CCXXI. THE INFLUENCE OF THE SOLVENT ON THE BIOLOGICAL EFFECT OF CAROTENE AND VITAMIN A.

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SINCE it was first shown by Euler *et al.* [1928] that carotene could replace vitamin A in causing the resumption of growth of rats depleted of their reserves of this vitamin, experiments have been described in which the amount of carotene stated as being capable of restoring growth has shown considerable variations. These differences were partly but not entirely dispelled when Drummond *et al.* [1930] found that the instability of carotene in ethyl oleate, the solvent employed by them in their original biological tests [Dulière *et al.*, 1929], accounted for its apparent ineffectiveness. Moore [1933] published some experiments in which he tried to define the "minimum doses" of carotene and of vitamin A; but his figures only represented "minimum doses" for the particular diet and technique employed by him. Since the experiments of Drummond *et al.*, it has been repeatedly confirmed that certain oils are quite unsuitable for the biological test. Smedley-MacLean and Hume [1930] and Drummond *et al.* [1930] concluded that the observed discrepancies were due to the rapid oxidation which carotene undergoes in certain solvents. Notwithstanding this, we have found that when oils are used in which carotene is reasonably stable, there is still a wide divergence in the quantitative results obtained both with carotene and with vitamin A, so that the rate of deterioration of the vitamin A or carotene does not afford a sufficient explanation of the results, and the suitability of an oil from the physiological point of view is a separate problem. Failure to realise this biological influence of the solvent explains some of the difficulties which workers have experienced in the estimation of vitamin A in terms of the International Standard carotene, and in November 1932 we communicated our results to the Vitamin A Sub-Committee of the Accessory Food Factors Committee. Dr Coward, working on behalf of the Committee, has confirmed many of them, and at their request we made an experiment comparing three selected coco-nut oils, the result of which is described in this paper.

In a paper published by one of us in 1933 [Culhane, 1933] attention was drawn to the fact that rats reared on the selected basal diet gave no response to a dose of 0.002 mg. of carotene dissolved in arachis oil, which was considered a sufficient dose by Moore. We also found it necessary to give as much as 10 times the dose of cod-liver oil recommended by Coward for use with her diet. Nelson and Jones [1928], using a diet containing very highly purified caseinogen, had published experiments which showed effective doses of about the same magnitude as ours. We concluded that the wide diversity in the quantity of vitamin A-containing substances necessary to restore growth was due to the differences in the

basal diet employed. A number of experiments in which the constituents of the basal diet were systematically varied, afforded valuable information regarding the incidence of xerophthalmia, but they failed to provide an explanation of the need for such very different quantities of vitamin A to be associated with the various diets. In continuation of these experiments we tried the effect of different oils as solvents and found that the result of varying these was much greater than that of changing any other constituent.

An experiment showed coco-nut oil to be greatly superior to arachis oil as a solvent for carotene. We followed this observation by quantitative comparisons of various oils used to dissolve both carotene and vitamin A concentrates, and we found that not only do oils differ, but different samples of the same oil may vary greatly in their suitability quite independently of the rate of decomposition of the substance dissolved. Certain samples of arachis oil we have found to give excellent results, whereas certain samples of coco-nut oil proved quite unsuitable.

The diet used throughout has been the same as the standard diet used in the paper referred to above (A 5 diet). The litters used in each comparison have been as large as possible, and only litter-mates of the same sex have been compared. The experiments have been closed after the rats have received oils for ten weeks, and average growth curves have then been constructed for comparable groups of rats. When an animal has died in the course of a test, we have omitted it and its litter-mates from the final result. We consider that this is the only justifiable procedure, since a dead animal cannot take part in a growth test. Upon occasions, however, when a number of rats have died, in addition to the comparison of the average growth curves of the survivors, we have made a comparison of the average periods of survival on the different doses.

Both in the case of carotene and vitamin A it is our practice to make a sufficient amount of the various dilutions for feeding to last for about five weeks. They are poured into small amber bottles containing about $2\frac{1}{2}$ g. and corked, care being taken that the bubble of air at the top of the bottle is as small as possible. The bottles are immediately placed in the cold room, and the one in use for feeding each day is returned to the cold as soon as possible. One bottle usually lasts two or three days only, but if there are only a few animals on test it lasts longer; in any case it is discarded and a fresh sample taken after one week.

