LXXXVIII. OBSERVATIONS ON THE ASSAY OF VITAMIN A.

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INTRODUCTION.

THE assay of vitamin A presents a number of difficulties that have not yet been satisfactorily overcome. On the one hand, the biological method is subject to error on the ground of the wide variations that may be encountered, and against which few take adequate precautions. On the other hand, it is still uncertain whether the colour reaction of Rosenheim and Drummond, on which the alternative method of estimation is based, is due specifically to the vitamin.

The biological method has been reviewed several times recently, and on each occasion emphasis has been laid on the very disturbing variations in response exhibited by the animals. Although this is now somewhat generally recognised, it is, unfortunately, exceptional to find that an investigator has employed a sufficiently large group of animals to minimise the error arising from this cause.

The biological method has in the past usually been carried out by observing the effect of a measured supplement of the foodstuff on young rats whose development has been retarded by a period of feeding on a diet devoid of, or seriously deficient in, the vitamin. It seems highly probable that Hume and Smith [1928] are correct in attributing in such tests some of the variations in response to the ill-health that may arise during the period of depletion. Nevertheless, they still find rather disturbing variations when the test is made by supplying the substance under investigation from the beginning.

The necessity of using large groups of animals in either form of the biological test renders the undertaking of numerous comparative studies a formidable task. Such a position arose when one of the authors (J.C.D.) undertook, in conjunction with Prof. T. P. Hilditch, an extended investigation into certain aspects of the vitamin content of cod-liver oils on behalf of the Empire Marketing Board. In the course of this study it became necessary to examine several hundred samples for vitamin A, and to have submitted them all to an adequate biological test would have proved a very costly and laborious matter. The alternative was to rely on other and less definitely specific tests if it could satisfactorily be proved that they give results in agreement with the animal experiments. The method of estimation based on

the colour reaction of Rosenheim and Drummond has, during several years, proved in our laboratory very reliable, particularly in the examination of cod-liver oils.

A number of statements have, however, appeared in the literature to the effect that the colorimetric method of assay is untrustworthy. It will be shown in a later section of this paper that these statements are generally founded on unsatisfactory evidence.

The Accessory Food Factors Committee of the Medical Research Council arranged a comparative test between the biological method of assay, as used in a number of different laboratories, and the colorimetric method. The report of this test [1928] stated that the latter method afforded evidence consistent with that derived from the animal experiments, but its greatest value was to demonstrate clearly for the first time the serious nature of the variations encountered in the biological method as usually employed. The Committee was forced to admit that the biological methods employed were not sufficiently accurate to permit the results being presented in numerical form.

In view of the task that faced us, it seemed desirable that another and much more severe comparative examination of the two methods should be made before finally accepting or rejecting the colorimetric method as a routine test. Furthermore, it seemed desirable that the comparison should be extended to cover a method based on the quantitative examination of the absorption band in the ultra-violet spectrum extending from 280 to 360 $\mu\mu$ (max. near 328 $\mu\mu$) believed by Morton and Heilbron [1928] to be characteristic of vitamin A.

For the purpose of making this extensive test six samples of cod-liver oil were selected at random from our stock of recently collected materials. The main part of this report records the investigations on these six oils, but a number of other materials containing vitamin A were also examined by the three methods, although in these cases the biological tests were not made as thoroughly. The tests were conducted in the following manner.

(a) BIOLOGICAL TESTS.

The "curative" or "recovery" test was employed throughout. The investigation was well advanced before Hume and Smith [1928] pointed out the disadvantages of this method, and advised dispensing with the depletion period.

The rats employed in these tests were bred in the laboratory from stock fed on a mixed diet that has supported satisfactory growth and reproduction for many generations. At an average weight of 45 g. they were placed on the vitamin A-free diet compounded as follows:

Purified caseinogen	...	...	20
Purified rice starch	...	...	72
Salt mixture	...	...	4
Yeast extract	...	...	4

Vitamin D was supplied in the form of irradiated ergosterol prepared in the laboratory. A dose of 0.01 mg. of this preparation dissolved in ethyl oleate was given twice weekly to each rat.

We have found ethyl oleate a convenient solvent for materials that must be diluted before administration. The choice of a suitable diluent has not proved an easy matter. It is, in our opinion, inexcusable to use natural oils, such as arachis oil, for such a purpose. To do so is to run a very grave risk of disturbances caused by the action of components having unsuspected physiological activity.

On the other hand, medicinal paraffin, used by Drummond, Coward and Handy [1925], is not entirely satisfactory, in view of the uncertainty that absorption of the dissolved substance is quantitative.

