CXXVI. THE DETERMINATION OF VITAMIN A IN COD-LIVER OILS (*a*) BIOLOGICALLY, (*b*) CHEMICALLY, (*c*) PHYSICALLY, WITH A STATISTICAL EXAMINATION OF THE RESULTS.

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(*Received June 26th, 1931.*)

A method for the biological estimation of vitamin A has been described by Coward *et al.* [1930, 1931] which appears to have a greater degree of accuracy than the methods previously used. It therefore appeared desirable to use it for measuring the vitamin A values of several samples of cod-liver oil and to compare these values with the "blue values" obtained by the antimony trichloride (or arsenic trichloride) colour test originally put forward by Rosenheim and Drummond [1925] as a measure of the same factor. Eleven samples of oil and two concentrates were examined:

(*a*) biologically (Pharmaceutical Society);

(*b*) chemically by the colour test ($SbCl_3$) on (1) the oils and (2) their unsaponifiable fractions (Pharmaceutical Society—by F. J. Dyer);

(*c*) physically—the intensity of the 328 $m\mu$ absorption and the intensities of the 572 $m\mu$ and 606 $m\mu$ bands of the blue colour obtained with $SbCl_3$ on the oils (The University, Liverpool—by R. A. Morton).

The results were examined statistically by J. H. Gaddum of the National Institute for Medical Research.

The oils and concentrates which were examined by the three methods are given in Table I.

It is evident from Table VII in which the various results have been collected that, in general, an oil with a high biological value has also the following properties.

(*a*) A high blue value obtained by the action of $SbCl_3$ on the oil itself, though the two values are by no means parallel.

(*b*) A high blue value obtained by the action of $SbCl_3$ on the unsaponifiable fraction of the oil. These values are almost parallel.

(*c*) A high value for the absorption bands 572 $m\mu$ and 606 $m\mu$ obtained by the action of $SbCl_3$ on the oil. The intensities of these two bands run parallel in these oils, though Morton has examined numerous oils in which they do not.

Table I.

Designation	Source	Quality	Date of catch
A	Scotch	Finest non-freezing	1928
B	—	Cattle and poultry	1930
C	Norwegian	Finest non-freezing	1930
D	Icelandic*	Finest non-freezing	1930
E	Icelandic*	Finest non-freezing	1930
F	Icelandic*	Unrefined	1930
G	—	Unrefined (?)	1930 (?)
H	Norwegian	Finest non-freezing	1926
J	—	Cod, suspected of being adulterated with shark-liver oil	1928
K	Scotch and Norwegian (mixed)	Finest non-freezing	1930
L	—	Suspected of being adulterated (Unsap. M. = 1·9 %) (F.F.A. = 4·1 %) (I.V. = 152)	1930
M	Concentrate		
N	Concentrate		

* Received direct from the manufacturers, Messrs I. Spencer, Aberdeen.

(*d*) A high value for the intensity of the 328 $m\mu$ absorption band of the oil itself. These values are almost parallel.

There are, however, certain exceptions to this: *e.g.* oil C has a higher biological value than oil A (about 3 : 2) whereas its chemical and physical values are lower than those of oil A (average about 3 : 4); similarly oil K has a biological value about 2·5 times that of oil A and its chemical and physical values are practically equal to those of oil A. It was obviously necessary, therefore, to have the results examined as carefully as possible, partly to see what faith we could place on the biological tests and then to determine which chemical or physical measurement was generally the truest measure of the vitamin A content. Dr J. H. Gaddum has done this and having calculated the probable range of each test on the severe criterion of $\pm$ twice the standard deviation where $\sigma = \sqrt{\frac{\Sigma d^2}{n-1}}$, he found that many of the physical measurements lie outside the range of the known error of the biological test and suggested that certain oils might contain substances which were inactive biologically but which might increase the blue value ($SbCl_3$) and the intensity of the absorption band 328 $m\mu$. On the other hand, certain oils might contain physiologically active substances other than vitamin A, or the biological test itself might be subject to unknown errors. On the whole the intensity of the 328 $m\mu$ absorption band gave the best agreement with the biological value, and the blue value obtained by the action of antimony trichloride on the unsaponifiable fraction of the oil was nearly as good. Both of these measures were found to be much more accurate than the Lovibond measure of the blue value obtained with the oil itself.

BIOLOGICAL EXAMINATION OF THE OILS.

