CXCVIII. A DISCREPANCY BETWEEN BIOLOGICAL ASSAYS AND OTHER METHODS OF DETERMINING VITAMIN A. I¹.

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THREE methods are at present widely used for the determination of vitamin A, *viz.* (i) the fundamental method of biological assay, (ii) the measurement of the ultraviolet absorption at $328m\mu$, and (iii) the evaluation of blue-colour intensity in the antimony trichloride test. The colour test and the ultraviolet absorption owe their origin to the alcohol $C_{20}H_{29}OH$ or its esters [Karrer *et al.*, 1931; 1933; Karrer and Morf, 1933; Heilbron *et al.*, 1932], and the biological activity shows a very high correlation with these tests. Refinement of all three methods has, however, revealed discrepancies, and the object of this paper is to show that some materials possess biological activity, indistinguishable from that of vitamin A, over and above that corresponding with the proportion of $C_{20}H_{29}OH$ (or its esters) present in these preparations.

The reliability of the physicochemical tests as measures of vitamin A potency has already been the subject of much investigation. The antimony trichloride colour test was introduced in 1926 [Carr and Price, 1926], but on account of the errors then obtaining in biological assays, its validity could not be readily assessed [*cf.* Medical Research Council Committee Report, 1928]. The spectroscopic test was introduced in 1928 [Morton and Heilbron, 1928], and the first attempt to correlate biological and spectroscopic data on a quantitative basis was made shortly afterwards [Drummond and Morton, 1929]. The extremes in the intensity of absorption at $328m\mu$ for the six cod-liver oils tested covered a range of only 3.25 to 1, and the biological assay could not be relied upon to differentiate clearly between oils differing in potency in a ratio of less than 2 to 1. Within the limitations of the comparison, however, the agreement was satisfactory.

A more comprehensive investigation was made by Coward *et al.* [1931]. Eleven cod-liver oils and two concentrates were studied, the range of potency being of the order 30 to 1. In this work Blue values in the colour test were determined in two ways: (i) in the ordinary way on the oils themselves, and (ii) on the unsaponifiable extracts from known weights of the oils. Ultraviolet data were recorded directly on the oils. Statistical methods showed that although there was a high degree of correlation between the physical and biological assays, the estimated errors of the biological test were not sufficient to account for the observed discrepancies. It was pointed out, however, that the biological test as carried out might have been liable to further error due to a variation in the average response of the animals to vitamin A at different

¹ The substance of this paper was read at the 166th meeting of the Biochemical Society (Liverpool, December 15th, 1934).

periods, and it has since been shown that the error from this source may be very large indeed [Coward *et al.*, 1933].

In further work on the same subject [Coward *et al.*, 1932] four oils from the same series were re-examined, and five new samples were studied. The results generally supported the conclusions of the first paper.

In the present work any error of the biological assay due to variation in the average response of rats to vitamin A at different periods has been eliminated by the use of the international carotene standard. Each assay has been made by direct comparison of the test substance with the standard preparation; for every rat that has received a dose of the test substance, another of the same litter and the same sex has received a comparable dose of the standard over the same period. It appears, therefore, that we are justified in assuming that the error of the biological assay, calculated from the variation in the difference of the growth of animals receiving doses in a known ratio, represents the total error of the assay.

It was pointed out in the previous papers of Coward *et al.*, noted above, that the ultraviolet assay carried out directly on liver oils is apt to give results erring on the high side owing to the presence of substances, other than vitamin A, which contribute to a variable extent to the observed absorption. This extraneous contribution is usually of the order 0.05–0.2 ($E_{1\text{ cm.}}^{1\%}$), which, although insignificant in comparison with the gross absorption of a rich oil (*e.g.* halibut, $E_{1\text{ cm.}}^{1\%} 328m\mu = 30$ or more), may introduce a serious error into determinations on weaker oils (*e.g.* cod, $E_{1\text{ cm.}}^{1\%} 328m\mu = 0.5\text{--}1.5$). When the antimony trichloride test is applied directly to a weak oil, there is an error in the opposite sense, which may be considerable, arising from the presence of substances which interfere with the development of the blue colour [*cf.* Morton, 1932]. If, however, the physico-chemical tests are, in such a case, applied not to the oil but to the total unsaponifiable matter from a known weight of oil, both these sources of error can be largely eliminated. In the present work, tests on the unsaponifiable fractions have accordingly been adopted in all determinations on cod-liver oils and materials of similar potency.

EXPERIMENTAL METHODS.

Blue values (Carr-Price-Lovibond)¹ were determined according to the method described in the British Pharmacopoeia (1932), the ether-extracted unsaponifiable fraction of the oil being employed where necessary. All Blue values were determined on more than one occasion.

Spectroscopic measurements were made of the intensities of absorption at the two characteristic maxima exhibited by the blue solution obtained in the antimony trichloride test (at 580–583, and 620 $m\mu$ for rich oils and unsaponifiable extracts; at 572 and 606 $m\mu$ for weaker oils), and of the intensity of absorption in the ultraviolet at or near 328 $m\mu$. For the measurements made on the blue solution, a visual spectrophotometer was employed, and each recorded measurement was the mean of 10–12 readings rapidly taken on freshly prepared solutions, before appreciable fading could occur. For the measurement of the absorption at 328 $m\mu$, a quartz spectrograph with long-focus sector photometer was employed.

For the preparation of unsaponifiable fractions required for spectroscopic examination, the following method, based on the recommendations of the Society

¹ For these determinations we are indebted to Mr H. Pritchard of the Research Department, Lever Brothers, Limited.

of Public Analysts for the determination of unsaponifiable matter [1933], was developed:

Saponify 0.2–1.0 g. of oil, according to potency, with 10 ml. *N*/2 alcoholic KOH for 5–10 minutes. Dilute with 20 ml. water, and extract twice with 25 ml. freshly distilled ether. Wash the combined ethereal extracts once with water (swirling), once with dilute alkali, and then twice more with water (gently shaking), using 10–20 ml. for each wash. For the colour test evaporate an aliquot portion of the ethereal solution and blow dry twice with nitrogen or carbon dioxide at 70–80°, after adding a few drops of pure ethyl alcohol. Make up the residue to suitable volume with pure dry chloroform and examine without delay. For the examination of ultraviolet absorption, evaporate a second aliquot portion of the ethereal extract in a stream of nitrogen or carbon dioxide and dissolve the wet residue in alcohol that has been tested spectrographically for transparency. The use of porous pot (to prevent “bumping”) should be avoided, since adsorption of appreciable quantities of vitamin A on the pot may occur.