EXPERIMENTAL.

A. Comparison of arachis oil and coco-nut oil.

(1) *As solvents for carotene.* The carotene employed consisted of a mixture of isomerides estimated to contain about 85 % of β -carotene. It melted at $177-179^{\circ}$. 85 rats were used in the comparison and the doses given were 1γ and 2γ in 0.02 ml. of each solvent.

In Fig. 1 (1) and (2), the average growth curves for groups of 7 male and 4 female litter-mates show clearly that with either solvent it is easy to distinguish between doses bearing a ratio of 1 : 2. 2γ in arachis oil has given the best growth, 1γ in arachis oil and 2γ in coco-nut oil are about equally effective and 1γ in coco-nut oil has given the least growth. The average curves of groups of 9 male litter-mates (Fig. 1 (3)) confirm the equivalence of 1γ in arachis oil and 2γ in coco-nut oil.

Hence of these two, the arachis oil is about twice as effective as the coco-nut oil.

(2) *As solvents for vitamin A.* In another experiment using different batches of oils, coco-nut oil and arachis oil were compared as diluents for a vitamin A

concentrate—Carr-Price figure of 49,000¹ and $E_{1\text{cm}}^{1\%} = 1100$. For feeding to the rats it was diluted with the oils under test to give values of 4 and 8 blue. 75 rats were used for the comparison. Fig. 2 gives the average growth curves of comparable groups of rats. Both males and females on the smaller and larger doses have given better results with the coco-nut samples. Hence, of these two, the coco-nut oil is superior to the arachis oil.

Fig. 1. Average growth curves for groups of rats (litter-mates) "run out" on A 5 diet. When growth ceased they were given daily doses of 0.02 ml. of solutions of carotene in arachis or coco-nut oils.

A. —●— 2γ daily in arachis oil   C. ——— 2γ daily in coco-nut oil
B. - - - - 1γ   "   "   D. - - - - 1γ   "   "

At the same time a few rats were given a dose of 4 U of standard dissolved in the coco-nut oil. Their average growth curves are shown in Fig. 3. Apart from the fact that they are so few in number, the comparison gives a fair assay of the vitamin A content of the coco-nut oil sample, the effect of the solvent having been eliminated. From Fig. 3 one can gain an idea of the very different results that would be obtained by assaying the vitamin A content of these concentrates in the arachis oil or in the coco-nut oil. The latter would give a result of 1,225,000 U/g., the former not more than 612,500 U/g. Further, had the standard been given in the arachis oil, the coco-nut oil solution would have appeared to contain 2,450,000 U/g.

(3) *Arachis and coco-nut oils compared with linseed oil and ethyl oleate.* Two other batches of these two oils were compared with each other and with ethyl oleate and linseed oil, using 102 rats in a test of the potency of a vitamin A

¹ All "blue" figures quoted in this paper were determined by the method described in British Pharmacopoeia 1932, p. 596, making appropriate allowances for dilution.

preparation giving a Carr-Price value of 23,000. The concentrate was diluted in the ratio 1 part in 4600 for test and the usual dose, 0.02 ml. of the solution, was fed daily to each animal.

The rats receiving their doses in ethyl oleate declined steadily, and all were dead long before the ten weeks of the experiment had passed. The growth was not very satisfactory on any of the oils and there were a number of deaths.

Fig. 2. Average growth curves for groups of rats (litter-mates).

A. ———	Vitamin A concentrate diluted to 8 blue with coco-nut oil; 0.02 ml. daily.
B. —●—	" A " " 8 " arachis oil " "
C.	" A " " 4 " coco-nut oil " "
D. -.-.-	" A " " 4 " arachis oil " "

Fig. 4 shows some of the comparisons between groups of litter-mates and it follows from them that the linseed oil has given the best growth, the arachis oil being slightly superior to the coco-nut oil.

In yet another comparison of two further specimens of coco-nut and arachis oils, in which vitamin A was dissolved at two different dose levels, we found the oils about equally effective whether the comparison was made by means of the growth rate of the survivors or by comparing the rate of mortality of those which died.

