ♂	49, 114, 153 (K)	71	,,	35	?	?	5,300	250	58,000

D = Died. K = Killed.

* These rats were first cured by very minute doses of carotene before the larger amounts were given.

In calculating the colour value of the ingested pigment in Table I the values for both samples of carotene have been taken as blue = 180 units per mg. per cc., yellow = 2000 units per mg. per cc.

Discussion.

The above results afford a complete picture of the conversion of carotene *in vivo* to the familiar vitamin A of liver oils, as defined by all the chromogenic and spectroscopic properties which have been found to run parallel to this factor. The details of the conversion may be summarised as follows:

Carotene	⟶	Vitamin A
Synthesised in plant		Stored in animal
Intensely yellow		Almost colourless
328$\mu\mu$ absorption band absent		328$\mu\mu$ absorption band developed
Greenish blue $SbCl_3$ reaction at 590$\mu\mu$		Vivid blue $SbCl_3$ reaction at 610–630$\mu\mu$

This evidence could be regarded as conclusive if it could be stated with certainty that the carotene used was completely pure. Upon this point, however, doubts might be raised, since Euler, Karrer and Rydbom [1929] have

advanced certain evidence, based upon supposed variations in the activity of different samples of carotene, which raises the question as to whether the activity of the pigment may not after all be due to the presence of a "stubbornly adhering" impurity active at about the same level as vitamin D. The danger of this possibility was indeed pointed out by the writer [Moore, 1929, 4] at a time when Euler, Euler and Hellström were satisfied with the chemical purity of their carotene, but it is now obvious that this view in its simplest form would imply a complete rejection of the chromogenic evidence, since it is obvious that the same chromogen cannot be present in both carotene and cod-liver oil. It should be noted that the evidence of Euler, Karrer and Rydbom [1929] is based on small variations in the growth rates of rats and in blue values determined by the tintometer technique. Results obtained by both these methods are notably open to considerable experimental errors, and must therefore be of very doubtful significance.

A point of greater interest, however, arises from the statement of Drummond and Baker [1929] that vitamin A probably constitutes only a minute fraction (10 % and probably much less) of even the most potent liver oil concentrates. Experiments described above (Part V) have shown that the minimal dose of carotene is only slightly lower than that of the most active concentrates, which may actually exceed the pigment in regard to chromogenic activity. From this evidence it might be expected that the activity of carotene would similarly be derived from a small fraction of the total pigment. Two alternative possibilities, however, may be borne in mind, either that the conversion of carotene may be of an inefficient character, or that the estimate of Drummond and Baker may be unduly low. For the present the evidence does not seem of sufficient weight to justify any serious doubt that the physiological activity of carotene is not derived from the pigment *per se*.

Summary.

Part V.

1. The $SbCl_3$ colour reaction given by a minimal physiological dose of carotene at 590 $\mu\mu$ is slightly less than that given by a minimal physiological dose of the vitamin A of liver oil at 610–630 $\mu\mu$.

2. It is therefore impossible that the colour reaction of carotene could conceal any underlying colour reaction due to the liver oil vitamin A in such amounts as would account for its physiological activity.

Part VI.

1. The liver oils of rats suffering from vitamin A deficiency invariably gave negative results when tested with the $SbCl_3$ reagent.

2. Rats after depletion of vitamin A were cured by the administration of large excess of carotene (12 times recrystallised from cyclohexane, M.P. 178° uncor.), red palm oil, or fresh carrots. Traces of yellow pigment now appeared

in the liver oil but the predominant chromogen present (99 %) was invariably vitamin A, as characterised by (*a*) absence of such intense yellow pigmentation as must have accompanied the storage of carotene as such; (*b*) an intensely positive $SbCl_3$ reaction showing a marked band at 610–630 $\mu\mu$; (*c*) the development of an absorption band in the untreated oil at 328 $\mu\mu$; (*d*) intense biological activity.

3. The conclusion must therefore be reached that carotene, or some part thereof, if it should later prove to be heterogeneous, behaves *in vivo* as a precursor of the vitamin.

My thanks are due to Dr L. J. Harris for his valuable criticism, to Mr T. A. Webster for advice on the extraction of liver oils and to Messrs Chivers and Sons, Limited, for drying the large quantities of carrots from which the carotene used in this experiment was derived. The care of the experimental animals was in the capable hands of Mr A. Ward.

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