Biological assays were carried out according to the method recently described [Morgan, R. S., 1934], using a diet with meat meal and coconut meal as sources of protein. The paper referred to contains an example showing how assays are calculated from growth data (Cod-liver oil II in the present series of tests). In this example the oil was considerably more potent than was expected, and the growth response exceeded that produced by the comparative dose of standard in 17 of the 18 pairs of animals. In many tests, however, the assays were very near the expected potencies, *i.e.* the growth responses produced by the standard and the test substance were nearly equal. In some tests, the experimental period was 6 weeks, growth being averaged at 4, 5 and 6 weeks, and in others the experimental period was 3 weeks. In these, the initial and final weights of the rats were taken as the average of the weights on three consecutive days.

In all assays the reference substance was the 1931 carotene standard of which $1\gamma = 1$ unit. The colour of every new solution of the standard was matched in the Lovibond tintometer, the solution being diluted with chloroform until a match of about 5 yellow was obtained in a 1 cm. cell. The matches on successive solutions were constant within the error of the determination and gave an average value, calculated to a 1 % solution of the carotene, of 12,250 yellow + 3050 red.

RESULTS.

Spectroscopic examinations.

The details of the results of the spectroscopic examinations are given in Table I. In calculating the percentage of vitamin A, the rich concentrate described by Carr and Jewell [1933] has been accepted provisionally as the pure substance. This material has a Blue value of 80,000 and the following spectroscopic characteristics: $E_{1\text{ cm.}}^{1\%}$ 620 $m\mu$ 5000, 583 $m\mu$ 2600, 328 $m\mu$ 1600. Percentages have been calculated by simple proportion. Those based on the extinction coefficients of the blue solution with antimony trichloride tend to represent lower limits, because of inhibition, whilst those based on the extinction at 328 $m\mu$ represent upper limits. It will be noticed that the two sets of values approach each other closely in many unsaponifiable extracts and other rich materials, in which the effects of inhibition and extraneous absorption are slight. The extinction at 583 $m\mu$ is, however, not always easy to determine accurately, and slight discrepancies here may be ignored.

Biological assays and Blue values.

The results of the biological assays and Blue value determinations are also summarised in Table I. The ratios assay/Blue value and assay/spectroscopic data have been brought to the same basis by expressing each as the assay extrapolated

to "pure vitamin A". In all assays thus extrapolated from data on low-potency oils, the figures obtained *via* the unsaponifiable fractions have been used.

Details of the biological assays of three of the substances are given below.

Assay No. 12. Vitamin A distillate. Doses were chosen on the expectation of a potency of about 1.2 million units per g., *i.e.* a dose of 1/1200 mg. was fed in comparison with 1 unit of the standard. The growth responses in 3 weeks were as follows (all the animals were bucks).

	Standard g.	Concen- trate g.		Standard g.	Concen- trate g.
$\frac{1}{2}$ -unit pairs	40.7	35.6	1-unit pairs (<i>cont.</i>)	40.7	49.0
	16.0	11.0		15.4	32.0
$\frac{3}{4}$ -unit pairs	31.3	51.4		21.6	34.0
	0	26.3		44.7	42.0
	22.7	30.0	2-unit pairs	53.0	57.7
1-unit pairs	55.6	57.7		83.7	63.0
	57.0	70.7		66.7	60.7
	56.0	57.0	Positive controls	71.3	—
	52.7	51.0		79.0	—
	52.6	56.0		88.3	—

The growth response produced by the concentrate was superior to that produced by the comparative dose of standard in 11 of the 17 pairs. The average value of the pair difference of the squares of growth is -186, corresponding to a dose ratio of 1.075.

Assay = 1.29 million units per g.

Assay No. 15. Liver oil from the fish Thyrsites atun. Doses were chosen on the expectation of a potency of about 16,750 units per g. The growth responses in 3 weeks were as follows (all bucks).

	Standard g.	Liver oil g.		Standard g.	Liver oil g.
$\frac{1}{2}$ -unit pair	26.3	16.7	2-unit pairs (<i>cont.</i>)	44.1	52.7
1-unit pairs	42.3	47.7		58.0	61.4
	57.3	53.3		41.0	45.7
	38.7	39.4		53.0	42.4
	30.0	38.3	Positive control	78.7	—
2-unit pairs	58.3	60.7			

The growth response produced by the liver oil was superior to that produced by the standard in 7 out of the 10 pairs. The average value of the pair difference of the squares of growth is -212, corresponding to a dose ratio of 1.085.

Assay = 18,200 units per g.

Assay No. 23. Commercial cod-liver oil concentrate II. Doses were chosen on the expectation of a potency of about 40,000 units per g. Growth responses in 3 weeks were as follows (all bucks).