Ethyl oleate has been found more satisfactory in this respect, although not wholly ideal. Many of the natural oils contain substances that appear to act as "anti-oxydants," capable to some extent of protecting labile substances such as vitamin A. The nature of these substances is unknown, but they are removed during our preparation of ethyl oleate, so that, unless care is taken to keep the solutions out of contact with oxygen, a more rapid destruction of vitamin A tends to occur in this solvent than in a natural oil.

The preparation of the ethyl oleate from a high-grade olive oil is a simple matter, the most important step being the complete removal of the unsaponifiable constituents by ether extraction of the soaps before separating the fatty acids. In view of the trouble caused in the past, this step is essential in case there should be present substances possessing physiological action. The ethyl esters prepared from the purified fatty acids are fractionated under reduced pressure, only the main fraction being used. It is an excellent solvent, and is readily taken by the rats. It is possible that the rate of loss of vitamin A dissolved in this solvent and exposed to air might be greatly retarded by the addition of a suitable "anti-oxydant" such as quinol. This is being investigated. Meanwhile we use brown glass ampoules of small capacity and store them while the contents are being used for feeding in a vacuum desiccator in a cold, dark cupboard. Unopened the contents keep well.

Each rat was kept in a separate cage and the supplements of vitamin A were in every case administered directly into the mouth. In the case of the experiments on cod-liver oil, groups of not less than seven rats were employed for each dosage and at least four different doses of each oil were given. Smaller groups of about four animals were used for most of the doses of the other substances investigated.

The problem of presenting the results of these biological tests is a difficult one, which raises afresh the much-discussed point as to the most satisfactory manner in which to express the response of the animals. Several workers of experience have criticised one method often used, namely, comparison of the rates of growth evoked by the same dose of different oils. It is obviously less misleading to compare the doses necessary to induce the same rate of growth,

provided that this is subnormal. In any case the test must not be too prolonged, for even on doses producing normal or nearly normal growth in the early stages for a time, the response may later show a failure which is not made good by subsequently increasing the amount of the supplement.

The opinion appears to be general that it is reasonably safe to compare the growth for a period of 5 to 6 weeks after giving the supplement.

In the experiments described in this paper it has proved difficult to decide at what stage to make the comparison. The majority of the experiments extended over 5 or 6 weeks, but in a few cases results based on shorter or longer periods have been included in the tables. Another difficulty concerns deaths during the course of the tests. In the results given below, records of animals that died before they had received the supplements for 3 weeks have been excluded. Such decisions are doubtless open to criticism, but it is not easy to decide on a more satisfactory plan.

The results on the six cod-liver oils are presented below.

Table I. *Oil C.*

Dose (mg.)	No. of rats	Reference No.	Period on test (weeks)	Change in wt. (g.)	Change in wt. per week (g.)	Average for group (g.)
10	6	L 20/74	6	+42	+ 7.0	+6.1
		L 20/77	6	+40	+ 6.6	
		21/71	5	+10	+ 2.0	
		L 99/75	5	+51	+10.2	
		L 99/76	5	+36	+ 7.2	
		L 99/74	6	+22	+ 3.7	
7	8	L 99/70	5	+12	+ 2.4	+4.4
		L 99/71	5	+27	+ 5.4	
		L 97/78	5	+27	+ 5.4	
		L 97/79	5	+16	+ 3.2	
		L 97/77	5.5	+32	+ 5.8	
		L 97/76	5	+30	+ 6.0	
		L 96/62	5	+22	+ 4.4	
		L 96/63	6	+16	+ 2.7	
5	6	L 96/61	5	+36	+ 7.2	+2.8
		L 96/60	6	-10	- 1.7	
		L 96/59	6	- 8	- 1.3	
		L 98/82	6	+22	+ 3.7	
		L 98/81	5	+12	+ 2.4	
		L 98/80	5	+16	+ 3.2	
2	8	L 100/92	5	+20	+ 4.0	+1.5
		L 100/93	6	+20	+ 3.3	
		L 100/94	6	+22	+ 3.7	
		L 100/95	6	-10	- 1.7	
		L 98/83	5	0	0	
		L 98/84	5.5	+ 5	+ 0.9	
		L 101/100	5.5	+ 2	+ 0.4	
		L 101/101	5	+ 6	+ 1.2	

The analysis of these results is not simple. If the principle of comparing the doses necessary to promote the same rate of growth (subnormal) be followed, it is convenient to adopt the rate of 3 g. per week suggested by Sherman and used in the official method of assay published in the United States Pharmacopoeia (X) 1926.

