CXXIV. THE INFLUENCE OF THE SOLVENT ON THE VITAMIN A ACTIVITY OF (a) CAROTENE AND (b) COD-LIVER OIL.

By FREDERICK JOHN DYER, KATHLEEN MARY KEY
AND KATHARINE HOPE COWARD.

*From the Pharmacological Laboratories, Pharmaceutical Society
of Great Britain, London.*

(Received April 30th, 1934.)

IN June, 1931, the Permanent Commission on Biological Standardisation of the Health Organisation of the League of Nations recommended [1931] the adoption of a particular sample of carotene as a provisional standard of reference for the estimation of vitamin A. This was to be prepared from the pooled contributions of five workers from different laboratories by the National Institute for Medical Research, London, where it would be kept and issued as required. The unit of vitamin A suggested was the vitamin A activity of 1γ (0.001 mg.) of this particular sample of carotene.

The standard of reference for vitamin A was adopted provisionally for two years, for it was not known how long carotene would retain its growth-promoting potency. As soon as the standard became available, workers in many laboratories began to use it and, from informal comparisons of results, it early became evident that widely different responses to the same dose of standard were being obtained in different laboratories. It was difficult to believe that the differences in response were entirely due to differences in basal diets of the rats, and it seemed most probable that the cause of the differences would be found in the different oils used for dissolving the carotene for administration to the rats. It was, therefore, thought desirable to make direct comparisons of different oils.

I. THE INFLUENCE OF THE SOLVENT ON THE VITAMIN A ACTIVITY OF CAROTENE.

A. *First comparison of oils as solvents for carotene.*

In the first comparison the standard carotene was dissolved in (a) hardened cotton-seed oil, (b) ethyl laurate (redistilled), (c) arachis oil, respectively, so that a daily dose of 5γ carotene could conveniently be given in 0.02 g. oil. In preparing the solutions, one of two methods was adopted; either (a) the ampoule containing the standard was weighed before and after washing out with benzene and the loss taken as the weight of carotene, or (b) after transferring the solution to a known weight of melted solvent, the benzene was evaporated *in vacuo* until constant weight was attained and the increase in weight recorded as the weight of carotene added. Solvent was then added to produce the required dilution. The solutions were distributed in amber-coloured glass ampoules which were twice evacuated, refilled with dry nitrogen and immediately sealed. The tests were carried out by our usual method of giving 3 doses on Tuesdays and

4 on Fridays [Coward and Key, 1934]. A fresh ampoule of solution was opened each day on which doses were given. The results of this comparison are seen in Table I. All ampoules of solutions were kept at 0°.

Table I. *Comparison of (a) hardened cotton-seed oil, (b) ethyl laurate and (c) arachis oil as solvents for carotene for use in vitamin tests.*

Dose: 5 γ carotene (International Standard) in 0.02 g. oil per day.

Solvent	No. of rats in test		No. of rats surviving		Mean increase in wt. in 5 weeks g.		Abscissae of Coward's curve of response corresponding to mean increase in wt.		Weighted mean of abscissae, i.e. relative potency of doses
	♂	♀	♂	♀	♂	♀	♂	♀	
Hardened cotton-seed oil	7	7	2	6	8.5	17.2	0.88	1.47	1.32
Ethyl laurate	5	6	4	6	9.2	22.0	0.91	2.24	1.71
Arachis oil	5	7	5	7	38.6	40.7	3.49	10.79	7.75

Thus the vitamin A value of 5 γ carotene dissolved in arachis oil may be five or six times as great as it is when dissolved in hardened cotton-seed oil or in ethyl laurate.

The three oils (without carotene) were tested on 3 groups of rats, 4 rats in each group, each rat being given a dose of 0.02 g. oil per day (3 doses on Tuesdays and 4 on Fridays). One rat given hardened cotton-seed oil lived for 35 days and was then killed; it was 5 g. less in weight than when first given the oil; the other three died in 11, 19 and 27 days respectively. The rats given ethyl laurate died in 6, 7, 17 and 17 days respectively. The rats given arachis oil died in 14, 17, 21 and 35 days respectively. Thus the superiority of the arachis oil solution of carotene cannot be attributed to the presence of vitamin A in the arachis oil.

Some comparisons were made of the yellow and red values of these solutions (5 γ carotene in 0.02 g. oil), (a) after the ampoules had been kept unopened in the refrigerator for 11 weeks after sealing, and (b) after a further week during which the open ampoule had been exposed to the air and warm temperature of the rat room for 20–30 minutes each day. The result may be seen in Table II. The colours were matched against the glasses of a Lovibond tintometer.