	Standard g.	Concen- trate g.		Standard g.	Concen- trate g.
$\frac{1}{2}$ -unit pairs	27.3	28.6	1-unit pairs (<i>cont.</i>)	63.7	43.7
	23.7	12.3	2-unit pairs	62.3	37.5
$\frac{3}{4}$ -unit pair	38.3	37.6		68.3	57.6
1-unit pairs	45.0	40.3		60.6	60.7
	30.0	25.0		55.7	62.6
	47.4	31.4	Positive controls	71.3	—
	30.3	29.3		76.6	—
	46.3	31.0		83.7	—

Table I. *Summary of vitamin A tests, by biological, chemical and physical methods.*

Potency of "100 %"
vitamin A (millions
of units per g.)
calculated from

No.	Date of assay	Description of sample	Biologica assay, in inter- national units per g.	Tests on oils						Tests on unsaponifiable fractions						Observed assay value	% from spectroscopic data				
				$E_{1\text{ cm.}}^{1\%}$ at						$E_{1\text{ cm.}}^{1\%}$ at											
				Blue value	606-620 $m\mu$	% vit.	572-583 $m\mu$	% vit.	328 $m\mu$	Blue value	620 $m\mu$	% vit.	583 $m\mu$	% vit.	328 $m\mu$						
1	July 1933	Commercial C.L.O. conc. I	15,800	—	—	—	—	—	—	—	—	1,027	61	1-22	42	1-61	23-3	1-46	1-23	1-22	
2	"	C.L.O. "Z"	975	12-7	1-14	0-023	0-92	0-035	0-79	33-2	1-84	0-037	1-05	0-04	0-70	0-044	0-70	0-044	2-36	2-22	
3	Aug.	Fish-liver oil "A"	3,390	139	10-6	0-21	6-6	0-25	3-6	139	—	—	—	—	—	—	—	—	1-95	1-54	
4	Sept.	Mammalian L.O. I	68,000	3438	315	6-3	230	8-9	100	—	—	—	—	—	—	—	—	—	1-58	1-08	
5	Nov.	Mammalian L.O. unsap. matter	410,000	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1-35	1-41	
6	Dec.	C.L.O. I	530	7-5	0-95	0-019	0-69	0-027	0-50	19-1	0-95	0-019	0-53	0-020	0-45	0-028	0-45	0-028	2-22	1-89	
7	"	C.L.O. II	800	10-5	1-3	0-026	1-0	0-038	0-70	24-2	1-37	0-027	0-73	0-028	0-55	0-034	0-55	0-034	2-65	2-35	
1934																					
8	Feb.	C.L.O. "X" (U.S.P. reference oil)	2,840	43	3-1	0-062	2-1	0-08	1-74	67	4-85	0-097	2-35	0-090	1-58	0-099	1-58	0-099	3-38	2-9	
9	Feb.	C.L.O. IV	670	9-4	0-88	0-018	0-70	0-027	0-57	23-2	1-20	0-024	0-70	0-027	0-51	0-032	0-51	0-032	2-31	2-09	
10	Apr.	Unsap. fraction from 9 (1st prep.)	600	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2-06	1-87	
11	Feb.	Arachis oil solution of mammalian L.O.	1,100	64	4-3	0-086	2-85	0-11	1-5	—	3-2	0-064	2-0	0-077	1-44	0-09	1-44	0-09	1-38	1-22	
12	May	Vitamin A distillate (B.D.H.)	1,290,000	—	—	—	—	—	—	67,300	3525	70-5	1940	74	1110	69-4	—	—	1-53	1-84	
13	"	Halibut-liver oil	41,000	1925	107	2-14	58-5	2-25	36	—	—	—	—	—	—	—	—	—	1-71	1-82	
14	July	Unsap. fraction from 9 (2nd prep.)	606	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2-09	1-89	
15	Aug.	L.O. from <i>Thyriscites atara</i>	18,200	670	30	0-60	16-2	0-62	11	—	30	0-60	16	0-61	11	0-69	11	0-69	2-2	2-7	
16	Sept.	C.L.O. "X" (2nd test)	2,468	50	—	—	—	—	—	62-5	—	—	—	—	—	—	—	—	3-16	—	
17	"	Unsap. fraction from 16	2,150	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2-76	—	
18	"	Mammalian L.O. II	42,500	2080	113	2-26	64	2-46	36-6	—	—	—	—	—	—	—	—	—	1-63	1-85	
19	"	Mammalian L.O. III	19,150	1065	62	1-24	39-5	1-5	21-6	—	—	—	—	—	—	—	—	—	1-44	1-42	
20	"	Mammalian L.O. plus commercial C.L.O. conc. (i)	9,550	537	30	0-6	20	0-77	10	—	—	—	—	—	—	—	—	—	1-43	1-53	
21	Nov.	Mammalian L.O. plus commercial C.L.O. conc. (ii)	19,000	1205	60	1-2	39	1-5	22-5	—	—	—	—	—	—	—	—	—	1-26	1-36	
22	"	Tunny-liver oil	95,000	4883	265	5-3	147	5-65	93	—	—	—	—	—	—	—	—	—	1-56	1-67	
23	"	Commercial C.L.O. conc. II	30,900	1910	—	—	—	—	—	1,915	70	1-4	50	—	1-9	32	2-0	32	1-29	1-55	
24	"	Crude C.L.O. "B" (used for 23)	930	23	1-39	0-028	1-06	0-041	1-05	44-2	2-43	0-049	1-30	0-05	0-83	0-052	0-83	0-052	1-68	1-86	

Three of the substances were also assayed in other laboratories, as part of the preparatory work for the 1934 meeting of the League of Nations Health Organisation Permanent Committee on Biological Standardisation. The average assay of Cod-liver oil "Z" (Assay No. 2) was 1500 units per g., a value about 50 % higher than the individual value in Table I, but the average assays of Cod-liver oil "X" (Assay No. 8) and the vitamin A distillate (Assay No. 12) were in close agreement with the individual values reported here.

Cod-liver oil IV was a bulk sample prepared from 64 samples of medicinal cod-liver oil, and Cod-liver oils I and II were bulk samples prepared from the oils below and above median blue value respectively, in the same series. The assays of these oils have been reported in full elsewhere [Morgan and Pritchard, 1935].

The sample referred to as Fish-liver oil "A" (Assay No. 3) was described as cod-liver oil, but the percentage of unsaponifiable matter was above the B.P. limit, and this, together with the high Blue value, suggests that the sample was not genuine cod-liver oil.

The growth response produced by the concentrate was inferior to that produced by the standard in 11 of the 13 pairs. The average pair difference of the squares of growth is 670, corresponding to a dose ratio of 1/1.297.

Assay = 30,900 units per g.

STATISTICAL EXAMINATION OF THE RESULTS.

Correlation between Blue value and biological assay.

