CXL. VITAMIN A AND CAROTENE.

II. THE VITAMIN A ACTIVITY OF RED PALM OIL CAROTENE. III. THE ABSENCE OF VITAMIN D FROM CAROTENE. IV. THE EFFECT OF VARIOUS DIETARY MODIFICATIONS UPON THE VITAMIN A ACTIVITY OF CAROTENE.

By THOMAS MOORE.

Report to the Medical Research Council.

From the Nutritional Laboratory, Cambridge.

(Received November 5th, 1929.)

II. THE VITAMIN A ACTIVITY OF RED PALM OIL CAROTENE.

IN a previous communication [Moore, 1929, 1], in which the literature was quoted, confirmation was afforded to the claim of Euler, Euler and Hellström [1928] that the pigment carotene, in a state of apparent purity, possessed intense vitamin A activity. In this work the carotene was derived exclusively from carrots. Collison, Hume, Smedley-MacLean and Smith [1929], on the other hand, have demonstrated the activity of carotene not only from carrots but also from the green leaves of cabbage and spinach. Since other pigments, such as xanthophyll, lycopin, α -crocetin, bixin and capsanthin have been reported by Euler, Euler and Karrer [1929, 1] to possess no growth-promoting powers¹, attention must tend to be focussed on carotene as affording some peculiar clue to the problem of vitamin A activity. Experiments by the writer [Moore, 1929, 2] have, in fact, indicated the possibility that carotene may behave *in vivo* as a precursor of the vitamin. It is therefore of interest to test the activity of carotene derived from as many different sources as possible. The present experiments demonstrate the activity of a sample of the pigment prepared from red palm oil.

EXPERIMENTAL.

A sample of the unsaponifiable matter of red palm oil was made available through the courtesy of Mr MacLennan of Lever Brothers, Limited. Colorimetric examination revealed the presence of considerable amounts of pigment, but when methods similar to those used in the isolation of carotene from carrot

It was, however, reported by these workers that dihydro- α -crocetin possesses vitamin A activity. Confirmatory evidence of this surprising claim has not yet been put forward (see Karrer [1929]).

fat were applied it was found that no satisfactory separation of the pigment could be effected. The problem of isolating the bulk of the carotene, indeed, has not yet been solved, but tests have been carried out on a small specimen obtained in the following manner. From about 10 cc. of the crude unsaponifiable matter in chloroform solution a small fraction of brown amorphous material was removed by the addition of excess of methyl alcohol. The filtrate, after evaporation of the solvent under diminished pressure, was stored for several weeks in a refrigerating chamber. A small yield of crystals was then noticed. These were removed, washed with methyl alcohol, and recrystallised twice from chloroform by the addition of methyl alcohol. Only about 5 mg. of the pigment (M.P. 162° in air) was obtained, an amount which was obviously too small to permit of further purification. The appearance, solubility and chromogenic value of the crystals, however, all indicated that the pigment was carotene.

Colorimetric examination. As a preliminary to biological tests both the crude unsaponifiable matter and the isolated pigment (the latter dissolved in arachis oil at the concentration of 0.01 mg. per 20 mg. drop of oil) were examined both in regard to their degree of natural yellow pigmentation in chloroform solution and in regard to the depth of the blue colorations which were given with the SbCl_3 reagent.

Colorimetric technique. The colorimetric methods used throughout this research would seem to be liable to inaccuracies when applied indiscriminately over wide ranges of concentration. It is desirable, therefore, to describe in detail the technique used and the mode of presentation of results. Observations were carried out in a Rosenheim-Schuster tintometer fitted with the usual circular tube of 1 cm. diameter. In estimations of natural yellow pigmentation, (a) the given material was dissolved in chloroform and diluted with the same solvent until a reading of about five yellow units was given, (b) this reading was then divided by the amount of material in mg. present in 1 cc. of solution thus giving the value in yellow units calculated for 1 mg. of the material dissolved in 1 cc. of solvent and viewed in a layer 1 cm. thick (for brevity "1 cm. cube"). In evaluating the blue colorations given with the SbCl_3 reagent, (a) a known volume (calculated by trial experiments to give a reading of about five units) of a standardised chloroform solution of the material was delivered into the tintometer tube by means of a blood pipette and the volume made up with washings to 0.5 cc., (b) 2 cc. of the SbCl_3 reagent was added and a reading immediately taken, (c) the mean of several readings was divided by the total amount of the material present and multiplied by the total volume (2.5) in cc. thus giving the value in blue units calculated for 1 mg. of material dissolved in "1 cm. cube" of reagent. It should be noted that the values so calculated would not necessarily agree with experimental findings at the same concentration, as depth of colour does not seem to bear a linear relation to the amount of material taken. The advantage of this uniform method of expression lies rather in its simplicity, and in its convenience when it is desired

