CLXXXIX. THE DETERMINATION OF VITAMIN A IN COD-LIVER OILS, (A) BIOLOGICALLY, (B) CHEMI- CALLY, (C) PHYSICALLY, WITH A STATISTICAL EXAMINATION OF THE RESULTS.

II. FURTHER EVIDENCE THAT THE INTENSITY OF ABSORPTION AT $328m\mu$ GIVES THE BEST AGREEMENT WITH THE BIOLOGICAL MEASURE OF VITAMIN A IN COD-LIVER OILS.

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IN a previous paper [Coward, Dyer *et al.*, 1931] it was shown from an examination of eleven samples of cod-liver oil and two concentrates that, taking into account the known sampling error of the biological test, the measurement of the absorption by the oil at $328m\mu$ gave the best agreement with the vitamin A value as determined by a biological test. The "blue value" of the unsaponifiable fraction of the oil gave rather less good agreement, while the "blue value" of the oil itself gave only a very crude measure of the biological activity. It was pointed out, however, that the intensity of absorption of certain oils lay outside the range of the possible biological value. In particular it was noted that two of these oils, C and G, having the same biological values (155 and 153) had absorption bands whose relative intensities were 103 and 257 respectively, and it was suggested that some unknown factor might have influenced the biological test and invalidated the result. Moreover the results of all the tests were evaluated by means of the curve drawn up in these laboratories by Coward, Key, Dyer and Morgan [1930; 1931] and expressed in terms of the oil A originally used for the making of the curve, without making a simultaneous test on this oil at the time of examination of oils C and G. The values obtained would be true in relation to oil A and to each other, only if the whole stock of rats was not varying in sensitivity during this time. As we had no information on this point, it was decided to test oils C and G again making a simultaneous test on oil A and using "litter-mate" animals in the three groups. This was done

twice, in June-July and in August, respectively, of 1931. In the August experiment, a re-test of oil L (of very high vitamin content) was also included. The agreement between oils C and G in biological value was confirmed but when the intensity of absorption at $328m\mu$ of oil C was re-examined it was found to have fallen about 20 %, and that of oil G to have fallen about 50 %. Thus the intensity of the $328m\mu$ band of oil G was only about 150 % of that of oil C. Tests on five fresh samples of cod-liver oil, each one with a simultaneous test on oil A, have also been made during the last nine months. Chemical and physical tests have been made on all the oils at the same time as the biological tests. The whole of the new tests on the old oils and the tests on the new samples have been collected and statistical examinations of the results have been made by the same method as was employed in the previous paper.

It is evident that in this series of oils there is less good agreement between the physical and the biological tests than in the previous series, the range of value ($\pm 2\lambda$) allowed being that shown by Coward [1932] to hold for this colony of rats. It is practically the same as that calculated from a smaller number of tests in the previous paper by Coward, Dyer, Morton and Gaddum [1931].

RE-EXAMINATION OF OILS C AND G.

Comparisons of these oils were made by the method employed in the earlier examinations, though with the added precaution of dividing each litter of rats between the different groups used for testing the different oils. In the first test (June-July, 1931) on oils, A, C and G, the doses chosen proved to be somewhat low and a second test was made using higher doses. The potencies of oils C and G were calculated relative to the contemporary values of oil A (Table I). They again appeared to be equal in vitamin A value, the finding reported in the previous paper being thus confirmed.