(In the calculations made in this section, the assays Nos. 16 and 17, for which there are no spectroscopic data, have been omitted, so that the results may be comparable with those of similar calculations made for the correlation between spectroscopic estimates and biological assays.)

In Table II, the assays extrapolated to "100 %" vitamin A from the Blue values have been arranged in order. The number of pairs of rats used in each assay is given as the equivalent number of pairs of male rats in a 3-week test, on the basis that an assay with 10 pairs of males will be of the same accuracy as one with 20 pairs of females, and that a 4, 5, 6-week assay with 10 pairs will be of the same accuracy as a 3-week assay with 12.2 pairs. The limits of error corresponding to twice the standard error (=three times the probable error) of the biological assays, have been calculated from the number of pairs of animals used in each test and the standard deviation of the difference in the squares of growth of male rats receiving doses in the ratio 2/1.

Table II. Assays extrapolated to "100 %" vitamin A (Blue value = 80,000) from the Blue values.

Assay No.	No. of pairs as males in 3-week tests	Substance assayed	Assay of "100 %" vitamin A, millions of units per g.	Limits of error corresponding to twice the standard error
1	10.4	Commercial C.L.O. conc. I	1.23	0.99-1.66
21	8	Mammalian L.O. + C.L.O. conc. (ii)	1.26	0.90-1.77
23	13	Commercial C.L.O. conc. II	1.29	0.99-1.69
5	7.9	Mammalian L.O. unsap.	1.35	0.96-1.90
11	17.6	Mammalian L.O. dilute solution	1.38	1.10-1.74
20	7	Mammalian L.O. + C.L.O. conc. (i)	1.43	0.99-2.06
19	7	Mammalian L.O. III	1.44	1.00-2.07
12	17	Vitamin A distillate	1.53	1.21-1.94
22	6	Tunny-liver oil	1.56	1.05-2.31
4	12.8	Mammalian L.O. I	1.58	1.21-2.07
18	8	Mammalian L.O. II	1.63	1.16-2.29
24	11	Crude C.L.O. "B"	1.68	1.26-2.24
13	7	Halibut-liver oil	1.71	1.19-2.46
3	9.2	Fish-liver oil "A"	1.95	1.43-2.66
10	8.5	Unsap. from C.L.O. IV, prep. 1	2.06	1.48-2.87
14	13	Unsap. from C.L.O. IV, prep. 2	2.09	1.60-2.73
15	10	L.O. from <i>Thyrsites atun</i>	2.2	1.62-2.98
6	17	C.L.O. I	2.22	1.76-2.80
9	13.7	C.L.O. IV	2.31	1.78-2.99
2	17	C.L.O. "Z"	2.36	1.87-2.98
7	17	C.L.O. II	2.65	2.10-3.35
8	8.5	C.L.O. "X"	3.38	2.43-4.70

Since the errors of the biological assay are calculated logarithmically (*i.e.* the range of error of an assay is expressed by the mean multiplied or divided by a factor, not *plus* or *minus* a difference), the average of the assays extrapolated to pure vitamin A is best calculated *via* the logarithms. The value so obtained is 1.766 million units per g. Seven assays of the twenty-two differ

from this mean by an amount which is equal to or greater than twice the standard error of the assay. It is evident, however, that if two assays differ significantly from the mean in opposite directions, they differ very significantly from each other. The distribution of the assays extrapolated to "pure vitamin A", and their errors, are shown diagrammatically in Fig. 1, but the error

Fig. 1. Biological assays extrapolated to "pure vitamin A" from the Blue values (Assay $\times$ 80,000/b.v.).

Each assay is represented by a point, and the limits of error corresponding to 1.41 times the standard error are shown by the unshaded area on either side of each point. The numbers on the left are the assay numbers given in Table I; they give the order in which the assays were carried out. The letters on the right indicate the class of substance assayed, thus: *M*=mammalian liver oils and concentrates; *CN*=commercial cod-liver oil concentrates; *C*=medicinal cod-liver oils and concentrates prepared from them. *F*=fish-liver oils other than medicinal cod-liver oils; *D*=vitamin A distillate.

The two assays marked * (for which no spectroscopic data are available) have been omitted in calculating the average, so that the averages of the assays of "pure vitamin A" calculated from Blue values and from extinction coefficients, may be comparable.

range in the diagram (shown by the unshaded area on either side of each point) is given by $\sqrt{2}$ times the standard error. If the errors of two assays, calculated in this way, are approximately equal (in a ratio of not more than 2/1), and just fail to overlap, then the difference between the two assays is approximately equal to twice the standard error of the difference and may be considered significant. Examination of Fig. 1 shows that there are 17 assays significantly lower than the highest, 11 assays significantly higher than the lowest *etc.*

The average of the logarithms of the assays calculated to "pure vitamin A" in millions of units per g. is $\log 1.766 = 0.247$. The standard deviation of this value is 0.117. Taking the limits of error as twice this error (*i.e.* to give approximately the 22/1 chance) the limits of error of the Blue value as an estimate of the biological assay are 58.3–171.4 % of the found value.

Correlation between spectroscopic estimations of vitamin A and biological assays.

The assays extrapolated to "pure vitamin A" from the spectroscopic estimations have been arranged in order in Table III. The limits of error corresponding to twice the standard error of the biological assays have been calculated as in Table II. The numbers of pairs of rats used for the assays have already been given in Table II.

Table III. Assays extrapolated to "100 %" vitamin A from the spectroscopic estimations.