to compare the degrees of natural yellow pigmentation of materials with their activity in the SbCl_3 reaction.

The following data were obtained:

	Lovibond yellow units per mg. per "1 cm. cube" CHCl_3	Lovibond blue units (at 590μ) per mg. per "1 cm. cube" of SbCl_3 reagent
Crude unsaponifiable fraction	75	16
Crystalline pigment	750	190

Physiological activity. Biological tests of both the crude unsaponifiable fraction and the isolated pigment were carried out, using the technique described in the preceding paper. It will be seen from the growth curves (Fig. 1) that 0.01 mg. of the pigment sufficed to restore slow growth in two rats, whereas the minimal dose of the concentrate lay at a higher level, 0.02 mg. giving no response and 0.2 mg. sufficing for irregular growth.

Fig. 1. The vitamin A activity of red palm oil carotene.
 Rat No. 1: negative control.
 Rats Nos. 2 and 3: 0.01 mg. carotene daily.
 Rat No. 4: 0.02 mg. crude unsaponifiable matter.
 Rat No. 5: 0.2 mg. crude unsaponifiable matter.

It may be recalled that carotene derived from carrots is of much greater activity than the fat from which it has been separated. It is noteworthy, therefore, that the concentration of activity in the crystalline pigment has now been confirmed in the case of a preparation of entirely different origin.

III. THE ABSENCE OF VITAMIN D FROM CAROTENE.

Although it was originally suggested by Euler, Euler and Hellström [1928] that the failure of previous investigators to obtain evidence of the vitamin A activity of carotene might have been due to the absence of vitamin D from the test diets employed, it has more recently been reported by Euler, Euler and Karrer [1929, 2] that omission of vitamin D from diets containing carotene does not cause cessation of growth, but leads to faulty calcification. From this result it has been inferred that carotene does not possess antirachitic power¹. It must be remarked, however, that in these experiments an ordinary complete basal diet, as opposed to a rachitogenic diet, seems to have been employed, and also that the antirachitic value of the pigment was tested only at relatively low levels. For these reasons it seemed desirable to confirm the inactivity of the pigment under more conventional conditions.

EXPERIMENTAL.

Young albino rats of 40–60 g. were placed upon the Steenbock rachitogenic diet No. 2965. Graded doses of carotene, dissolved in arachis oil, were administered daily from the commencement of the experiment. After 35 days the rats were killed and the rib junctions examined macroscopically. The following results were obtained:

Sex and weight (g.)	Daily supplement	Condition of ribs	Relative severity of rickets
♀ (59–114)	200 mg. arachis oil (negative control)	Arch good, no fractures or angulation. Costochondral junctions slightly enlarged. Lines slightly hazy	+
♂ (54–126)	0.0001 mg. carotene	Arch good, etc. Costochondral junctions greatly enlarged with cupping and almost complete blurring of lines	+++
♂ (64–143)	0.001 "		
♂ (45–150)	0.01 "		
♂ (47–132)	0.1 "	Arch good, etc. Costochondral junctions moderately enlarged with lines hazy	++
♂ (57–140)	0.75 "		
♀ (36–103)	1 mg. cod-liver oil concentrate (positive control)	Normal	0

The carotene, therefore, did not prevent the development of rickets when administered at a level equivalent to about 100 times the minimal dose for vitamin A².