Table II. *Oil F.*

Dose (mg.)	No. of rats	Reference No.	Period on test (weeks)	Change in wt. (g.)	Change in wt. per week (g.)	Average for group (g.)
10	7	210	6	+48	+ 8.0	+8.5
		213	6	+45	+ 7.5	
		214	6	+44	+ 7.3	
		L 40/180	5	+58	+11.6	
		L 39/174	5	+35	+ 7.0	
7	8	L 39/175	5	+49	+ 9.8	+5.5
		L 31/132	5.5	+15	+ 2.7	
		L 37/167	5.5	+16	+ 2.9	
		209	7	+34	+ 4.9	
		212	5.5	+40	+ 7.3	
		L 39/172	5.5	+15	+ 2.7	
		L 39/173	5	+20	+ 4.0	
		215	5	+60	+12.0	
		L 38/168	5	+32	+ 7.4	
		218	4.5	+27	+ 6.0	
5	9	L 34/135	5	+23	+ 4.6	+4.3
		L 35/152	6	+14	+ 2.3	
		L 20/78	5.5	+25	+ 4.5	
		L 21/83	6	+21	+ 3.5	
		208	5.5	+17	+ 3.1	
		L 34/144	5	+20	+ 4.0	
		207	5.5	+32	+ 5.8	
		L 29/123	6	+31	+ 5.2	
		L 20/75	6	+19	+ 3.2	
		L 21/91	5	+14	+ 2.8	
2	6	231	5	+ 9	+ 1.8	+2.3
		232	5	+28	+ 5.6	
		L 20/74	3	-10	- 3.3	
		L 21/82	5.5	+19	+ 3.5	

Table III. *Oil G.*

Dose (mg.)	No. of rats	Reference No.	Period on test (weeks)	Change in wt. (g.)	Change in wt. per week (g.)	Average for group (g.)
10	6	L 47/210	5.5	+29	+ 5.3	+5.8
		L 48/219	5	+34	+ 6.8	
		L 46/203	5	+19	+ 3.8	
		L 46/207	7.5	+40	+ 5.3	
		L 49/223	7.5	+38	+ 5.1	
		298	5	+43	+ 8.6	
7	6	L 41/185	5	+57	+11.4	+4.7
		L 43/195	4	-24	- 6.0	
		L 46/206	5	+21	+ 4.2	
		297	5	+35	+ 7.0	
		L 43/197	5	+34	+ 6.8	
		299	5	+25	+ 5.0	
5	4	L 43/192	5.5	+18	+ 3.3	+3.7
		L 49/221	5	+38	+ 7.6	
		L 49/222	6	+22	+ 3.7	
		301	5.5	+ 1	+ 0.2	
2	12	L 40/182	6	+17	+ 2.8	+1.6
		L 40/178	5	+10	+ 2.0	
		L 39/176	5	+29	+ 5.8	
		L 39/177	5	- 2	- 0.4	
		L 39/170	5.5	+19	+ 3.4	
		L 38/171	5	-10	- 2.0	
		L 7/17	5	+ 3	+ 0.6	
		L 7/16	5	- 4	- 0.8	
		L 34/146	5	+22	+ 4.4	
		L 34/147	5.5	+ 7	+ 1.3	
		L 37/166	4	-11	- 2.8	
		L 37/165	5	+25	+ 5.0	

Table IV. *Oil J.*

Dose (mg.)	No. of rats	Reference No.	Period on test (weeks)	Change in wt. (g.)	Change in wt. per week (g.)	Average for group (g.)
10	7	L 36/158	5	0	0	+2.6
		L 36/156	5	+13	+ 2.6	
		L 35/152	6	+14	+ 2.3	
		224	6	+26	+ 4.3	
		226	5	+25	+ 5.0	
		227	5	+20	+ 4.0	
		L 36/157	5	- 6	- 1.2	
7	8	L 36/161	6	+ 3	+ 0.5	+4.0
		L 37/162	5	+26	+ 5.2	
		L 36/160	5	+ 7	+ 1.4	
		L 36/159	5	+ 7	+ 1.4	
		220	6	+27	+ 4.5	
		223	5	+ 3	+ 0.6	
		221	6	+37	+ 6.1	
5	8	222	5	+63	+12.6	+2.8
		L 30/125	6	+11	+ 1.9	
		L 30/127	6	+43	+ 7.1	
		L 32/134	6	+34	+ 5.7	
		L 32/136	6	+42	+ 7.0	
		L 37/163	5	- 7	- 1.4	
		L 37/164	5	-12	- 2.4	
		217	4	+12	+ 3.0	
		218	3	+ 6	+ 2.0	