Assay No.	Substance assayed	Assay of "100 %" vitamin A, millions of units per g.	Limits of error corresponding to twice the standard error
4	Mammalian L.O. I	1.08	0.83-1.41
1	Commercial C.L.O. conc. I	1.22	0.91-1.64
11	Mammalian L.O. dilute solution	1.22	0.97-1.54
21	Mammalian L.O. + C.L.O. conc. (ii)	1.36	0.97-1.91
5	Mammalian L.O. unsap.	1.41	1.00-1.99
19	Mammalian L.O. III	1.42	0.99-2.04
20	Mammalian L.O. + C.L.O. conc. (i)	1.53	1.06-2.20
23	Commercial C.L.O. conc. II	1.54	1.18-1.86
3	Fish-liver oil "A"	1.54	1.13-2.10
22	Tunny-liver oil	1.67	1.13-2.48
13	Halibut-liver oil	1.82	1.27-2.62
12	Vitamin A distillate	1.84	1.46-2.33
18	Mammalian L.O. II	1.85	1.32-2.60
24	Crude C.L.O. "B"	1.86	1.39-2.48
10	Unsap. from C.L.O. IV, prep. 1	1.87	1.35-2.60
14	Unsap. from C.L.O. IV, prep. 2	1.89	1.45-2.46
6	C.L.O. I	1.89	1.49-2.39
9	C.L.O. IV	2.09	1.61-2.71
2	C.L.O. "Z"	2.22	1.75-2.81
7	C.L.O. II	2.35	1.86-2.97
15	Liver oil from <i>Thyrstites atun</i>	2.7	1.99-3.66
8	C.L.O. "X"	2.9	2.08-4.03

The distribution of the assays calculated to "pure vitamin A" and their errors calculated as $\sqrt{2}$ times the standard error, are shown diagrammatically in Fig. 2.

Fig. 2. Biological assays extrapolated to "pure vitamin A" from the spectroscopic estimates of the percentage present. The significance of the letters and the numbers is the same as in Fig. 1.

The logarithmic mean of the assays calculated to "pure vitamin A" is 1.73 million units per g. The standard deviation of the logarithm of this value is 0.1098, and thus the limits of error of the spectroscopic value as an estimate of the biological assay are calculated as 60.3-165.8 % of the found value.

Correlation of Blue value and extinction coefficient at 328m μ .

The values of the ratio Blue value/extinction coefficient are arranged in ascending order in Table IV.

Table IV. *Values of the ratio Blue value/extinction coefficient at 328m μ .*

	B.V./E.		B.V./E.
Mammalian L.O. I	34.4	Mammalian L.O. III	49.3
Fish-liver oil "A"	38.6	Halibut-liver oil	52.5
C.L.O. I	42.3	Tunny-liver oil	52.5
C.L.O. "X"	42.3	Crude C.L.O. "B"	53.2
C.L.O. II	44.0	Mammalian + C.L.O. conc. (ii)	53.5
Mammalian L.O. unsap.	44.0	Mammalian + C.L.O. conc. (i)	53.7
Commercial C.L.O. conc. I	44.1	Mammalian L.O. II	56.9
Mammalian L.O. dilute solution	44.4	Commercial C.L.O. conc. II	59.9
C.L.O. IV	45.5	Liver oil from <i>Thyrsites atun</i>	60.9
C.L.O. "Z"	47.5	Vitamin A distillate	61.3

The logarithmic mean of these values is 48.5, and the standard deviation of the logarithm is 0.0683. The limits of error of the Blue value as a means of predicting the extinction coefficient that will be found are 73–137 % of the found value (twice the standard error of a single determination with one sample).

If it is assumed that Blue value and extinction coefficient at 328m μ are really measures of the same thing, the error calculated above represents the sum of the independent errors of the Blue value and the extinction coefficient determinations. It will be somewhat greater than the sum of the errors of the Blue value determination and the spectroscopic estimates, since the latter take into account the absorption of the blue solution as well as the absorption at 328m μ .

The possible sources of error are:

(i) Instrumental, *viz.* from ± 3 % in routine spectrophotometry to ± 10 % in Lovibond readings.

(ii) Failure to effect 100 % recovery of vitamin A in the unsaponifiable fraction. This source of error is not significantly operative in careful work.

(iii) The presence of substances other than vitamin A absorbing in the near ultraviolet and remaining in the unsaponifiable fraction. This source of error is normally of no consequence, but in certain preparations of crude oils and mammalian extracts, a real difficulty arises.

(iv) The depth of blue colour in the antimony trichloride test may be subject to variations depending on the exact composition of the reagent, and the personal factor in matching a somewhat variable blue tint against standard Lovibond glasses may be a further source of error.

(v) In the colour test, inhibitors may not be completely eliminated from the unsaponifiable extract.

(iii) and (v) operate in opposite senses, but the main conclusions of this paper are the same, whether the biological assays are compared with colour tests or with ultraviolet absorption.

The most significant source of error, when it occurs, is (iii) (*vide infra*).

Allowance for the errors of the biological assay, the Blue value and the spectroscopic estimate in the above correlations.

The following values of the standard errors of the logarithms of ratios and the limits of error of one variable estimated from another have already been calculated:

Table V.

Ratio X/Y	Standard deviation of the logarithm λ	Limits of error of X predicted from Y (2 λ range %)
(1) Assay/Blue value	0.117	58.3–171.4
(2) Assay/spectroscopic estimate	0.1098	60.3–165.8
(3) Blue value/extinction coefficient	0.0683	73.0–137.0

The error of the biological assay has been dealt with in the paper describing the technique [Morgan, R. S., 1934]. The limits of error given there have been substantially confirmed by subsequent data, and the value of the standard error of the logarithm of an assay with 11.2 pairs of male rats in a 3-week test (the average number used in this series) is calculated as 0.064. This figure is calculated from the variation in the difference of the squares of growth of animals receiving doses of vitamin A in the ratio 2/1 and gives an overestimate of the average error, since the ratio of the doses of vitamin A supplied by the standard and the test substance was generally much nearer 1/1. (This point is discussed in the paper referred to above.) The amount of variation in the ratio assay/Blue value that is due to the error of the biological assay may now be allowed for, by subtracting the logarithmic variances, thus:

$$\lambda^2 \text{ of assay/Blue value} - \lambda^2 \text{ of assay} = \lambda^2 \text{ of vitamin A potency/Blue value} \\ 0.01372 - 0.0041 = 0.00962.$$

$$\lambda \text{ of vitamin A potency/Blue value} = \sqrt{0.00962} = 0.0981.$$

The limits of error corresponding to twice this value are 63.6–157.1 %. This gives the error of the found Blue value as an estimate of true vitamin A potency. In the same way the logarithmic variance of the ratio vitamin A potency/spectroscopic estimate is calculated as 0.00794, giving the standard error of the logarithm as 0.0891, and limits of error of 66.5–150.8 %.