The vitamin A value of oil A has been very thoroughly examined and the result described in papers by Coward *et al.* [1930, 1931]. It was found that the relation between the dose of oil given and the mean increase in weight of a group of rats given the daily dose of oil for 5 weeks, after having become steady in weight on a diet deficient in vitamin A, was expressed by the equation:

$$y = 12{\cdot}1 + 40{\cdot}24 \log x,$$

where y equals the mean increase in weight in grams of the group in 5 weeks, and x equals the daily dose of cod-liver oil in mg. This relation held when the numbers of bucks and does in each group were approximately equal. The behaviour of the bucks and does in each group was also calculated separately, and the following equations obtained:

$$y = 11{\cdot}3 + 50{\cdot}3 \log x \text{ (bucks)},$$
$$y = 12{\cdot}4 + 27{\cdot}4 \log x \text{ (does)}.$$

The latter equations have now been used for making comparisons of the biological values of ten samples of cod-liver oil and two concentrates.

Rats were prepared for the test by giving them a diet consisting of:

Caseinogen, light-white (B.D.H.) non-extracted	15 %
Dextrinised rice starch	73 ,,
Dried yeast	8 ,,
Salt mixture (Steenbock's 40)	4 ,,

About 2 units of vitamin D were given to each rat twice a week. Each sample of cod-liver oil was so diluted with olive oil that the required daily dose was always contained in 1 drop of the solution. Daily doses were given by dropping directly into the rat's mouth from tubes selected to deliver not more than 21 mg. and not less than 19 mg. oil in 1 drop. Fresh dilutions were made every 7 days and kept in cold store when not actually in the rat room for feeding. Each sample of cod-liver oil was tested in 1–4 doses. Each dose was tested on large groups of animals (9–26), each animal of a group receiving the same daily dose for 5 weeks. The average increases in weight of the bucks and does of each group were then calculated separately, and, by substitution in the corresponding equation, the dose of oil A which would have brought about this increase in weight was determined. The inverse ratio of this dose to the actual dose of the oil under test is the ratio of the vitamin A contents of the oils. The potency of the fresh oil was then calculated as a percentage of that of oil A. The average of the results from bucks and does on all doses of an oil was taken as the measure of its vitamin A potency. In addition the percentage deviation of each group of bucks and does from the average was calculated. Results are collected in Table II. An analysis of the percentage deviations of the groups reveals a surprising degree of accuracy in the results. Twenty different doses of oils were tested, giving 40 results, as the bucks and does were calculated

Table II. *Biological examination of the oils. The vitamin A value of each oil and concentrate is expressed as a percentage of the vitamin A value of oil A.*

Cod-liver oil	Dose mg.	No. of bucks on test	Av. inc. in 5 weeks g.	Deviation from mean of each group $\sigma = \sqrt{\Sigma d^2/(n-1)}$ bucks	Dose of oil A which would bring about this inc. mg.	No. of does on test	Av. inc. in 5 weeks g.	Deviation from mean of each group $\sigma = \sqrt{\Sigma d^2/(n-1)}$ does	Dose of oil A which would bring about this inc. mg.	Vitamin A potency (%) relative to oil A according to bucks	Vitamin A potency (%) relative to oil A according to does	Weighted mean	Percentage deviation of each group from the average bucks	Percentage deviation of each group from the average does	Date at which each test was begun
B	1·0	10	4·2	10·2	0·723	9	11·8	11·3	0·95	72·3	95	—	− 7·8	+21·2	14. vii. 30
	4·0	4	35·75	12·7	3·06	5	24·6	7·2	2·79	76·5	69·7	—	− 2·4	−11·1	10. vi. 30
										Av. = 78·4		79·7			
C	0·5	7	−0·9	7·5	0·571	10	−0·8	11·6	0·33	114·2	66·0	—	−26·3	−57·4	10. x. 30
	1·0	6	27·7	11·3	2·118	13	20·2	5·1	1·9275	211·8	192·75	—	+36·7	+28·4	25. vii. 30
	5·0	13	61·4	18·3	9·91	4	37·25	11·8	8·075	198·2	161·5	—	+27·9	+ 4·3	10. iv. 30
	5·0	7	54·9	18·2	7·357	11	36·2	11·6	7·393	147·1	147·9	—	− 4·1	− 4·5	20. iii. 30
										Av. = 154·9		156·6			
D	0·5	8	2·5	7·6	0·6685	12	6·33	7·8	0·599	133·7	119·8	—	+ 3·0	− 7·7	21. x. 30
	1·0	8	15·0	13·8	1·185	11	17·0	14·5	1·472	118·5	147·2	—	− 8·7	+13·4	22. viii. 30
										Av. = 129·8		130·1			
E	0·5	10	19·2	12·0	1·436	16	9·4	15·2	0·777	287·2	155·5	—	+63·7	−11·4	23. x. 30
	1·0	10	18·0	11·13	1·358	11	14·9	8·5	1·233	135·8	123·3	—	−22·6	−29·7	17. ix. 30
										Av. = 175·45		171·8			
F	0·1	10	1·7	6·9	0·6442	11	9·8	16·4	0·8037	644·2	803·7	—	+ 2·7	+28·1	26. ix. 30
	0·5	8	40·4	18·4	3·787	12	27·2	8·9	3·4682	757·4	693·6	—	+20·7	+10·6	24. x. 30
	1·0	14	42·4	9·6	4·15	6	30·3	15·7	4·498	415·0	449·8	—	−33·8	−28·3	25. viii. 30
										Av. = 627·3		625·8			
G	1·0	8	18·4	12·2	1·384	8	18·5	4·4	1·671	138·4	167·1	—	− 9·4	+ 9·4	11. ix. 30
										Av. = 152·75		152·75			
H	1·0	10	9·7	12·1	0·929	9	7·0	11·1	0·635	92·9	63·5	—	+18·8	−18·8	25. ix. 30
										Av. = 78·2		78·9			
J	1·0	10	14·6	11·4	1·163	12	15·3	6·9	1·276	116·3	127·6	—	− 4·6	+ 4·6	19. ix. 20
										Av. = 121·95		122·5			
K	1·0	2	31·5	0·5	2·5212	8	22·8	8·72	2·3960	252·1	239·6	—	+ 2·5	− 2·5	4. xi. 30
										Av. = 245·8		247·1			
L	0·067	7	13·7	10·1	1·116	7	12·0	7·6	0·9670	1674·0	1450·5	—	+ 7·1	− 7·1	9. i. 31
										Av. = 1562·25		1562·25			
M	0·02	6	−4·0	17·4	0·49636	4	−2·0	6·96	0·29854	2482·0	1493·0	—	+24·9	−24·9	15. xii. 30
										Av. = 1987·5		2086·4			
N	0·04	5	1·8	6·1	0·6473	5	6·2	1·4	0·5939	1618·25	1484·75	—	+ 4·3	− 4·3	20. i. 31
										Av. = 1551·5		1551·5			

