CCXCIII. VITAMIN A DETERMINATION: RELATION BETWEEN THE BIOLOGICAL, CHEMICAL AND PHYSICAL METHODS OF TEST.

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AFTER the introduction of the International Standard carotene for vitamin A in 1931 the variations that occurred in the results obtained by different workers in the assay of oils and other substances containing vitamin A appeared to be quite outside the range of experimental error. For this reason, as manufacturers, we (B.D.H., Ltd.) preferred to adhere to the practice of describing the potency of our products in terms of the Carr-Price colour test. At the same time some doubt was cast upon the value of the colour test, notably by Coward *et al.* [1932], who stated that "the blue colour value of an oil cannot be considered as a measure of its vitamin A potency." This conclusion of Coward *et al.* not being in accord with our own experience, we made a careful examination of a number of oils, comparing them with each other when diluted to the same colour value and assaying the diluted samples in terms of the International Standard. It may be stated at once that, using the International Standard with our technique for the biological test, consistent results have been obtained. The limited number of cod-liver oils that we have examined have shown a constant ratio to the Carr-Price figures, using the Smith and Hazley method, and also to those obtained by the spectrophotometric method. Especially we have found that purified vitamin A concentrates give a constant ratio between the results of the biological, chemical and physical methods, and there seems to be no reason to doubt that the three are interchangeable. In order that we may not be the only judges of the validity of our interpretations of the biological test, a number of these are here described in some detail.

Three of the oils originally examined were crude liver oils, the first two (oils A and Z) having been given to us by Dr Coward for the purpose of making an assay the results of which could be compared with her own and with those of various other workers to whom samples were also given. The third oil was subjected to further treatment, yielding concentrates C, D and E, and the last sample F was a concentrate obtained by distillation from a totally different source. All these samples were compared directly with the International Standard with the exception of D.

EXPERIMENTAL.

At a weight of about 40 g. young rats were placed on our vitamin A-free diet (A 5), which has the following percentage composition:

Extracted and heated caseinogen	...	...	20
Rice starch	...	...	60
Marmite...	...	...	5
Salt mixture	...	...	5
Hardened fat	...	...	9.9
Radiostol (3000 u./ml.)	...	...	0.1

The rats were weighed twice a week until they started to decline in weight. At this stage they were given doses of the oils, diluted with coconut oil to give blue values of 2.5 and 5.0, and of the solutions of the Standard in coconut oil containing 50 or 100 u./ml., 0.02 ml. being the volume of the dose invariably used. The coconut oil was not the same throughout but in every test oil from the same batch was used to dilute the sample and to dissolve the Standard. Each litter was divided up so that for every rat on a dose of oil there was a corresponding animal of the same sex given a dose of Standard. Individual members of the same litter practically always "ran out" at the same time, but where this was not so, only 2 or 3 days elapsed before all were ready for the oils. One from every litter was kept upon the basal diet without oil as a negative control. Out of the 335 rats employed in the examination of these oils all the negative controls started to decline at the time when their litter-mates were given oils and died soon after. We encountered none of the irregularities among the negative controls that are described by Bacharach [1933].

Fig. 1. Comparison of oils A and Z with the International Standard. Average growth curves of litter-mates. *Negative controls.* Since in every litter the animal that grew for the longest time on the deficient diet was taken as negative control, these averages illustrate the time of survival of the most vigorous animals in the litters used.

Daily doses:

- Oil A. 5.6 mg. (oil diluted to 2.5 blue on the colour test). × × ×
 11.2 mg. (oil diluted to 5.0 blue on the colour test). × - - - - - × - - - - -
 Oil Z. 2.5 mg. (dilution at 3.5 blue reckoned on non-saponifiable fraction). ● ●
 5.0 mg. (dilution at 7.0 blue reckoned on non-saponifiable fraction). ● - - - - - ● - - - - -
 Standard. 1.0 unit. 2.0 units. - - - - -

1. *Comparison of oils A and Z with International Standard.*

Doses. Oil A. 5.6 mg., 11.2 mg. Oil Z. 2.5 mg., 5.0 mg.

Fig. 1 shows average growth curves of directly comparable animals. From these we may conclude:

1. That the smaller doses of both oils contain more than 1 unit of International Standard vitamin A activity, oil Z being more active at this dilution than oil A.