Since the logarithmic variance of the found Blue value, as a measure of vitamin A potency, exceeds that of the spectroscopic estimate, as the same measure, by $0.00962 - 0.00794 = 0.00168$, it may be assumed that the logarithmic variance of the Blue value determinations exceeds that of the extinction coefficient determination by the same amount. Thus the variance of the ratio true/found extinction coefficient may be calculated as

$$(0.0683^2 - 0.00168)/2 = 0.0015,$$

and the variance of the ratio true/found Blue value as

$$0.0015 + 0.00168 = 0.00318.$$

If all the above values are adjusted for the number of observations, the values given in Table VI are obtained.

Table VI. *Variation in ratios of biological, chemical and physical assays of vitamin A.*

Ratio X/Y	$4\lambda^2$ adjusted for No. of observations	X predicted from Y. Range within which 21 out of 22 obser- vations will lie %
Assay/Blue value	0.06126	56.6–176.8
Assay/spectroscopic estimate	0.05382	58.6–170.6
Blue value/extinction coefficient	0.02126	71.5–139.9
Assay (i.e. vitamin A potency/Growth of animals)	0.01636	74.5–134.3
Vitamin A potency/found Blue value	0.04490	61.3–162.9
Vitamin A potency/spectroscopic estimate	0.03746	64.0–156.2
True/found Blue value	0.01435	76.0–131.7
True/found extinction coefficient	0.00691	82.5–121.2
Vitamin A potency/true Blue value	0.03055	66.9–149.6
Vitamin A potency/true extinction coefficient		

(In calculation of the above figures, the error of the spectroscopic estimate has been taken as equal to that of the determination of the extinction coefficient at 328mμ.)

Thus, even after allowing for the computed errors of the biological and the chemical or physical assays, the value of the ratio vitamin A potency/Blue value or extinction coefficient covers a range of about 2 to 1; the observed variation cannot be accounted for by the known errors of the biological, chemical or physical assays.

DISCUSSION.

The values of the individual assays extrapolated to "pure vitamin A" from either the Blue values or the spectroscopic estimates cover a range of about 2.7 to 1. It has been shown that many of the assays differ significantly from one another, and that even after allowing for the computed errors of the tests, a range of about 2 to 1 remains to be accounted for. Further evidence that this discrepancy is a real one is obtained from consideration of two assays which were carried out at the same time and both repeated after an interval of 6 months. The U.S.P. reference oil dated June, 1933 (Oil "X"), was assayed in February, 1934, at 2840 units per g. The Blue value was 67, indicating the presence of 0.084 % "pure vitamin A", and the spectroscopic estimate was 0.098 %. A second biological assay was made in September, 1934, and a value of 2468 units per g. was obtained (87 % of the original value); the Blue value had fallen to 62.5 (93 % of the original value)¹. Thus the second assay may be regarded as a confirmation of the first.

The dilute solution of mammalian liver oil (Assay No. 11) had a Blue value of 64, indicating the presence of 0.08 % of "pure vitamin A", and the spectroscopic estimate was 0.09 %. It would therefore be expected to have about the same potency as the cod-liver oil, Oil "X", but the assay (in February, 1934) was 1060 units per g., only 37 % of the potency of Oil "X". When the assay was repeated in September, 1934, the value obtained was 1140 units per g., a confirmation of the first value, and the Blue value had not fallen.

Examination of Tables II and III and Figs. 1 and 2 shows that the highest values of the assays calculated to "pure vitamin A" are generally those of cod-liver oils of medicinal grade, and that the lowest values are those of mammalian liver oils and commercial cod-liver oil concentrates. Potent fish-liver oils occupy an intermediate position. These observations might suggest the following possibilities:

(i) That the vitamin A of cod-liver oil undergoes a loss of activity on saponification not associated with any change in its ultraviolet absorption or chromogenic properties.

(ii) That the Blue values and extinction coefficients of the medicinal grade cod-liver oils have been grossly underestimated because of failure to effect complete recovery of vitamin A in the unsaponifiable fractions prepared for the determinations.

(iii) That the extinction coefficients of the mammalian liver oils and commercial cod-liver oil concentrates have been grossly overestimated because of the poor persistence² of the 328 $m\mu$ absorption band.

¹ $E_{1\text{ cm}}^{1\%}$ 328 $m\mu$ on another sample of the same oil has recently (April, 1935) been found to be 1.33 (*i.e.* 84 % of original value).

² Persistence is taken as the difference between the absorption intensities at 328 $m\mu$ (the maximum or head of the vitamin A absorption band) and at *ca.* 260 $m\mu$ (the minimum in the same curve). In a normal unsaponifiable fraction the persistence is approximately 60–70 % of the value at 328 $m\mu$.

It does not appear likely, however, that the observed variation in the assays calculated to "pure vitamin A" can be accounted for by these causes. The following comments on each possibility may be made:

(i) The cod-liver oil "B", used in the preparation of commercial cod-liver concentrate II, gave an assay calculated to "pure vitamin A" close to the mean of the whole series and lower than that of any of the medicinal grade cod-liver oils. Preparations of unsaponifiable matter from two of the oils of medicinal grade gave assays, calculated to "pure vitamin A", higher than those of any oils except the medicinal oils and the liver oil of the fish *Thyrstites atun*.