Table II. *Colours of solutions (a) after 11 weeks' storage in nitrogen-filled ampoules in refrigerator, and (b) same solutions after exposure to air and warm temperature of rat room for 20–30 minutes daily for one week.*

	Colour of solution from freshly opened ampoule after storage for 11 weeks		Colour of same solution after exposure to air of rat room	
	Yellow	Red	Yellow	Red
Hardened cotton-seed oil	19.6	7.0	20.3	6.4
Ethyl laurate	23.0	3.9	24.0	4.0
Arachis oil	24.0	5.7	24.0	5.7

Thus the colour of the solution seems to be very little indication of its vitamin A activity, for the solution in the hardened cotton-seed oil was only slightly less yellow and was a deeper red than the solution in arachis oil, and the solution in ethyl laurate had the same yellow value and a somewhat lower

red value than the solution in arachis oil. The exposure to the air and warm temperature of the rat room for 20–30 minutes daily for one week did not reduce the colour values of these solutions.

B. Second comparison of oils as solvents for carotene.

The second experiment consisted of a comparison of arachis oil and coconut oil as solvents for the International Standard carotene. A private communication from one worker that the activity of carotene in arachis oil had been found to be much less than in coconut oil made us decide to use a dose of 2 γ standard in arachis oil and a dose of 1 γ standard in coconut oil for this comparison. One ampoule of the standard was used for each solution and the dilution made by the same method as in the last experiment. Three doses were given to the rats on Tuesdays and 4 on Fridays as before, a fresh ampoule of solution being opened each day (Table III).

Table III. *Comparison between (a) arachis oil and (b) coconut oil (161441) as solvents for the standard carotene.*

Solvent	Dose of standard	No. of rats used in the test		Mean increase in wt. in 5 weeks g.		Abcissae of Coward's curve of response corresponding to mean increase in wt.		Weighted mean of abcissae, <i>i.e.</i> relative potency of doses
		♂	♀	♂	♀	♂	♀	
Arachis oil	2 γ	4	5	33.5	23.6	2.75	2.56	2.64
Coconut oil (161441)	1 γ	5	5	25.0	12.6	1.79	1.00	1.40

The relative potency of the doses as determined from the abcissae corresponding to the mean increases in weight of the groups of rats in Coward's curve relating increase in weight to dose of vitamin A given is 2.64:1.4 which is almost the ratio of the doses of carotene given, 2:1. Therefore, the activity of the standard in these samples of arachis oil and coconut oil (161441) is the same.

C. Third comparison of oils as solvents for carotene.

A third experiment was carried out (a) to compare different samples of coconut oil and (b) to see whether the addition of quinol to the solution would make any appreciable difference to the activity of the carotene within the duration of the experiment¹. The British Drug Houses, Ltd., kindly supplied three samples of coconut oil known to be of different origin. The test was made in the usual way on each of these samples, each one being tested with and without 0.1 % quinol respectively. The solutions were made in the usual way, one ampoule being opened on each day of dosing. The dose was at the rate of 1 γ carotene per day (Table IV).

It is evident that the addition of quinol has made no difference to the activity of the carotene dissolved in each of these samples of coconut oil. In two of the samples the activity of the carotene appears to be equal, while in the third it appears to be less than in the other two. In order to determine whether this difference is significant a calculation was made on the basis of the increases in weight of (a) the bucks which received each oil, whether with or without quinol, and (b) the does which received each oil whether with or without

¹ A comparison suggested by the Accessory Food Factors Committee of the Medical Research Council and Lister Institute and undertaken by three laboratories.

Table IV. *Comparison between three samples of coconut oil, each one with and without 0.1 % quinol respectively, as solvents for carotene.*

Solvent	No. of rats used in test		Mean increase in wt. in 5 weeks g.		Abscissae of Coward's curve of response corresponding to mean increase in wt.		Weighted mean of abscissae, i.e. relative potency of doses
	♂	♀	♂	♀	♂	♀	
C.O. 161441 + quinol	5	7	21.8	21.7	1.62	2.19	1.95
C.O. 161441 alone	3	8	16.7	19.3	1.28	1.79	1.65
C.O. 162398 + quinol	3	7	28.3	17.7	2.18	1.56	1.75
C.O. 162398 alone	6	7	30.5	23.3	2.41	2.5	2.46
C.O. 162750 + quinol	4	8	7.2	15.3	0.83	1.28	1.13
C.O. 162750 alone	3	7	-2.7	16.4	0.53	1.40	1.08
							Av. 1.8
							Av. 2.1
							Av. 1.1