Table I. *Re-examination of oils C, G and L in comparison with simultaneous test on oil A.*

Oil	Date of test 1931	Dose mg.	No. of ♂ rats on test	Av. incr. in wt. in 5 wks. g.	$\sigma = \sqrt{\frac{\sum d^2}{n-1}}$	Abscissa of curve of response corresponding to this increase	No. of ♀ rats on test	Av. incr. in wt. in 5 wks. g.	$\sigma = \sqrt{\frac{\sum d^2}{n-1}}$	Abscissa of curve of response corresponding to this increase	Av. abscissa of curves of response for bucks and does	Vitamin A potency (%) relative to oil A (contemporary test)
A	June-July	2	9	11.4	18.55	1.0	4	9.25	19.98	0.77	0.93	100
	Aug.	2	6	9.3	13.58	0.9	6	14.5	17.09	1.2	1.05	100
C	June-July	1	4	-2.5	7.04	0.53	6	-2.8	9.86	0.28	0.38	82
	Aug.	3	4	27.7	8.43	2.12	5	18.4	7.38	1.65	1.86	118
G	June-July	1	8	-3.6	19.78	0.51	8	4.0	8.87	0.51	0.51	110
	Aug.	3	5	18.0	13.64	1.36	5	11.4	7.60	0.92	1.14	72
L	Aug.	0.1	6	-0.3	7.56	0.59	5	0.6	9.06	0.37	0.49	933

Weighted mean =

Weighted mean =

APPARENT DETERIORATION OF VITAMIN A VALUE OF OILS A,
C, G AND L.

From column 12 of Table I it may be seen that a dose of 2 mg. of oil A produced, in June–July, 1931, a mean increase in weight in the rats equal to that which would have been brought about by 0.93 mg. when the curves relating increase of weight to dose of oil A were made. In August, 1931, 2 mg. of oil A appeared to be equivalent to 1.05 mg. of original value. Thus oil A seemed to have lost about half its vitamin A potency. Similarly oils C, G and L all seemed to have lost two-thirds of their original potency, which made it appear in August, 1931, that oils A, C and G were equal in vitamin A value and oil L was only nine times as potent as oil A, whereas, originally oils C and G had 150 % and oil L had 1560 % of the vitamin A value of oil A.

It is quite possible, however, that a difference in sensitivity of the rats accounted for these apparent decreases in vitamin A value. We have some information on this point from the successive tests on oil A which have been made for comparison with other oils (see later part of paper) but until we have more information, we consider it safer to make a simultaneous test on the standard oil with each test of a fresh sample. More satisfactory evidence on this point will be available if carotene proves to be a suitable standard of reference for vitamin A tests. Meanwhile, we have confined our attention to the relation between the biological (vitamin A) values of the oils and their colorimetric and spectrometric values as determined at the same time as the biological value.

CORRELATION BETWEEN THE BIOLOGICAL, COLORIMETRIC AND SPECTRO-
METRIC VALUES OF OILS A, C, G AND L AT DIFFERENT TIMES.

In Table II are collected the various values of oils A, C, G and L, each biological value being given relative to that of oil A (= 100) as determined by a simultaneous test on that oil. The chemical and physical measurements were made by the same methods as were described in the previous paper.

Oil A has proved to be very constant in chemical and physical values and oils C, G and L show losses in intensity of absorption at $328m\mu$ reconcilable with their losses of vitamin A potency relative to oil A. But the fact remains that oils C and G had equal vitamin A potency in 1930 when the intensity of absorption at $328m\mu$ of oil C was about $2/5$ of that of oil G, and they still had equal vitamin A potency in 1931 when the intensity of absorption at $328m\mu$ of oil C was $3/5$ of that of oil G. It seems impossible to avoid the conclusion that the measurement of the intensity of absorption at $328m\mu$ has failed to measure the potency of oils C and G.

Oil G has shown curious behaviour in its colour tests also. The "blue value" of the oil itself has fallen to about two-thirds of its former value, the "blue value" of the unsaponifiable fraction to about two-fifths. The "blue values" measured spectrophotometrically have also shown serious falls. Oil L showed a

Table II.