Table V. *Oil K.*

Dose (mg.)	No. of rats	Reference No.	Period on test (weeks)	Change in wt. (g.)	Change in wt. per week (g.)	Average for group (g.)
10	6	L 41/187	5	+39	+ 7.8	+5.8
		L 42/193	6	+39	+ 6.5	
		L 47/211	5	+50	+10.0	
		L 48/216	5	+24	+ 4.8	
		L 47/213	5.5	+33	+ 6.0	
		L 42/198	5	0	0	
7	6	L 43/196	5	+12	+ 2.4	+3.2
		L 46/204	7	+46	+ 6.6	
		L 48/215	6	+28	+ 4.6	
		L 49/224	6	+29	+ 4.8	
		L 76/284	6	- 4	- 0.7	
		L 76/299	5	+ 7	+ 1.4	
5	6	L 41/186	5	+42	+ 8.4	+2.5
		L 42/192	4	- 4	- 1.0	
		L 50/227	5	+15	+ 3.0	
		L 50/228	5.5	+ 5	+ 1.0	
		L 55/237	5	- 6	- 1.2	
		L 72/278	5	+23	+ 4.6	
2	6	L 55/238	5	- 8	- 1.6	-0.6
		L 55/239	5	- 6	- 1.2	
		L 72/277	5	+22	+ 4.4	
		L 63/268	4	-13	- 3.2	
		L 72/279	5	- 4	- 0.8	
		L 67/282	5	- 5	- 1.0	

The individual variations are surprising, particularly when it is observed that many of the animals showing wide differences in behaviour on the same dosage are litter mates. Numerous examples will be seen (*e.g.* ♂ 195 (Litter 43) given 7 mg. of oil G loses 24 g. in 4 weeks, whilst a sister (♀ 197) grows 34 g. in 5 weeks).

Table VI. *Oil L.*

Dose (mg.)	No. of rats	Reference No.	Period on test (weeks)	Change in wt. (g.)	Change in wt. per week (g.)	Average for group (g.)
10	6	L 41/189	5	+20	+4.0	+2.0
		L 47/212	5	- 1	-0.2	
		L 33/139	6	+31	+5.0	
		L 22/122	6	+12	+2.0	
		L 30/126	5.5	+ 7	+1.2	
		L 30/128	5.5	0	0	
7	3	L 41/188	5	+20	+4.0	+2.9
		L 46/208	5	+13	+2.6	
		L 49/225	3	+ 7	+2.3	
5	4	L 48/217	5.5	+ 9	+1.6	+0.9
		L 48/213	4.5	+ 4	+1.0	
		L 33/140	5	- 7	-1.7	
		L 33/138	5	+13	+2.6	
2	6	L 31/130	5	- 5	-1.0	+0.4
		L 32/133	5	- 3	-0.6	
		L 32/135	6	+ 1	+0.2	
		L 32/137	5.5	+ 5	+1.0	
		L 35/150	5	+12	+2.4	
		L 35/151	5	+ 2	+0.4	

But even when the results for each group are averaged, a more serious type of discrepancy remains. This is shown clearly in the lower average rate of growth on 10 mg. of the oil J than on the dose of 7 mg. The same difficulty arises in the case of oil L. Which of the values for these oils should be accepted? The choice presents an opportunity for doubling the possible vitamin value of the oil in the former case. If, however, as seems most reasonable, we accept the still lower dose (5 mg.) of the oil J, the following doses are found to promote a rate of growth of approximately 3 g. a week for a period of 5 to 6 weeks:

	mg.	Relative vitamin value
C	5	2
F	2	5
G	<5	>2
J	5	2
K	7	1.5
L	10*	1

* The value of 10 is accepted in this case because of the small number of animals in the group to which the dose of 7 mg. was given. Four deaths occurred in this group before 3 weeks had passed. The oil was undoubtedly the poorest in vitamin A of the six examined.

A survey of the figures in Tables I-VI does not encourage complacency with regard to the value of the biological test. Obviously even larger groups than 7 to 12 animals and a more extensive range of doses would have to be employed if the accuracy of the method were to be increased so as to distinguish with certainty between two oils showing less than a 100 % difference in vitamin A content.

If the figures we have presented are examined on the alternative basis, namely, a comparison of the rate of growth produced by the same doses of the different oils the following results are obtained¹.

¹ The values for the 2 mg. doses have been excluded as they are obviously unsuitable for treatment on these lines.

Table VII. *Comparison of six oils (biological tests).*

Normal rate of growth at period covered by tests (80-130 g.): ♂ 14.5 g./wk.; ♀ 10.3 g./wk.