separately. Of these, two results only showed more than 50 % deviation (57·4 and 63·7 respectively) from the average value of the sample of oil; two results showed between 30 and 40 % deviation, eleven between 20 and 30, six between 10 and 20, and nineteen less than 10 %. It is, of course probable that, if more doses of each of the last seven oils or concentrates had been tested, the average would have been somewhat different and some of the percentage deviations correspondingly greater. The results on the whole support our claim that a group of about 10 rats used in a test in this manner will give a result almost always within ± 30 % of the true value. Hence, we claim that the biological values which we have assigned to these oils as the result of this method of testing on much larger numbers of rats must be very near to the true values for the oils.

CHEMICAL EXAMINATION OF THE COD-LIVER OILS.

By F. J. DYER.

The oils used in the biological investigation described above were subjected to the following chemical tests.

A. *The oils themselves.*

The chief values determined were:

(1) antimony trichloride blue value by comparison with Lovibond blue glasses;
(2) natural tint;
(3) acid value;

and the opportunity was taken for carrying out also the following tests:

(4) Fearon's reaction;
(5) phosphomolybdate test;
(6) trichloroacetic acid test.

For suggesting to me these three tests and for kindly advising me concerning the conditions under which he applies them in his own laboratory, I am greatly indebted to Dr O. Rosenheim, of the National Institute for Medical Research.

B. *Unsaponifiable fractions of the oils extracted by the method of Smith and Hazley [1930].*

Antimony trichloride blue value (Lovibond measure) and other tests applied to a chloroform solution of the unsaponifiable fractions.

Experimental.

A. *Examination of the oils.*

(1) *Antimony trichloride blue value (Lovibond).* This determination was made by the Carr and Price [1926] modification of the Rosenheim and Drummond test [1925], standardised to a greater degree of uniformity and precision

by the undermentioned conditions recommended by the Cod-liver Oil Colour Test sub-committee of the Pharmacopoeia Commission [1931]. It is well known that the relation between intensity of colour developed and concentration of an oil is not linear but is represented by a line which falls away at higher concentrations to a curvilinear function. For the final colour values of an oil the concentration was chosen so that readings were made between the Lovibond blue figures 4 and 6, a range over which for most oils intensity of colour produced bears an approximately linear relationship to the concentration. Blue values were, however, taken for other concentrations in order that curves for each oil might be constructed if desired.

The conditions under which the test was carried out were as follows.

Reagent. A solution of antimony trichloride was prepared in pure alcohol-free chloroform, saturated at 20°, and found to contain 23 % $SbCl_3$ immediately before use. It was stored in a stoppered amber-coloured glass bottle.

Procedure. Two grams of oil were weighed into a narrow-necked 10 cc. measuring flask, and the flask filled to the mark with chloroform at 20° and mixed.