2. That the larger doses of both oils contain more than 2 units of vitamin A activity, oil Z again being more effective than oil A.

3. That the smaller doses of both oils appear to be more effective than 2 units of Standard.

It will be seen that all comparisons are in agreement except that of Z with 2 units of Standard. The averages of two pairs of males on this dose suggest that Z contains less than 2 units in 5.0 mg., whereas according to all the other evidence it contains more. From the fact that agreement is not perfect with regard to these larger doses, we may conclude that the estimates 320 U. for oil A, and 720 U. for oil Z are not far removed from the true values. These figures were obtained by calculation from the actual dilutions made by assuming equality of effect between the larger doses of the two oils and 1 unit of Standard. Actually oil Z was more active at the same dilution than oil A, and it therefore follows that if oil A contains 320 U./g., oil Z contains more than 720 U./g.

The evidence of the colour test is in agreement with that of the biological method. The antimony trichloride test on these oils gave values of 8.0 and 12.0 respectively. For oil A the figure obtained by calculation from the non-saponifiable fraction was the same as that obtained from the oil itself, but for oil Z it was much greater, 25.0. The doses given were based upon the values 8.0 and 18.0 respectively, dilutions being made to 2.5 and 5.0 in accordance with our experience that 0.02 ml. of oils of these colour values gives reasonable but not maximum growth. If the value 12.0 obtained for oil Z gives the true indication of its potency, then the dose given would be rather less effective than the corresponding dose of oil A, but, if the figure for the non-saponifiable fraction gives the truer estimate of the biological value, then the dose would be more effective than the corresponding dose of oil A, which is what we found. Hence, in all other assays of cod-liver oils we have based our dose upon the blue figure obtained after saponification.

2. *Comparison of a highly active liver oil with the product of its hydrolysis after separation of sterols.*

Seventy-six rats were used in the test which was carried out in exactly the same way as that described above. The colour values found for the two samples were 460 (B) and 30,000 (C). The first gave the same value by calculation from the figure obtained after saponification.

Doses. Oil B. 0.0977 mg., 0.1953 mg. Concentrate C. 0.0015 mg., 0.0030 mg.

These doses were based upon the colour test and correspond to dilutions giving colour values of 2.5 and 5.0 blue respectively.

Fig. 2 gives the average growth curves of comparable animals. These show:

1. That 0.0977 mg. of B is more effective than 1 unit and probably more than 2 units of International Standard vitamin A.

2. That 0.1953 mg. of B is more effective than 2 units of International Standard.

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Vol. XXVIII, p. 2256, line 19 from top of paper

for larger doses of the two oils and 1 unit

read smaller doses of the two oils and 2 units

3. That 0.0015 mg. of concentrate C is similar to or rather more effective than 1 unit of Standard.

4. That 0.0030 mg. of concentrate C is more effective than 2 units of Standard.

Fig. 2. Comparison of oil B with the product of its hydrolysis after separation of sterols and with the International Standard. Average growth curves for litter-mates.

Daily doses:

Oil B. 0.0977 mg. (i.e. dilution giving blue value of 2.5).	• • • • •
0.1953 mg. (i.e. dilution giving blue value of 5.0).	• - - - - •
Oil C. 0.0015 mg. (i.e. dilution giving blue value of 2.5).	× • • • • ×
0.0030 mg. (i.e. dilution giving blue value of 5.0).	× - - - - ×
Standard. 1 unit.	2 units. - - - - -

The results on the lower doses show that the original oil diluted to a given colour is more active than its concentrate diluted to the same colour value. The results on the higher doses are less definite.

3. *Comparison of two highly active concentrates.*

Seventy-eight rats were used in the test. The samples were obtained by high vacuum distillation of the non-saponifiable fractions of fish-liver oils. E, prepared by distillation from C, gave a blue value of 48,000, and F gave a blue value of 63,820.

Doses. E. 0.0019 mg., 0.0009 mg. F. 0.0014 mg., 0.0007 mg.

The average growth curves from comparable groups of rats are shown in Fig. 3.

Fig. 3. Comparison of samples E and F with International Standard.
Average growth curves of litter-mates.