Oil	Average weekly weight gain (g.)			Relative values L = 1		
	on dose			on dose		
	10 mg.	7 mg.	5 mg.	10 mg.	7 mg.	5 mg.
C	6.1	4.4	2.8	3.0	1.5	3.1
F	8.5	5.5	4.3	4.2	1.9	4.8
G	5.8	4.7	3.7	2.9	1.6	4.1
J	2.6	4.0	2.8	1.3	1.4	3.1
K	5.8	3.2	2.5	2.9	1.1	2.8
L	2.0	2.9	0.9	1	1	1

These figures are entirely unsatisfactory from a quantitative standpoint, but they could be interpreted as indicating that L is the poorest oil, G and F the best with 2 to 4 times the activity of L, whereas the other three, K, J and C are intermediate in value. As a matter of fact, if the average of the relative values for each oil on the three doses of 10, 7 and 5 mg. are taken, a rather questionable procedure, the figures are more or less of the same order as those yielded by the comparison of the doses required to produce a weekly gain of 3 g.

Oil	Relative value deduced from dose necessary to promote growth at approximate rate of 3 g. a week	Relative value deduced from average of relative values on 10, 7 and 5 mg. doses in Table VII
C	2	2.5
F	5	3.6
G	>2	2.9
J	2	1.9
K	1.5	2.3
L	1	1

The biological assay of these six oils has been carried out with more care than is usually bestowed on such tests. No less than 190 animals were employed, and yet the results do not enable us to judge the relative values of the oils more than approximately. F would certainly appear to be the oil richest in vitamin A, probably about 4 or 5 times more potent in this respect than the poorest oil L. Of the other four it is only possible to state that they possess intermediate values, and that G is probably second richest.

Our own conclusion from this study of the biological test is that by using reasonably large groups of rats (7-12) drawn from a uniform stock, it is possible to detect differences of the order of 100 % in the vitamin A content of cod-liver oils. This refers of course only to the "curative" or "recovery" form of the test. It is possible that the alternative method advised by Hume and Smith would be more sensitive.

(b) COLORIMETRIC TESTS.

The six oils were examined by the Carr and Price modification [1926] of the Rosenheim and Drummond test. At least three independent determinations by the Lovibond tintometer were made on each oil. Determina-

tions of the blue colours were also made by means of a Hilger-Nutting spectrophotometer, attached to a constant deviation wave-length spectro-scope. This is probably the most accurate method available for the absolute measurement of the intensity of absorption at the head of the band responsible largely for the blue colour.

The blue colour to be measured suffers from the disadvantage of being transient, and special precautions had to be taken to ensure the recording of the maximum intensity of absorption. The type of cell used is specially well adapted for spectroscopic work with transient colours. It is made in one piece of fused quartz with flat fused-on end plates. Two such cells are placed empty in the cell holders and the zero adjustment of the photometer checked. The comparison cell, without being removed from the cell holder, is then filled with the solution of antimony trichloride. Using a specially calibrated pipette with a fine jet, 0.3 cc. of a solution of the cod-liver oil, at an appropriate dilution in chloroform, is run into the other cell. The observer then adjusts the drum of the wave-length spectrometer to $608\text{ }\mu\mu$, the head of the sharp absorption band, in preparation for making rapid measurements. A second worker then allows 3 cc. of the 30 % solution of antimony trichloride to run into the absorption cell, tapping it to secure a homogeneous mixture. The observer matches the intensity of the blue colour every 15 seconds, the readings being recorded by his colleague.

The photometer readings are in terms of $\log I_0/I$; I_0 being the intensity of the light incident on the absorption cell and I that of the emergent light. When $\log I_0/I$ is plotted against time, the absorption at $608\text{ }\mu\mu$ reaches a maximum after about one minute. If the maxima in these curves are compared for different samples (equating the concentrations and cell thicknesses), the relative values express the relative intensities of the blue colours for the different samples. This procedure, with practice, gives trustworthy results.

The relative values obtained by the two modifications of the colorimetric test are given in Table VIII, the value for the poorest oil L being taken as unity.

Table VIII.

Oil	Relative intensities of blue colours measured by	
	Lovibond tintometer	spectrophotometer
C	2.5	2.65
F	3.3	3.30
G	3.0	3.14
J	1.7	1.70
K	1.8	1.77
L	1.0	1.0

The agreement is satisfactory.

(c) MEASUREMENTS OF RELATIVE INTENSITIES AT THE MAXIMUM ($328\ \mu\mu$)
OF THE ABSORPTION BAND OF COD-LIVER OILS.

The intensity of absorption at $328\ \mu\mu$ can be measured to within about 2 % by the ordinary methods of quantitative spectrophotometry using the photographic technique, and to a definitely greater degree of accuracy by means of a photoelectric method. The former method was sufficiently accurate for our requirements, because a much larger source of uncertainty exists and has to be allowed for. The absorption spectrum of a material containing vitamin A is not as a rule due at $328\ \mu\mu$ to vitamin A solely, but the observed curve is made up of two super-imposed curves. If a number of samples of cod-liver oil are being compared, it is reasonable to suppose that the fatty acid glycerides and other normal oil constituents will generally absorb to approximately the same extent.