B. Comparison of three samples of coco-nut oil.

The comparison of three samples of coco-nut oil was carried out at the request of the vitamin A Sub-Committee of the Accessory Food Factors Committee. Two of the oils were from Cochin (referred to as Cochin 1 and 2), and the third from Ceylon.

152 rats (19 litters) were used in the comparison of these oils. The standard was dissolved in each at a level of 100 units per g. so that the rats received 2 units per day. All doses were duplicated by making up additional solutions containing 0.01 % of quinol. The rats were distributed as evenly as possible between the six doses, one or two rats from every litter being left as negative controls. In Fig. 5 are shown the comparisons of the solutions containing no quinol: they show that the Cochin 1 oil is definitely less effective than the other two. Those of

Fig. 3. Average growth curves for groups of rats (litter-mates).

- A. ——— Vitamin A concentrate diluted to 8 blue with coco-nut oil; 0.02 ml. daily.
 B. —●—●— " A " " 8 " arachis oil " "
 C. —×—×— 4 units of standard carotene in 0.02 ml. of coco-nut oil daily.

the solutions with quinol are similar but are omitted for the sake of space. The order of the two superior oils is somewhat indefinite, but there is no doubt that the Cochin 1 oil is inferior, and the rate of mortality confirms the fact that it is less satisfactory than the other two oils. Thus, on this oil, twenty died out of twenty-five, on the Cochin II oil, one died out of thirty-one, and on the oil from Ceylon, five died out of twenty-seven.

In comparing the solution containing quinol with those that did not, we had groups of twelve litter-mates on each of the six samples. With every pair the result was the same on the males and females, there being a slight difference in favour of the oils without quinol.

The chemical properties in which the Cochin 1 oil differed from the other two are shown in Table I, but none of these differences was sufficient to account for the different actions of the oils.

Fig. 4. Average growth curves for groups of rats (litter-mates). After "running out" on A 5 diet they were given 0.02 ml. daily of vitamin A concentrate diluted 1 part in 4600 (*i.e.* to 5 blue) with different oils:

A. ○—○ Linseed oil   B. ●—● Arachis oil   C. —·—· Coco-nut oil.

Fig. 5. Average growth curves for groups of rats (litter-mates). After "running out" on A 5 diet they were given 2 units of international standard carotene in 0.02 ml. of three different coco-nut oils:

A. —·—· Cochin I oil.   B. ⊗—⊗ Cochin II oil.   C. ×—× Oil from Ceylon.

Table I*.

Colour	Coco-nut oils		
	Cochin I White	Cochin II Slightly coloured	Ceylon Slightly coloured
Acid value	3.0	3.5	3.4
Iodine value	9.1	8.3	8.0
Peroxide test, ml. <i>N</i> /200 thiosulphate	0.05	0	0
After 190 hours' exposure to 60 Watt lamp	0.35	0.35	0.40
After 840 hours' exposure to 60 Watt lamp	0.70	1.60	1.60
Repeated 4 months later	0.10	0.05	0.05

* For these figures and for other chemical estimations quoted in this paper we are grateful to the Analytical Department of The British Drug Houses, Ltd.

DISCUSSION.

Considering only the results with the different coco-nut and arachis oils, which are the varieties of oils most commonly used in this country in the testing of vitamin A, we have obtained the following results:

A. *As solvents for carotene.*

(1) Original experiment (not described here).

The coco-nut oil was superior to the arachis oil.

(2) Quantitative test (described above).

Carotene dissolved in arachis oil was twice as potent as in solution in coco-nut oil, different batches of the oils being used.

B. *As diluents for vitamin A concentrates.*

(1) Vitamin A dissolved in coco-nut oil was more effective than when dissolved in arachis oil.

(2) A vitamin A concentrate dissolved in another arachis oil was superior to its solution in coco-nut oil (linseed oil was better than either, ethyl oleate was greatly inferior).

(3) A vitamin A concentrate was equally active when tested in arachis oil or in a coco-nut oil. (Not described.)

The most obvious suggestion to explain these apparently contradictory results is that carotene and vitamin A deteriorate more rapidly in some oils than in others, but colour tests, where these have been carried out, show that this is not the true explanation. Unfortunately, the earlier experiments were not checked by colour tests, but we have sufficient data to show that there is some other factor involved.