Daily doses:

- E. 0.0019 mg. (i.e. dilution giving blue value of 5.0). × - - - × - - - ×
 0.0009 mg. (i.e. dilution giving blue value of 2.5). × × ×
 F. 0.0014 mg. (i.e. dilution giving blue value of 5.0). • - - - - - •
 0.0007 mg. (i.e. dilution giving blue value of 2.5). • • •

Standard. 1 unit. 2 units. - - - - -

Results. 0.0009 mg. of E=0.0007 mg. of F=1 unit;
 0.0019 mg. of E=0.0014 mg. of F=2 units.

We may conclude that with these highly purified concentrates the colour test gives a very good indication of the activity of the oils. The ratio between growth and colour test appears to be less with distilled oils than with the original oil or its non-saponifiable fraction.

4. *Direct comparison of samples B, C, F and D, when diluted to the same colour value (5.0 blue).*

The samples are the same as those used in 2 and 3 with the exception of D (50,000 blue) which replaces E (48,000 blue). D was another distillate from C. Fifty-eight rats were used in the comparison.

Doses. B. 0.1954 mg. C. 0.0030 mg. D. 0.0018 mg. F. 0.0014 mg.

The average growth curves from comparable groups of rats are given in Fig. 4.

Fig. 4. Direct comparison of samples B, C, F and D when diluted to the same colour value. Average growth curves of litter-mates.

Daily doses:

B. 0.1953 mg.	• - - - • - - - •	F. 0.0014 mg.	■ - - - ■ - - - ■
C. 0.0030 mg.	x - - - x - - - x	D. 0.0018 mg.	• - - - • - - - •

Results. The activities of the samples fall into the following order: oil B is more effective than C: D and F are probably about equal when diluted to the same blue colour value.

Note on the interpretation of results.

In the tests reported here, all comparable rats have been included in the averages just considered, but when one has died they have been recalculated entirely omitting the dead animal and its litter-mates. Using such small numbers of animals this is the only practicable course, as well as the only fair one. If an animal is allowed to reach such a state of ill health as to be unable to give any response to a dose of vitamin A adequate for the majority, it ceases to comply with the fundamental principles of the test in that it is suffering from something more than vitamin A deficiency. All sorts of secondary effects come in which must have a profound influence and the animal therefore becomes an unsuitable test object. Results have been taken from live animals only, and in the curves given here live animals only were included. At the same time we have kept careful notes of animals dying in the course of a test, which might give a conclusion of some significance if we were to find that a large number of deaths occurred regularly upon one particular sample rather than another.

Relation between the number of biological units and the result of colour test.

The results of these few preliminary tests are summarised in Table I. They lead to the following observations:

1. The ratio between the number of International Units per g. and the result from the colour test is greater in the case of a natural oil than of one which has been distilled.

Table I. *Relation between results of biological tests and of colour tests on liver oils and concentrates.*

Sample	Blue value	Blue value on non-sap. fraction	Approximate number of International Units per g.	Ratio between results
				Biological test Colour test (on non-sap. fraction)
A	8	8	320	40 : 1
Z	12	25	1,000	40 : 1
B	460	460	18,400	40 : 1
C	30,000	—	600,000	20 : 1
D	50,000	—	1,000,000	20 : 1
E	48,000	—	960,000	20 : 1
F	63,820	—	1,276,400	20 : 1

2. The ratio for a distilled oil is about 20 : 1, that for a natural oil is about 40 : 1, based on the value calculated from the non-saponifiable fraction.

Hence, the ratio for natural oils is about as twice as great as that for purified concentrates.