(ii) To reduce the average assay, calculated to "pure vitamin A", of the five cod-liver oils at the end of Table IV to the average of the whole series, it would be necessary to assume a 24 % loss of vitamin A in the extraction of the unsaponifiable matter for the extinction coefficient determination. For these oils, however, the average ratio of the extinction coefficient determined directly to that determined *via* the unsaponifiable matter, was 1/0.87, *i.e.* the combined effects of (a) any loss of vitamin in the extraction process, and (b) the elimination of substances, other than the vitamin, capable of contributing to the gross absorption at 328 $m\mu$ account for a difference of only 13 %. Tests with richer oils show that (b) is reduced to negligible dimensions by the technique here used. The feeding experiments referred to above, with unsaponifiable fractions of two of the oils, furnish further evidence that the Blue values and extinction coefficients of cod-liver oils determined *via* the unsaponifiable matter are not likely to suffer from any considerable error that does not affect direct determinations made on potent oils and concentrates. Two experiments were made with Cod-liver oil IV, and one with Oil "X". For these experiments, about 10 g. of the cod-liver oil were saponified and the unsaponifiable matter was extracted with peroxide-free ether (distilled after standing over sodium wire); an aliquot part of the ether extract was taken for the determination of Blue value, and the remainder was mixed with arachis oil containing quinol and freed from ether by evaporation in a stream of carbon dioxide or nitrogen. The determination of the Blue value of an aliquot part of the unsaponifiable matter, without addition of arachis oil, showed that the recovery was about equal to that obtained in the routine determination of Blue value *via* unsaponifiable matter. The arachis oil solution of unsaponifiable matter was then fed in comparison with the original oil and the percentage recovery of vitamin A determined from the comparative growth responses. In the first and third experiments the animals were divided into litter-mate triads, one of each triad receiving the standard, another receiving the oil and the third receiving the unsaponifiable matter; in the second experiment, the standard was omitted. The respective assays showed recoveries of 89.4, 90.5 and 87 % (all within the limit of 13 % loss indicated by the comparison of extinction coefficients determined directly and *via* the unsaponifiable matter), but the recoveries were probably rather better than these, for in the first two experiments there was some loss of Blue value on storage of the arachis oil solution of the unsaponifiable matter.

(iii) In some of the commercial cod-liver oil concentrates and mammalian-liver oils, the 328 $m\mu$ band showed poor persistence or was entirely masked by other more intense absorption further in the ultraviolet. This absorption was not eliminated by saponification and cannot at present be accounted for. Generally, the ultraviolet results were in satisfactory agreement with the extinction at 620 $m\mu$; in cases of appreciable discrepancy the latter values were taken in extrapolating to "pure vitamin A". It must not be overlooked, however, that the substance(s) responsible for the extraneous absorption in the

ultraviolet, although possibly biologically inactive or less active than vitamin A, may yet conceivably be chromogenic towards antimony trichloride in a similar manner to vitamin A and so account for the unusually low values extrapolated from both the ultraviolet and colour test figures. There is some evidence that the $583m\mu$ band is stronger than usual whenever the ultraviolet absorption is anomalous.

It will be observed that the biological assays are expressed in terms of International Units, the 1931 standard carotene having been used as reference substance. This material, according to physical tests, contained about 68 % of carotene, the remainder being probably impurities or decomposition products. The 1931 standard might thus be expected to show about two-thirds of the activity of pure β -carotene. This is in good agreement with the observation reported to the 1934 League of Nations Commission that 0.6γ β -carotene was biologically equivalent to 1γ of the 1931 standard preparation. The redefinition of the International Unit in terms of 0.6γ pure β -carotene, thus involves 1.66×10^6 I.U. per g. for β -carotene.

A simple calculation is here instructive. If the reaction:

proceeds quantitatively *in vivo* in the avitaminotic rat, 536 parts by weight of β -carotene yield 572 parts of vitamin A, and if this vitamin A is responsible for the growth effect, we have:

$$1 \text{ g. } \beta\text{-carotene} \rightarrow 1.067 \text{ g. vitamin A} \equiv 1.66 \times 10^6 \text{ I.U. per g.}$$

Hence 1 g. of vitamin A corresponds, on this basis, to 1.56×10^6 I.U.

Now let us consider the vitamin A distillate (No. 12 in Table I) containing 70 % of vitamin A by the physicochemical assays. The potency in I.U. observed in different laboratories leads to a value very near to 1.3×10^6 I.U. per g., corresponding to 1.86×10^6 I.U. per g. for the pure vitamin. This result suggests that the conversion of carotene into vitamin A is substantially quantitative *in vivo*, at the experimental dosage used.

The whole validity of the carotene standard rests upon the assumption of a constant efficiency in this conversion, and if the basis of the redefinition of the international standard is borne in mind, there is little room for doubt that the reaction can be quantitative at the levels of dosage used in vitamin assays. This is supported by the fact that β -carotene, which from its structure can yield two molecules of vitamin A, is twice as active as α -carotene, which can yield only one.

If now we turn to Oil "X", emphasis must be laid on the excellent agreement between the spectroscopic colour test and ultraviolet absorption, both carried out on the unsaponifiable extract. The percentage vitamin A is 0.098 to within ± 2 %. The biological assay rests on as rich a body of data as any vitamin A assay yet made, namely nine assays carried out in U.S.A. (mean 3000 I.U. per g.) and several concordant assays in this country. If we accept the figure given in this work, namely 2840 I.U. per g., the calculated figure for 100 % vitamin A must be 2.9×10^6 I.U. per g. This is so much higher than the theoretical value of 1.56×10^6 I.U. per g. as to be outside the known range of experimental error.

There seems, therefore, to be no escape from a real discrepancy, which does not appear to be due to biological differences between vitamin A as an alcohol and vitamin A as an ester. The simplest assumption is that cod-liver oil "X"

contains a biologically active substance distinct from the vitamin A which is usually associated with the formula $C_{20}H_{29}OH$.

It is not unlikely that in the decomposition of vitamin A, biological activity may disappear earlier than selective absorption and chromogenic power. Although the inactive substances formed might show selective absorption and chromogenic power differing qualitatively from those shown by the original active substance, they might interfere with the measurement of the absorption and chromogenic power of any active substance with which they were admixed and thus lead to an anomalous physical assay erring on the high side. A physical assay lower than would be expected from the biological assay, cannot be accounted for in this way.

Note on a paper by Lathbury [1934].