		Blue value, SbCl ₃ test					Ultraviolet band 328mμ; oil itself
Oil	Date of test	Biological value relative to oil A (=100) simul- taneous test	Lovibond measure		Spectrophotometric		
			Oil	Unsap. fraction	572mμ	606mμ	
A	Nov. 1929–May 1930	100	11.6	—	—	—	—
	Dec. 1930	—	9.0	17.25	0.70	0.92	0.78
	Mar. 1931	—	9.0	17.1	—	—	—
	Apr. 1931	—	—	—	0.75	1.04	0.80
	June–July, 1931	(100)	—	—	—	—	—
	Aug. 1931	(100)	—	—	—	—	—
	Sept. 1931	—	—	17.25	0.72	0.93	0.82
	Oct. 1931	—	12.2	—	—	—	—
	Dec. 1931	—	10.5	—	—	—	—
C	Mar. 1930	147	—	—	—	—	—
	Apr. 1930	180	—	—	—	—	—
	July 1930	202	—	—	—	—	—
	Oct. 1930	90	—	—	—	—	—
	Dec. 1930	—	7.6	12.9	0.50	0.61	0.67
	June–July, 1931	82	—	—	—	—	—
	Aug. 1931	118	—	—	—	—	—
	Sept. 1931	—	—	11.9	0.46	0.64	0.52
	Oct. 1931	—	8.5	—	—	—	—
G	Sept. 1930	153	—	—	—	—	—
	Dec. 1930	—	—	—	1.45	1.92	1.68
	Jan. 1931	—	28.0	42.0	—	—	—
	June–July, 1931	110	—	19.6	—	—	—
	Aug. 1931	72	—	—	—	—	—
	Sept. 1931	—	—	16.4	1.05	1.26	0.87
	Oct. 1931	—	10.1	—	—	—	—
L	Jan. 1931	1562	305.0	—	—	—	—
	Apr. 1931	—	—	266	7.5	12.15	8.0
	Sept. 1931	933	—	236	6.7	11.0	6.1
	Oct. 1931	—	197.0	—	—	—	—

fall of about 30 % in the blue value of the oil itself, but practically none in that of the unsaponifiable fraction. The colour values of oil C have not appreciably changed. The fluctuations in the "blue value" of oil A are indicative of the differences that may be obtained even by the same workers under supposedly standardised conditions.

It is evident that the chemical and physical values are not always measurements of the same substance.

A comparison between the recently obtained biological, colorimetric and

Table III. *Showing the results of the different tests all reduced to the same units, and the probable limits of error of the biological test (probability 21/22).*

Oil	Biological value		Blue value, SbCl ₃ test				Ultra-violet band 328 mμ; oil itself
	Most probable	Possible range (±2λ)	Lovibond measure		Spectrophotometric		
			Oil	Unsap. fraction	572 mμ	606 mμ	
I	II	III	IV	V	VI		
A	100	69-144	97	98	99	93	114
C	100	66-153	68	68	63	64	72
G	90	62-130	81	93	144	126	121
L	930	534-1620	1572	1345	922	1103	846
Factors by which readings in			7.98	5.70	137.6	100.3	138.7

Table IV. *Showing estimates of the logarithmic variance of the different tests.*

	Blue value, SbCl ₃ test					Ultra-violet band 328 <i>mμ</i> ; oil itself
	Biol. value	Lovibond measure		Spectrophotometric		
		Oil	Unsap. fraction	572 <i>mμ</i>	606 <i>mμ</i>	
I	II	III	IV	V	VI	
Biological test λ_1^2	0.0089	—	—	—	—	—
Ratio of biol./phys. results λ^2	—	0.0276	0.0189	0.0272	0.0238	0.0137
Phys. results $\lambda^2 - \lambda_1^2$	—	0.0187	0.0100	0.0183	0.0149	0.0048
Percentage range corresponding to $\pm 2\sqrt{\lambda^2 - \lambda_1^2}$ within which the result may be expected to lie 21/22 times	65–154	53–188	63–158	54–186	57–175	73–137

spectrometric values of these oils has been made by the same method as in the previous paper. It is summarised in Tables III and IV. It is evident from the values of λ (the logarithmic variance of the different tests) that again the 328 m μ band gives the best agreement with the biological value and the blue value of the unsaponifiable fraction of the oil gives the next best measurement, though this is not as near to the 328 m μ measurement as in the last series of oils.