Table II. *Cod-liver oils.*

Blue value	Blue value on unsap. fraction	$E_{1.0\text{ cm.}}^{1\%}$ 328 $m\mu$	B/E
	B		
7.6	12.0	0.4	30 : 1
7.2	15.0	0.65	23 : 1
8.8	18.8	0.8	26 : 1
9.2	32.0	0.85	38 : 1
17.6	20.0	1.0	20 : 1
10.0	30.0	1.0	30 : 1
22.0	30.0	1.1	27 : 1
28.0	40.0	1.2	33 : 1
13.2	22.5	1.2	19 : 1
32.0	46.0	1.6	29 : 1
Average			27.5 : 1

Table III. *Concentrates.*

Blue value	$E_{1.0\text{ cm.}}^{1\%}$ 328 $m\mu$	Blue value/E
530	8.0	66 : 1
550	12.0	45 : 1
720	16.0	45 : 1
900	18.0	50 : 1
1,000	20.0	50 : 1
1,400	28.0	50 : 1
2,000	44.0	45 : 1
2,900	60.0	48 : 1
3,000	45.0	55 : 1
4,800	100.0	48 : 1
5,760	94.0	61 : 1
7,000	146.0	50 : 1
10,000	180.0	55 : 1
1,050	28.0	39 : 1
3,000	37.5	80 : 1
4,800	100.0	48 : 1
7,000	141.0	50 : 1
11,000	225.0	50 : 1
22,000	480.0	46 : 1
27,000	550.0	49 : 1
40,000	800.0	50 : 1
50,000	1100.0	49 : 1
57,000	1160.0	49 : 1
63,000	1128.0	55 : 1
76,000	1360.0	56 : 1
76,000	1440.0	53 : 1
80,000	1600.0	50 : 1
Average		52 : 1

Relation between the extinction coefficient and the colour test.

At the time when the biological work had reached this stage, Mr Crews of our Analytical Department had already accumulated a considerable number of results of routine testing, and these provide interesting data upon the relationship between the extinction coefficient and the colour test. The figures given in Tables II and III are taken from his results, many of which have already been published [1934]. They show:

1. That the ratio between the result of the blue colour test and the extinction coefficient is greater in the case of a purified vitamin A preparation than in that of a natural oil.

2. The average ratio for a concentrate is 52 : 1, that for a natural oil is 27.5 : 1.

Hence, the ratio for natural oils is about half that for purified oils.

Relationship between the number of biological units, the result of the colour test and the value of the extinction coefficient.

When we combined the two sets of observations given above it followed that the extinction coefficient must be proportional to the number of biological units per g. for all oils whether crude or purified. Thus:

For concentrates:

$$\text{If } \frac{\text{Number of units}}{\text{Blue value}} = K_1$$

and

$$\frac{\text{Blue value}}{\text{Extinction coefficient}} = K_2,$$

then

$$\frac{\text{Number of units}}{\text{Extinction coefficient}} = K_1 K_2 \quad \dots\dots(1).$$

For natural oils:

$$\frac{\text{Number of units}}{\text{Blue value}} = 2K_1,$$

$$\frac{\text{Blue value}}{\text{Extinction coefficient}} = \frac{1}{2}K_2.$$

Therefore, again,

$$\frac{\text{Number of units}}{\text{Extinction coefficient}} = K_1 K_2 \quad \dots\dots(2).$$

We proceeded to verify this relationship by determinations of the biological value and the extinction coefficient of as many different oils as possible. These figures are summarised in Tables IV and V. Fig. 5 shows graphically the results of comparing the vitamin A contents of 17 oils by the three different methods, the total number of rats used being over 2000. In the diagram the vitamin A content of oil Z which assayed at about 1000 units/g. was taken as unity. Bearing

Table IV. *Relation between the results of the biological, chemical and physical tests on liver oils.*

Oil	Blue value (non-sap.) B	Extinction coefficient E	Approximate number of biological units per g. U	Ratios	
				U/B	U/E
A	8	0.8	320	40 : 1	400 : 1
Z	25	0.8	1,000	40 : 1	1250 : 1
B	460	10.8	just > 18,400	just > 40 : 1	just > 1704 : 1
X	54	1.7	2,400	44 : 1	1412 : 1
Y	13.4	0.55	600	45 : 1	1091 : 1
G	46.0	1.6	1,840	40 : 1	1150 : 1
H	12.0	0.8	> 344	> 29 : 1	> 430 : 1

Table V. *Relation between the results of the biological, chemical and physical tests on vitamin A concentrates.*