Fig. 1.

The curves in Fig. 1 represent diagrammatically the absorption of two different samples of cod-liver oil. *EFCG* represents the most probable curve for a typical cod-liver oil minus vitamin A, so that the relative intensities of the "vitamin" band are given by *AC* and *BC*. In actual practice, the distance *CD* has been determined from the mean of a large number of curves obtained with spent oils from which the vitamin has been extracted, very poor cod-liver oils, and oils in which the vitamin had been destroyed by photochemical or chemical means. It must, however, be pointed out that the measurements on the absorption band at $328\ \mu\mu$ lose some of their quantitative value when the persistence of the band is small, but gain very much in accuracy as the persistence of the band increases.

The values obtained in the examination of the six cod-liver oils are given in Table IX.

Table IX. *Spectrographic examination of oils.*

1 % solutions in alcohol, cell thickness 1 cm.				
Oil	<i>AD</i>	Correction <i>CD</i> minus	Persistence of the ultra-violet band alone	Relative intensity <i>L</i> = unity
C	0.96	0.22	0.74	2.65
F	1.13	0.22	0.91	3.25
G	1.13	0.22	0.91	3.25
J	0.62	0.22	0.40	1.5
K	0.66	0.22	0.44	1.7
L	0.50	0.22	0.28	1.0

The curves for the six oils are given, together with those obtained on samples of the oils after 12 months storage, in Figs. 2, 3 and 4.

We are now in a position to assemble our results.

Table X. *Comparison of biological and physical measurements of the relative vitamin A value of the six cod-liver oils.*

Oil	Biological method	Colorimetric method		Ultra-violet band
		Tintometer	Spectrometer	
C	2	2.5	2.65	2.65
F	5	3.3	3.3	3.25
G	> 2	3.0	3.15	3.25
J	2	1.7	1.7	1.5
K	1.5	1.8	1.77	1.7
L	1	1.0	1.0	1.0

All things considered, the agreement is remarkably good, particularly when the weaknesses of the biological method are borne in mind.

Examination of the six cod-liver oils after a year's storage. The six oils that were studied in the main part of this work were re-examined after they had been stored for just over a year. Two samples of each had been kept, one preserved in the dark, the other exposed to the daylight of a London laboratory. Both samples were contained in white glass bottles of 250 cc. capacity, closed with ground glass stoppers, and with an air space of about 20 cc. The examination by the biological test was not conducted as thoroughly as in the original tests. Four samples only were tested, and the animal groups were not sufficiently large. The results of the colorimetric and spectrographic tests are given in Table XI. The curves representing the latter are given in Figs. 2, 3 and 4.

Biological tests were made on oils C₁, C₂, L₁ and L₂. These represent the original oils C and L after storage for a year in light and dark respectively. The results are given in Table XII.

To attempt to estimate from these animal experiments—which were after all as complete a test of the vitamin A content as is often made—to what extent the vitamin has been destroyed during storage is almost impossible. The most that can be said is that destruction has proceeded further in the samples stored in light than in those kept in the dark, as would be expected,

and that in the former it is probably of the order of 50–60 % of the active substance originally present.

Doubtless a more thorough biological examination would have provided slightly more reliable evidence, but there is even in this admittedly superficial examination a general agreement between the results of the animal tests and the two physical methods of measurement.

Experiments on other materials. In the Medical Research Council Committee's report [1928] stress was laid on the importance of testing the colorimetric method on other products than cod-liver oil before drawing conclusions as to its general validity.

Although we have not investigated oils of plant origin, as was there suggested, we have submitted a variety of products to careful biological test and to examination by the two physical methods. The results are presented in Table XIII.