Considering the pair of carotene solutions in coco-nut oil and arachis oil, there appeared to be no difference in their colours, either when they were first made up, or after the test had been proceeding for some weeks. Further, there was no sudden spurt of growth when they were made up freshly, which one would have expected of the coco-nut oil solution if its comparative inactivity had been due to more rapid deterioration of carotene. Colour tests on some of the same carotene dissolved in coco-nut oil carried out six months previously had shown only a very slight loss in four months: 4.8–4.5 in the solution containing 100 γ per g., and 2.5–2.2 in the solution containing 50 γ per g. In all probability these differences are not significant in view of the error inherent in the tintometer reading.

When carotene was again tested in the three different coco-nut oils, we carried out colour tests on the actual solutions used. These are shown in Table II, from which it can be seen that none of the samples had suffered any change whatsoever in six weeks. These figures show that our method of keeping carotene solutions is satisfactory, that quinol is unnecessary, and that we must seek for some other explanation of the differences between the oils than that of the deterioration of the carotene.

Table II. *Yellow colours read in the Lovibond tintometer.*

0.01 mg. carotene in 0.1 g. of oil diluted to 2 ml. with chloroform.

Oil	At 2 weeks	At 8 weeks
Cochin I	4.6	4.8
Cochin I + 0.01 % quinol	4.8	5.3
Cochin II	5.2	5.8
Cochin II + 0.01 % quinol	5.6	5.9
Ceylon	4.9	5.1
Ceylon + 0.01 % quinol	4.4	4.8

In the comparison of coco-nut oil, arachis oil, linseed oil and ethyl oleate as solvents for vitamin A, colour tests after the conclusion of the experiment showed that all had lost significantly, the ethyl oleate sample giving a Carr-Price value of 1.2 blue representing a loss of three-quarters of its vitamin content. Although this is a very substantial loss, it is our experience that rats receiving a dosage at so low a level as 1.2 blue would have given some response to the dose. However, it is not possible to draw conclusions from absolute values but only from comparisons. We found that the linseed oil and the arachis oil solutions were equal by the colour test, both having lost about one-third of their value, but the result of the growth test was far removed from equality. Referring again to Fig. 4 there is no doubt of the superiority of the solution in linseed oil. The coco-nut oil sample had lost about half its value by colour test, which shows a sufficient difference in vitamin A content to account for its inferiority.

Baumann and Steenbock [1933] have shown that quinol retards the decoloration of carotene in coco-nut oil, and the stabilising power of quinol for vitamin A solutions is well known. Thus, quite apart from our observation that the carotene solutions did not change over a period of six weeks, if the deterioration of the dissolved substance had been the cause of the differences that we have found, then the solutions of carotene in the inferior oil to which quinol was added should have given a growth result better than that obtained without addition of quinol and equal to that obtained with the other two oils. In actual fact this sample containing quinol gave the worst result of all.

The fact that sometimes the sample in arachis oil is the better and sometimes the one in coco-nut oil, whether carotene or vitamin A concentrate be used for the vitamin, precludes the possibility that carotene, before it can replace vitamin A, needs supplementing with some substance generally found in one of the varieties of oil. That an oil which allows the best growth may contain some factor necessary to supplement these highly purified preparations of vitamin A and its probable precursor is not unlikely, but if that is the case, the experiments show that it is not necessarily present in oils of any one kind. If this is the explanation, the substance is present in the sample of linseed oil tested, but absent from ethyl oleate; it is more abundant in one arachis oil than in the coco-nut oil with which it was compared, whilst with another pair the reverse is true. Further, of the three coco-nut oils compared directly, one contained much less than the other two.

We are continuing this work with a view to finding some physical or chemical means of selecting an oil that will be satisfactory in the biological test, and also to discovering the cause of the difference to the animal of oils that appear to be similar.

SUMMARY.

1. Different batches of arachis oil and of coco-nut oil vary in their suitability as solvents for the biological testing of vitamin A or carotene.
2. The suitability of a particular oil can only be determined biologically since it is a property independent of the stability of the dissolved substance in the oil.
3. Quinol is not effective in rendering an inferior oil suitable for the biological test.

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