quinol. The difference in mean increase in weight of the groups of bucks receiving oils 162398 and 162750 respectively was found to be significant, P (the probability that a correlation should arise by random sampling from an uncorrelated population) being <0.01 . When, however, the calculation was made not from the increase in weight of each rat, but from the doses corresponding to the individual increases in weight (found from Coward's curve of response) P was >0.05 and <0.1 . Therefore the difference indicated by the male rats is of doubtful significance. For the female rats P lies between 0.2 and 0.3 as calculated by the former method and by the latter method P is >0.02 and <0.05 . The difference indicated by the female rats is therefore insignificant. The latter method must be accepted as the more accurate as the response (increase in weight) is proportional to the log of the dose given, not to the dose itself. When the results from both male and female rats are calculated as one result by the use of the abscissae of the curve only (not the increase in weight), P becomes very slightly less than 0.02. Thus there is some indication that there is a difference between the two samples of coconut oil but these results cannot be considered to have proved it.

II. THE INFLUENCE OF THE SOLVENT ON THE VITAMIN A ACTIVITY OF COD-LIVER OIL.

When the early experiments indicated that the activity of carotene was influenced by the oil in which it was dissolved, it appeared to be possible that the oil with which a sample of cod-liver oil was diluted for a test would influence the vitamin A activity of the cod-liver oil. This was investigated, using our cod-liver oil Z for the tests.

A. Comparison of oils as diluents for cod-liver oil.

The cod-liver oil, Z, was diluted so that the solution contained the daily dose of 1 mg. in 0.02 g. oil (± 0.001 g.). Fresh dilutions were made once a week during the test. The solvents tested were (a) hardened cotton-seed oil (the sample already used for the carotene experiments), (b) olive oil (sample bought in the open market) and (c) coconut oil (sample 161441 from B.D.H.). A simultaneous test of 2 γ carotene (International Standard) in the same sample of coconut oil was also made (Table V).

Thus coconut oil may enhance the apparent vitamin A value of cod-liver oil or, alternatively, hardened cotton-seed oil and olive oil may lessen the apparent

Table V. *Comparison of (a) hardened cotton-seed oil, (b) olive oil and (c) coconut oil (161441) as diluents for cod-liver oil in a vitamin A test; and also a simultaneous test of the International Standard for vitamin A in coconut oil (161441).*

Dose (daily)	Solvent	No. of rats		Mean increase in wt. in 5 weeks g.		Abscissae of Coward's curve of response corresponding to mean increase in wt.		Weighted mean of abscissae, i.e. relative potency of doses
		♂	♀	♂	♀	♂	♀	
1 mg. cod- liver oil Z	Hardened cotton-seed oil	3	7	1.0	13.1	0.62	1.06	0.93
„	Olive oil	4	7	12.5	15.7	1.06	1.32	1.23
„	Coconut oil (161441)	5	7	17.8	30.9	1.35	4.73	3.32
2γ carotene (Int. St.)	Coconut oil (161441)	5	7	37.6	33.7	3.33	5.99	4.88

vitamin A value of cod-liver oil. The significance of the difference between the results for olive oil and coconut oil is expressed by the value found for P , < 0.01 .

A simultaneous test of the coconut oil (161441) on 6 rats 0.02 g. per day resulted in the death of five of the rats within 3–28 days. One rat was alive at the end of 5 weeks and had gained 15 g. Thus the coconut oil cannot be suspected of itself containing vitamin A.

B. *Estimation of the vitamin A potency of a sample of cod-liver oil (Z).*

(a) A test of a daily dose of 2γ carotene (International Standard) in the same sample of coconut oil (Table V) was made at the same time as the comparison of different oils as solvents for cod-liver oil, in order to determine directly the value of our cod-liver oil Z. It proved to be 1400 International Units per g.

(b) At the time of the test summarised in Table III, a simultaneous test was made of cod-liver oil Z. The daily dose was 1 mg. dissolved in 0.02 (± 0.001) g. arachis oil. Fresh dilutions were (as usual) made each week (Table VI). From this comparison, cod-liver oil Z was found to contain 1750 International Units vitamin A per g.

Table VI.