Since a recent paper by Lathbury reports work similar to that described in the present paper, but gives conclusions somewhat different from those arrived at here, it is necessary to consider it in some detail. Her technique differed from ours in the following particulars:

(i) A quantitative interpretation of the biological tests, based on the actual growths of the animals, was not attempted. The standard and the oil to be examined were each fed at two levels, and if the growths produced by the comparative doses were of the same order, the standard and test substance were considered to supply equal amounts of vitamin A at the levels fed. The ratio assay/Blue value was usually a round number (40/1, 25/1 or 20/1) following apparently from the use of the Blue value as guide to dosage level.

(ii) Blue values *via* the unsaponifiable matter were determined by the method of Smith and Hazley [1930] in which chloroform is used to extract the unsaponifiable matter.

(iii) Extinction coefficients of cod-liver oils were determined directly instead of *via* the unsaponifiable matter.

For purposes of comparison, the values given in Tables IV and V in Lathbury's paper have been converted into the form in which the data of this paper are presented, those assays which are given only as limits being omitted.

		Assay extrapolated to "pure vitamin A" (millions of units per g.)	
		From the Blue value	From $E_{328m\mu}$
Liver oil	A	3.20	(0.64)*
	Z	3.20	2.00
	G	3.20	1.84
	B	3.20	2.73
	X	3.55	2.26
	Y	3.58	1.75
Logarithmic average (omitting oil A)		3.34	2.09
Concentrate	E	1.60	1.68
	F	1.60	1.42
	J	1.60	—
	K	1.60	1.69
	Q	1.60	1.60
	D	2.00	2.33
	N	2.00	2.00
	R	2.00	2.09
Logarithmic average (omitting conc. J)		1.76	1.81

* The extinction coefficient of oil A is given as 0.8. This value is abnormally high because of the interfering effect of absorbing substances which can be removed by saponification. The net value, *via* the unsaponifiable matter, determined by one of us (J. R. E.) was 0.36.

The extreme variation in the assays extrapolated to "pure vitamin A" from the Blue values, is from 1.6 to 3.58 million units per g. The values calculated from the data on cod-liver oils are generally higher than those obtained in the present work, but this can be attributed to the difference in the techniques of determining the Blue value *via* the unsaponifiable matter. The use of ether instead of chloroform for extracting the unsaponifiable matter usually results in higher Blue values being obtained, because destruction of vitamin A by chloroform during the concentration of the extract is avoided.

The extreme variation in the assays calculated to "pure vitamin A" from the spectroscopic data is from 1.42 to 2.73 million units per g., compared with 1.08–2.9 in the present work. The mammalian-liver oils and commercial cod-liver oil concentrates, however, are not represented in Lathbury's series, and if they are omitted from the present series, the extreme variation is 1.54–2.9. It is not possible to calculate whether the substantial variation in Lathbury's series is significant of a real discrepancy, since data for the sampling error of the biological assay are not available. The average value for the oils (2.09 million units per g.) is slightly higher than that calculated for the concentrates (1.81 million units per g.), and presumably if the extinction coefficients of the oils had been determined *via* the unsaponifiable fractions, the average value for the oils would have been raised to about 2.4 million units per g.

Thus Lathbury confirms a discrepancy between biological assays and Blue values, and her results cannot be held to exclude the existence of the discrepancy between biological and physical assays noted here.

There is a very close agreement between the average assays of concentrates calculated to "pure vitamin A" in Lathbury's series and the average assays calculated for all oils examined in the present work:

	Assays extrapolated to "pure vitamin A" (millions of units per g.)	
	From Blue values	From spectroscopic data
Lathbury's concentrates	1.76	1.81
All oils in the present work	1.77	1.73

SUMMARY.

Biological assays for vitamin A, Lovibond Blue values, and spectroscopic estimates of the percentage of vitamin A ($C_{20}H_{29}OH$) present, are given for a series of 22 oils and concentrates covering a range of potency of 530 to 1,290,000 international units per g.

From the assay of each substance, and the physicochemical tests made, the potency of "100 %" vitamin A has been calculated, the characteristics of the rich concentrate described by Carr and Jewell [1933] being accepted as those of the pure substance.

The assays so expressed vary from 1.23 to 3.38 million units per g. when calculated from the Blue values (mean 1.77), and from 1.08 to 2.9 million units per g. when calculated from the spectroscopic estimates (mean 1.73). This variation is greater than can be accounted for by the known errors of the assays and the physicochemical measurements; allowance for these errors leaves a range of variation from 67 to 150 % of the mean value to be accounted for.

Generally the highest values are given by cod-liver oils of medicinal grade. The highest of all in the series examined was given by the U.S.P. reference oil; this has been assayed in several other laboratories and the results are in close

agreement with that given in the present work. Evidence is presented that the high values obtained are not due to gross underestimation of the Blue values or the spectroscopic absorptions.

From the recent redefinition of the unit of vitamin A as the activity of 0.6 γ of β -carotene¹, it is calculated that if β -carotene is converted efficiently into vitamin A at the levels of dosage used in assays, the potency of pure vitamin A would be 1.56 million units per g.; this is of the same order as the mean for the whole series of oils and concentrates examined in the present work. Values lower than this might be accounted for by the presence of biologically inactive material showing selective absorption and chromogenic power; but values significantly higher suggest the existence of a biologically active material without selective absorption or chromogenic power, or with these characteristics much weaker than in the vitamin C₂₀H₂₉OH.

One of us (J. R. E.) is indebted to the Medical Research Council for a full time personal grant.

The biological assays reported in this paper were made in the Research Department of Lever Brothers, Limited; the spectroscopic work was carried out in the Chemistry Department of Liverpool University. One of us (R. S. M.) is responsible for the statistical analysis.

¹ According to recent experiments in these laboratories (i) the absorption intensity of pure β -carotene (from grass) is to the absorption intensity of 1934 standard carotene as 100:86, and (ii) the comparative feeding values of 1934 standard carotene and 1931 standard carotene are in the ratio 1 1934 unit (0.6 γ of 1934 carotene)=0.86 1931 unit (0.86 γ 1931 carotene). Thus by compensation of errors, 0.6 γ pure β -carotene=1 1931 unit.

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