Table XI. *Ageing of cod-liver oils.*

Oil (1)	Date of examination (2)	Con- dition of storage (3)	Log I_0/I at 328 $\mu\mu$, 1 % solution 1 cm. cell (4)	Persistence of band $\times 100$ (correction)*		Antimony trichloride blue Log I_0/I at 608 $\mu\mu$, 1 % oil in 30 % SbCl ₃ , 2 cm. cell (7)		Rela- tive in- tensity blue colour (8)	Percentage loss (averages from figures in columns 5 and 8)	
				0.13 (5)	0.22 (6)				In light	In dark
C	C Aug. 1928	—	0.96	83	74	2.75	83			
	C ₁ May 1929	Light	0.54	41	32	1.41	42	50		
	C ₂ May 1929	Dark	0.73	60	51	1.96	59			28.3
F	F Aug. 1928	—	1.13	100	91	3.3	100			
	F ₁ May 1929	Light	0.83	70	61	1.8	55	37.5		
	F ₂ May 1929	Dark	0.95	82	73	2.25	68			25
G	G June 1928	—	1.13	100	91	3.11	94			
	G ₁ May 1929	Light	0.82	69	60	1.84	56	35.5		
	G ₂ May 1929	Dark	1.05	92	83	2.25	68			17.5
J	J Aug. 1928	—	0.62	49	40	1.85	56			
	J ₁ May 1929	Light	0.36	23	13	0.8	24	55.2		
	J ₂ May 1929	Dark	0.53	40	31	1.2	36			27.7
K	K June 1928	—	0.66	53	44	1.76	53			
	K ₁ May 1929	Light	0.39	26	17	1.04	31	46.2		
	K ₂ May 1929	Dark	0.51	38	29	1.28	39			27.4
L	L June 1928	—	0.50	37	28	1.0	30			
	L ₁ May 1929	Light	0.21	8	—	0.4	12	70		
	L ₂ May 1929	Dark	0.39	26	17	0.84	25			24
								Av. 49		25

* 0.22 seemed to be the most reasonable allowance to make for the absorption of the fresh oils at 328 $\mu\mu$ in respect of constituents other than vitamin A. This appears to be rather too high for the oils after standing. The differences may quite possibly be connected with the vitamin D potency. Both corrections have been inserted for all the oils, as the two figures for each oil demonstrate the real limitations of the method.

The agreement between the results obtained with the three tests is on the whole satisfactory, and strengthens confidence in the colorimetric and spectrographic methods of assay. Either vitamin A itself shows selective ultra-violet absorption from 280–360 $\mu\mu$ and is responsible for the blue colour,

Table XII.

Oil	Dose mg.	No. of rats in group	Average rate of growth g. weekly	Estimated loss due to storage	Estimated percentage loss detected by physical methods Table XI
C	5	6	2.8	—	—
C ₁ (light)	5	4	1.0	About 50 %	50
	10	4	2.9		
C ₂ (dark)	5	4	1.6	Probably less than 50 %	28
	10	3	2.9		
L	10	6	2.0	—	—
L ₁ (light)	10	3	-0.6	More than 50 %, prob- ably nearer 70 %	70
	20	3	1.0		
	30	3	2.2		
L ₂ (dark)	10	3	-0.2	More than 50 %	24
	20	4	1.9		

Table XIII.

Material tested	Biological test ("recovery" test)		Colorimetric test	Spectrographic test
	Dose mg.	Result		
(1) Caplin oil (oil extracted from whole body)	10	No growth	No colour	No band
	20	No growth		
(2) Squid (body oil)	20	Very slight growth	Slight transient blue colour, head of band about 598 μ	Small inflexion 306-336 μ
	40	Growth about 5 g. weekly		
3) Oil extracted from <i>Nephrops norvegicus</i>	30	No growth	No colour	No band with max. near 328 μ
	60	No growth		
(4) Oil extracted from <i>Meganyctiphanes norve- gicus</i>	30	No growth	Little if any colour	Small inflexion near 320 μ
	60	Very slight growth		
(5) Oil extracted from <i>Calanus finmarchicus</i>	30	No growth	No colour	
	60	No growth		
(6) Unsaponifiable fraction from sample of plank- ton oil (5)	30	No growth	No colour	
	60	No growth		
(7) Oil extracted from a mixed plankton sample consisting largely of copepods	30	No growth	No colour	
	10	No growth		
(8) Oil extracted from sheep liver	0.1	Growth about 3-5 g. weekly	About 14 times as rich as cod-liver oil "G" by both methods	
	0.3	Growth about 8 g. weekly		
(9) Unsaponifiable fraction from sample of sheep- liver oil (8)	0.005	Growth 2-3 g. weekly	About 9 times (8)	About 12 times (8)
	0.01	Growth 5-7 g. weekly		
	0.03	Growth 10 g. weekly		

with an absorption maximum near $608\text{ }\mu\mu$, in the antimony trichloride test, or else these are properties of other substances contained in cod-liver oil and exhibiting a remarkable similarity to the vitamin as regards distribution and behaviour.

Fig. 2.

Objections to the specificity of the colour test for vitamin A. A number of papers have appeared recently in which the value of the colour test as an indication of the presence of vitamin A has been questioned. The relation of this test to the colour reactions of carotene and certain other substances is discussed in another paper [Dulière, Morton and Drummond, 1929] and is not directly related to the subject of this section.

Schmidt-Nielsen [1928] states that samples of herring oil gave vitamin A values of 50–180 units when tested by the U.S. Pharmacopoeia method, but failed to give the blue colour with antimony or arsenic trichloride.