Sample	Blue value (non-sap.) B	Extinction coefficient E	Biological units per g. U	Ratios	
				U/B	U/E
C	30,000	600	>600,000	>20:1	>1000:1
D	50,000	858	<1,200,000	<40:1	<2000:1
E	48,000	915	1,250,000	25:1	1457:1
F	63,820	1440	960,000	20:1	1049:1
J	30,000	—	1,276,400	20:1	886:1
K	25,000	475	600,000	20:1	—
L	500	10	500,000	20:1	1053:1
M	1,000	20	<12,500	<25:1	<1250:1
N	1,000	20	<25,000	<25:1	<1250:1
P	49,000	1100	25,000	25:1	1250:1
Q	1,000	20	>612,500	>12.5:1	>557:1
R	60,000	1150	<1,225,000	<25:1	<1114:1
			20,000	20:1	1000:1
			1,500,000	25:1	1304:1

Fig. 5. Results of the determination of the vitamin A contents of eleven concentrates and six liver oils by the three different methods.

▨ Biological method. □ Colour test. ■ Spectrophotometric method.

in mind the errors inherent in the biological and in the colour tests the general parallelism between the results by the three methods leaves very little room for doubt that all three are estimating the same substance. In view of the possibility of the attainment of the highest degree of accuracy by means of the spectrophotometer, this is clearly the best method of test. It has the additional advantage that its application to concentrates and to natural oils is the same.

Degree of accuracy of the biological test.

In all the tests reported the Standard was given in doses of 1 and 2 units, and the oil to be assayed was diluted to 1 and 2 blue, to 2 and 4 blue, or to $2\frac{1}{2}$ and 5 blue, and 0.02 ml. was the volume of every dose administered. The results were inferred from the observation that an oil of the given blue value was equivalent to 50 or 100 units per ml. or that it was more active, or that its activity lay between the two. Hence, every biological result may be subject to an error not greater than 50 %. We believe that the method is really susceptible of a more quantitative interpretation than this, but, since we have not carried out experiments to prove it, this limited standard of accuracy is all that is claimed.

The evaluation of an oil by means of the extinction coefficient and by the colour test.

The figures given in Tables IV and V were intended rather to establish a principle than to fix the absolute values and, since several of them are unfinished results expressed as limits, the average of these is clearly not the best value from which to calculate the number of biological units corresponding to any given value for the extinction coefficient.

The purest preparation of vitamin A for which physical and chemical constants are known gave a blue value of 80,000 and an extinction coefficient of 1600, and contained 2.25×10^6 biological units per g.; *i.e.*

1600 *E* corresponds to 2.25×10^6 ,

therefore 1 *E* corresponds to 1250–1563 units per g.

If we take this figure as 1400 it will be sufficiently near the truth for any practical purpose. Therefore to convert the value of the extinction coefficient into units per g., it should be multiplied by the factor 1400. For any purpose for which the utmost accuracy is not required, the value for the antimony trichloride colour test can be converted into biological units by multiplying by 40 for a cod-liver oil or by 20 for a purified vitamin A preparation. The test is carried out by the method described in the British Pharmacopœia on the diluted

Table VI. *Determination of vitamin A. Differences between results predicted from the spectrophotometric test and those obtained by biological estimation.*

Sample	Biological result	Result calculated from spectrophotometric test	% error
A	320	1,120	>100
Z	1,000	1,120	12
B	>18,400	15,120	>18
X	2,400	2,380	10
Y	600	770	28
G	1,840	2,240	22
H	>344	1,120	—
C	600,000	840,000	—
	–1,200,000		
D	1,250,000	1,201,200	4
E	960,000	918,660	4
F	1,276,400	2,016,000	58
K	500,000	665,000	33
L	<12,500	14,000	>12
M	<25,000	28,000	>12
N	25,000	28,000	12
P	>612,500	1,560,000	—
	<1,225,000		
Q	20,000	28,000	40
R	1,500,000	1,610,000	7

non-saponifiable fraction, allowance being made for the dilution in calculating the result.

Table VI shows the error involved in using the spectrophotometric method to determine the number of biological units. Two of the cod-liver oils have shown anomalous results, but the maximum error in the case of the concentrates is 58 %. If we are right in our conclusion that the extinction coefficient and the growth test are actually estimating the same substance, then Table VI shows that the error of our biological test is usually much less than 50 %.

We have no experience of the application of either of the three methods discussed to the determination of the vitamin A in butter or other dilute natural sources.

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