IV. THE EFFECT OF VARIOUS DIETARY MODIFICATIONS UPON THE VITAMIN A ACTIVITY OF CAROTENE.

It has been suggested [Moore, 1929, 1] that the cause of the divergent results of previous workers upon the activity of carotene might lie in some

¹ The position was further complicated by the claim that vitamin A (carotene) was essential for the promotion of normal calcification even on diets considered by these authors to contain an adequate amount of vitamin D (2 mg. of irradiated arachis oil).

² The carotene tested in this experiment was prepared from carrots. A similar sample has subsequently been examined by Mr A. L. Bacharach, to whom my thanks are due. No vitamin D activity could be detected at 0.11 mg. by the faecal p_H method, nor at 0.045 mg. by the line test method.

unsuspected difference in biological technique rather than in the degree of purity of the various samples examined. In particular it was considered that the presence of fat in the basal diet might be of some importance. Experiments to decide this point, and also the effect of some other minor alterations in technique, have now been carried out.

Fig. 2. The vitamin A activity of carotene under various dietary modifications.

————: Arachis oil diet.

-----: "Fat-free" diet.

(a): 1 drop radiostol daily.

(-a): Radiostol withdrawn.

(b): 1 drop irradiated ergosterol in paraffin (same conc.) daily.

EXPERIMENTAL.

For these experiments two sets of young albino rats were used.

Set I. Of nine rats five (including one as the negative control) received the basal diet containing 15 % of arachis oil which has been generally employed in this research, the remaining four received a similar, but "fat-free," diet differing only in the substitution of additional rice starch in place of arachis oil, and in the substitution of medicinal paraffin for arachis oil as a solvent for

the irradiated ergosterol in those cases in which an addition of vitamin D was made. When growth had failed carotene (prepared from carrots; m.p. 174°) was administered at the level of 0.01 mg. daily at the points shown, being dissolved either in arachis oil (by warming) or in medicinal paraffin (by addition of the pigment in chloroform solution and concentration under diminished pressure).

It will be seen from the growth curves (Fig. 2) that the negative control rat declined and died, whereas in all the rats receiving carotene decline was prevented and growth restored. Poor growth responses, however, were given in some cases, and notably in those in which the carotene was administered in medicinal paraffin.

Set II. Of six rats, three received the ordinary basal diet containing arachis oil and vitamin D, three received the "fat-free" diet without the addition of vitamin D. After growth had ceased in both groups 0.01 mg. of carotene dissolved in arachis oil was administered in all cases and simultaneously the diets given were interchanged as indicated in the growth curves. Except in the case of one animal, which died suddenly, growth was restored in both groups.

The results indicate that carotene is active even in the absence of substantial amounts of fat in the basal diet. The definitely positive responses obtained when carotene was administered in paraffin solution demonstrate, moreover, that the process previously adopted of dissolving the pigments in warm arachis oil was not an essential in promoting physiological activity. It is clearly a matter of minor importance, in this connection, that activity tended to be weakened by the use of paraffin, since in any case it has been stated by various workers that the administration of paraffin leads to a less ready utilisation of vitamin A.

SUMMARY.

1. Carotene (m.p. 162°) obtained from red palm oil was active as a source of vitamin A activity to rats in doses of 0.01 mg. daily.

2. Carotene (m.p. 174°) obtained from carrots was inactive as a source of vitamin D to rats in doses greatly in excess of the minimal dose for vitamin A.

3. Carotene (m.p. 174°) at the level of 0.01 mg. daily displayed activity even when a basal diet of the "fat-free" type was used. The pigment still displayed activity when medicinal paraffin replaced arachis oil as a solvent for the test doses, although this modification tended to weaken the growth responses observed.

My thanks are due to Dr L. J. Harris for his most helpful criticism.

REFERENCES.

- Collison, Hume, Smedley-MacLean and Smith (1929). *Biochem. J.* **23**, 634.
Euler, Euler and Hellström (1928). *Biochem. Z.* **203**, 370.
— — — and Karrer (1929, 1). *Helv. Chim. Acta*, **12**, 278.
— — — (1929, 2). *Biochem. Z.* **209**, 240.
Karrer (1929). *Z. angew. Chem.* **42**, 923.
Moore (1929, 1). *Biochem. J.* **23**, 803.
— (1929, 2). *Lancet*, ii, 830.