It is also stated that the oils of the flesh of sprat and mackerel are good sources of vitamin A, but do not give the blue colour test, and that the oil of the caplin, whilst failing to respond to the test, "ought to be rather rich" in vitamin A. We have examined three samples of caplin oil and have found them uniformly inactive in doses of 10 and 20 mg.

Fig. 3.

No details are given in these papers of the animal experiments beyond the statement that vitamin D was supplied, so that it cannot be said whether they were in all respects satisfactory, particularly as regards the length of the test and the number of animals employed. At present we are unable to explain the results of Schmidt-Nielsen. We are preparing for examination a number of body-oils of fish similar to those used by him¹. In addition, we hope to obtain for comparative tests samples of the oils actually examined by him.

¹ Dr Ahmad, working in the laboratory at University College, is making this examination with salmon-body oil and salmon-liver oil. The results of the biological test so far are in agreement with the colorimetric test.

The comparative examinations of cod-liver oils by the biological and colorimetric methods recorded by Bailey, Cannon and Fisher [1928] and by Jones, Briod, Arzoomanian and Christiansen [1929] appear to be without significance, as the biological method employed was that recommended in the 1926 edition of the U.S. Pharmacopœia. It has been repeatedly pointed out that this method is liable to give highly misleading results, owing to the absence of vitamin D during the depletion period.

Fig. 4.

The adverse criticisms of Steudel [1927, 1929] regarding the specificity of the blue colour reaction do not rest on a firm basis. From the growth curves given in his paper it is apparent that the vitamin A "preparations" with which he was working were of so low an activity that from 1 to 2 g. were required daily to promote a rather poor rate of growth in rats. It is not to be expected that the colour reaction would be exhibited except very faintly by a material so poor in vitamin A. The colour reaction, even using the

more sensitive arsenic trichloride reagent, is given but feebly by samples of butter that promote growth in daily doses of from 0.1 to 0.4 g. In investigating such materials it is necessary, before applying the colour test, to concentrate the chromogenic substance by preparing the unsaponifiable fraction.

We have found it difficult to apply the colour test to the estimation of vitamin A in butter because of the similar blue colours produced by the lipochrome pigments. No entirely satisfactory method of removing these pigments or of making an allowance for the blue colour produced by them has yet been evolved. It is possible that determinations of the intensity of the 328 $\mu\mu$ band will provide a simple way out of the difficulty. Quite recently, however, Andersen and Nightingale [1929] have reported success in applying the colorimetric method to margarines containing vitamin A. The test was applied to the unsaponifiable matter and the results showed general agreement with those of the biological test.

It is also necessary to bear in mind that the colour reaction is inhibited by water and the lower alcohols.

Further work is needed to establish definitely whether the colour reaction and the absorption band at 328 $\mu\mu$ are in reality due to vitamin A, or whether they are caused by one or more than one substance closely resembling the vitamin in distribution and properties. A final decision on this point will not be reached until the substances concerned have been isolated. The absorption band at 328 $\mu\mu$ by itself is probably not a reliable index of vitamin A as such [Rosenheim and Webster, 1929], but we are satisfied that for cod-liver oils the colour test and the measurement of the intensity of the 328 $\mu\mu$ band taken together are more reliable indications of the relative vitamin A potencies than the biological tests, unless the latter are performed on an unusually large number of animals.

SUMMARY.

1. Six samples of cod-liver oil have been examined for vitamin A by the biological method. In spite of the use of much larger groups of rats (7-12) for each dose than are usually employed, the individual responses of the animals showed such wide variations as to make it a very difficult matter to detect quantitative differences in vitamin A potency of less than 100 %.

2. As far as the relative values of the six oils could be estimated by the biological test, they were in agreement with the response to the colour reaction of Rosenheim and Drummond, and with the relative intensities of the absorption band (328 $\mu\mu$) believed by Morton and Heilbron to be characteristic of vitamin A. The agreement between the results of the colorimetric and spectrographic methods was very close.

3. A comparison of the biological assay with the two physical methods was also made in the case of a number of other oils, and general agreement between the results found.

4. In view of the statement of other investigators that certain oils (fish-body oils) may be rich in vitamin A without showing the colour reaction, it seems still undesirable to claim that the colorimetric method can generally replace the tedious and inaccurate biological tests. Actually, in a wide experience, we have not encountered a single instance in which there has been disagreement between the animal tests and the intensity of the blue colour showing maximum absorption near $608\text{ }\mu\mu$. As far as cod-liver oils are concerned we have no hesitation in recommending the colorimetric and spectrographic methods as giving rapid, and reliable, quantitative results.

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