EXAMINATION OF FIVE FRESH SAMPLES OF COD-LIVER OIL.

Five further samples of cod-liver oil have been examined by these methods, each one in comparison with oil A by a simultaneous test on that oil. The results are collected in Table V and analysed in Tables VI and VII. In Table

Table V. *Biological, chemical and physical measurement of five fresh samples of cod-liver oil, each biological value being expressed as a percentage of the biological value of oil A as determined by a simultaneous test on "litter-mate" animals.*

Oil	No. of rats on test		Biol. value relative to oil A (=100)	Blue value, SbCl ₃ test				Ultra- violet band 328 m μ ; oil itself
				Lovibond measure		Spectrophotometric		
	♂	♀		Oil	Unsap. fraction	572 m μ	606 m μ	
R	5	5	570	17.1	32.7	1.07	1.26	0.65
S	7	3	314	23.0	42.8	1.24	1.46	0.75
T	5	4	440	19.6	25.5	0.92	0.94	0.60
U	9	0	314	23.7	34.3	1.10	1.34	0.67
V	4	6	532	13.1	19.8	0.61	0.81	0.52

VI it may be seen that the 328 m μ measure of each of the oils falls within the range of the biological test though in three of the oils it only just does so. It is obviously a rather better measure of the biological value than either of the other measures. In Table VII it is again seen from the values of λ that the 328 m μ measure is the best measure of the biological value. The other measures seem to be of approximately equal, but low, value.

Table VI. *Showing the results of tests on oils R, S, T, U, V, all reduced to the same units and the probable limits of error of the biological test (probability 21/22).*

Oil	Biological value		Blue value, SbCl ₃ test				Ultra-violet 328 $m\mu$; oil itself
	Most probable	Possible range	Lovibond measure		Spectrophotometric		
			Oil	Unsap. fraction	572 $m\mu$	606 $m\mu$	
S	314	195-506	513	599	538	541	498
U	314	216-456	529	480	477	497	445
T	440	257-753	437	357	399	349	398
V	532	316-895	282	277	265	300	345
R	570	343-948	311	458	464	467	432
Factors by which readings in Table V were multiplied to obtain the above results			22.3	14.0	433.7	370.8	664.0

Table VII. *Showing estimates of the logarithmic variance of the different tests on oils R, S, T, U, V.*

		Blue value, SbCl ₃ test				Ultra-violet band
		Lovibond measure		Spectrophotometric		328 m μ ; oil itself
	Biological value	Oil	Unsap. fraction	572 m μ	606 m μ	
Biological test λ_1^2	0.01281	—	—	—	—	—
Ratio of biol./phys. test λ^2	—	0.04870	0.05250	0.04442	0.04375	0.02865
Phys. result $\lambda^2 - \lambda_1^2$	—	0.03589	0.03969	0.03161	0.03094	0.01584
Percentage range corresponding to $\pm 2\sqrt{\lambda^2 - \lambda_1^2}$ within which the result may be expected to lie 21/22 times	59-168	42-239	40-250	44-227	44-225	56-179

DISCUSSION.

By making estimations of the vitamin A content of cod-liver oils by comparison with a simultaneous test on a standard oil of reference, it has been shown that the 328 m μ band of the oil gives the best agreement with the biological value. The "blue values" obtained by the action of antimony trichloride on the oil itself, or on the unsaponifiable fraction of the oil, give less good agreement. The 328 m μ values of some of the oils recently tested, however, only just fall within the range of possible biological values. It was thought to be at least possible that the method of estimating a sample of oil in terms of the value of the standard determined by a simultaneous test made on a reasonable number of animals (ten to twelve) might introduce a further error; for the value of the standard found in that particular test might be somewhat low owing to the normal variation in the response of the animals, whereas the value of the unknown might, for the same reason, be high. On the other hand, the normal variation of the animals might give a high value for the standard and a low one for the unknown. Then the value of the unknown would appear to be much less in the second event than in the first. The actual variation found

in testing the standard seven times showed that it might have consequences of the kind suggested. The seven values for the standard oil found during ten months were:

Date of test	Biological value		No. of animals used	
	Most probable	Range $\pm 2\lambda$	♂	♀
June-July, 1931	46.5	31-71	9	4
August, 1931	52.5	33-83	6	6
October, 1931	59.0	38-89	7	7
November, 1931	36.7	23-58	6	6
Jan.-Feb. 1932	76.2	46-126	5	5
March, 1932	64.3	39-107	0	13
April-May, 1932	55.4	32-97	3	6
Weighted mean	55.8	46-67	36	47

The distribution of these values proved to be normal [Fisher, 1930].

$$\gamma_2 = -0.58 \text{ (standard error} = 1.85\text{).}$$

$$\gamma_1 = 0.118 \text{ (standard error} = 0.925\text{).}$$

Thus it seemed possible that we might get truer values for the vitamin content of oils by ignoring the fluctuations in results found in the simultaneous tests, and state the values of the oils in terms of the same (average) value of the standard oil.

This calculation has been made. A summary of it is given in Table VIII. When the values of λ are compared with those of λ in Table VII it is obvious that the "fit" of the $328m\mu$ band with the biological value is distinctly less

Table VIII. *Estimates of the logarithmic variance of the tests on oils R, S, T, U, V, ignoring the fluctuations found in simultaneous tests of the standard oil.*

		Blue value, SbCl_3 test				Ultra-violet band 328 $m\mu$; oil itself
	Biological value	Lovibond measure		Spectrophotometric		
		Oil	Unsap. fraction	572 $m\mu$	606 $m\mu$	
Biol. test λ_1^2	0.01281	—	—	—	—	—
Ratio of biol./phys. test λ^2	—	0.06781	0.09032	0.07840	0.07850	0.05465
Phys. result $\lambda^2 - \lambda_1^2$	—	0.05500	0.07751	0.06559	0.06569	0.04184
Percentage range corresponding to $\pm 2\sqrt{\lambda^2 - \lambda_1^2}$ within which the result may be expected to lie 21/22 times	59-168	34-294	28-360	31-325	31-326	39-257

good when the average value of the standard is used for comparisons, than when a contemporary value of the standard is used for comparisons. It would, therefore, appear that we have evidence that in making an estimation of the vitamin A content of a substance, a simultaneous test on the standard should always be made.

SUMMARY.

The re-examination of four oils and the examination of five further samples of cod-liver oil by the biological, chemical and physical methods already described in a previous paper indicate again that the intensity of absorption at

328 $m\mu$ by the oil itself gives the best measure of the vitamin A value of the oil.

In the re-testing of the old oils, the blue value of the unsaponifiable fraction again gave better agreement with the biological value of the oil than did the blue value of the oil itself, but in the tests made on freshly prepared oils, there was no evidence that any one method of measuring the blue value was better than the rest. They all gave approximately the same results.

Some evidence is given that a more accurate estimation of the vitamin A content of an unknown oil in relation to a given (standard) oil is obtained if a simultaneous test on the standard is made and the respective responses compared, than if the response to the unknown is compared with an average response of the standard.

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Note added 26th September, 1932. A. Chevallier (Marseille) reported at the Physiological Congress held in Rome (September, 1932) that the intensity of absorption at 328 $m\mu$ gave good agreement with the biological value only in oils having a low free fatty acid value; the free fatty acids showed strong absorption at 317 $m\mu$ which interfered with the measurement of the 328 $m\mu$ band. Thus the intensity of absorption at 328 $m\mu$ is reliable as a measure of the vitamin A content only in fresh oils, or in older oils having a low free fatty acid value.