1. *Estimation of the vitamin A content of cod-liver oil Z, the oil and the standard both dissolved in arachis oil.*

2. *Comparison of the vitamin A activity of a sample of β-carotene with that of the International Standard, both dissolved in coconut oil (161441).*

Dose	Solvent	No. of rats		Mean increase in wt. in 5 weeks g.		Abscissae of Coward's curve of response corresponding to mean increase in wt.		Weighted mean of abscissae, i.e. relative potency of doses
		♂	♀	♂	♀	♂	♀	
1 mg. cod- liver oil Z	Arachis oil	5	5	25.2	24.8	1.82	2.84	2.33
2γ carotene (Inter. St.)	Arachis oil	4	5	33.5	23.6	2.76	2.56	2.64
1γ carotene (Inter. St.)	Coconut oil (161441)	5	5	25.0	12.6	1.79	1.00	1.40
1γ β-carotene	Coconut oil (161441)	5	5	30.0	23.6	2.36	2.56	2.44

There is no significant difference between the results (a) and (b). Thus the same estimate of the vitamin A potency of a sample of cod-liver oil was obtained by dissolving both oil and standard in either arachis oil or in coconut oil.

C. *Estimation of the vitamin A potency of a sample of β -carotene.*

Part of a sample of β -carotene kindly supplied to the Medical Research Council by Prof. Karrer was compared with the International Standard for vitamin A, both samples of carotene being dissolved in coconut oil, distributed in ampoules and used for feeding as in the previous tests. The result may be seen in Table VI. The β -carotene has about 175 % of the vitamin A potency of the International Standard.

DISCUSSION.

Baumann and Steenbock [1933] have examined the influence of various solvents on carotene with respect to changes in colour intensity of the solutions.

By measuring spectrophotometrically the intensity of $E_{1\text{ cm.}}^{1\%}$ at 485 and 460 $m\mu$, they found that, among edible oils, refined cotton-seed oil was outstanding as a stabilising solvent for carotene; esters of lauric and sebacic acids alone were unsatisfactory, but with quinol were as effective as cotton-seed oil. Although reduced by exposure to light, air and high temperature, solutions of carotene in cotton-seed oil were not rendered more stable by exclusion of oxygen or presence of quinol or the antioxidant factor of wheat germ oil. Under the conditions usually obtaining during feeding tests, carotene in cotton-seed oil suffered 26 % loss in the first month. Baumann and Steenbock recommend that the main supply of solution should never be removed from the ice-chest, but that a portion necessary for a week's feeding only should be removed for the short time while doses are given to the animals. Actually this is the procedure that we have always followed in our feeding tests with carotene and vitamin concentrates.

While we have confined our observations almost wholly to the biological activity of our solutions, our one physical comparison of different oils, hardened cotton-seed oil, ethyl laurate and arachis oil, indicated that, after 11 weeks, there was very little difference in the intensities of yellow colour of the solutions, although, during that period, biological tests had shown the solution in arachis oil to be more active than either of the other two solutions. Moreover, exposure of the solutions to the air and warm temperature of the animal room for 20–30 minutes daily for one week did not diminish the colour intensity of the solutions. Thus, while we have demonstrated biologically that different oils used in dissolving carotene for administration to the rats may influence greatly the activity of the carotene, our results with the physical measurements, so far as they go, do not indicate that the intensity of colour of the solutions can be taken as an indication of their vitamin A potency.

SUMMARY.

1. The oil used for solution of the provisional International Standard for vitamin A may greatly influence its biological activity as shown in ordinary vitamin A tests.
2. A solution of the Standard in arachis oil had 5–6 times the potency of solutions in hardened cotton-seed oil and ethyl laurate, though made of the same strength, *w/v*.
3. Solutions of equal strength of the Standard in one sample of arachis oil and in coconut oil (161441) had the same vitamin A potency.

4. Two samples of coconut oil gave solutions of the same potency. A third sample appeared to give a solution of less potency but the result was not conclusive proof of a difference.

5. The oil used to dilute cod-liver oil for dosing may have some influence on the vitamin A potency of cod-liver oil also. Cod-liver oil Z dissolved in coconut oil (161441) was about three times as active as when dissolved in hardened cotton-seed or olive oil. The coconut oil itself (161441) was shown to be without vitamin A activity.

6. When cod-liver oil Z and the Standard were both dissolved in arachis oil for testing, the same estimate of the vitamin A content of oil Z was obtained as when both were dissolved in coconut oil.

7. An estimation of the vitamin A potency of a sample of β -carotene showed it to have about 175 % of the vitamin A potency of the International Standard.

8. The intensities of the yellow colour of different solutions of the Standard did not correspond with the vitamin A activities of the solutions but this comparison was made in only one experiment.

REFERENCES.

- Baumann and Steenbock (1933). *J. Biol. Chem.* **101**, 561.
Coward and Key (1934). *Biochem. J.* **28**, 870.
Permanent Commission on Biological Standardisation, Health Organisation of the League of Nations (1931). Official No. C.H. 1056 (1), Geneva.