0·2 cc. of the solution was delivered from a 1 cc. burette, the graduated portion of which measured 16 cm., into a rectangular cell of 1 cm. in the direction of observation as described below. 2 cc. of antimony trichloride reagent were added, the solutions allowed to mix, and the maximum blue colour was immediately matched in a tintometer by Lovibond glasses. Several readings were taken on each oil to ensure that the maximum intensity had been recorded.

Samples of six of the oils were also distributed to three other laboratories and similar determinations to the above were made under the same conditions.

(2) *Natural tint.* The colour of the oils was considered of importance in view of the statement of Drummond and Hilditch [1930] that "a yellow oil is probably richer in vitamins than one paler in colour" if freshly prepared, and even when old, provided that proper precautions have been taken in the making to remove all traces of liver tissue and accompanying enzymes.

The natural tint was determined by placing some oil in a tintometer cell measuring 1 cm. in the direction of observation and matching the colour as seen by diffused daylight with Lovibond yellow and red glasses in a tintometer.

(3) *Acid value.* The importance of a medicinal cod-liver oil possessing a low acid value, preferably below 0·3 % of free fatty acids calculated as oleic acid, has been emphasised by Drummond and Hilditch [1930]. It is also of importance in helping to determine how far the yellow tint of the oil may be regarded as significant, for a high acid value would indicate the presence of fat-splitting enzymes in accompanying liver tissue which would darken the oil.

Free acidity was determined in the customary manner by titration of a weighed quantity of oil in hot neutral alcohol with $N/10$ potassium hydroxide

using phenolphthalein (1 % in alcohol) as indicator. The percentage of free fatty acid was calculated as oleic acid.

(4) *Phosphomolybdate test.* When a solution of cod-liver oil in chloroform or light petroleum is shaken with a solution of sodium phosphomolybdate (10 %) in nitric acid (5 parts strong acid with 95 parts water), and, after allowing the mixture to stand, a strong solution of ammonia (sp. gr. 0·880) is added to the upper layer, a blue colour is developed. As the intensity of the colour is influenced by the time of shaking and by the length of the interval before adding the ammonia, the arbitrarily-chosen conditions set out below were adopted for each oil examined.

Method. To 1 cc. of 20 % solution of cod-liver oil in chloroform (or such other concentration as to give readings between 4–6 blue units as before), contained in a 10 cc. stoppered cylinder were added 2 cc. reagent. The mixture was shaken for exactly 10 seconds, allowed to stand for 1 minute, and then 3 cc. strong ammonia added and allowed to mix with the upper green layer. The maximum blue colour produced was read in a Lovibond tintometer in a cell measuring 1 cm. in the direction of observation using diffused daylight.

There is no evidence that the colour produced in this reaction bears any relation to vitamin activity, but it was thought to be worth while to determine whether there existed any relation between this chromogenic substance and the intensity of blue colour produced by the same oil with antimony trichloride.

(5) *Trichloroacetic acid test.* When trichloroacetic acid is shaken with a chloroform solution of cod-liver oil or preferably with a solution of its unsaponifiable matter, a blue colour is produced, and it was thought to be of interest to compare the intensities of the colours given under standard conditions by the cod-liver oils under investigation. The reagent for the test was prepared by mixing together:

Trichloroacetic acid (A.R.)	100 g.
Distilled water	10 cc.

allowing to stand overnight and using the resulting clear solution. The blue colour produced was measured in exactly the same manner as that described under the antimony trichloride test, using 2 cc. reagent and 0·2 cc. of a solution of the oil in chloroform of such strength that the range of Lovibond blue colour was between 4 and 6. It was thought that this colour, which is usually intensified after saponification of the oil, might bear some relationship to the antimony trichloride blue colour.

(6) *Fearon's Reaction.* Fearon [1925] using the converse of a test devised by Whitby [1923] for detecting sterols, claimed that the rose-pink colour produced in the presence of a condensing agent by the addition of a polyphenol, such as pyrogallol or resorcinol, to a chloroform solution of cod-liver oil was a measure of vitamin A potency. This claim was refuted by Rosenheim and Webster [1926], who showed that unsaponifiable matter of cod-liver oils in which vitamin A was abundantly present failed to give Fearon's reaction.

Further, they regenerated the chromogenic substance from the lead soaps of the unsaturated fatty acids of the original oil, and from other evidence suggested that the chromogen was probably an aldehydic oxidation product of an unsaturated acid (*e.g.* of the clupanodonic series). It was, therefore, considered of interest to apply the test to the oils under examination to see whether any relationship existed between this chromogen and the unsaturated fatty acids to which Norris and Church [1930] and others attribute interference with the blue colour produced by antimony trichloride with cod-liver oils at higher concentrations.

The method used was as follows.

To 0·5 cc. of 20 % solution of cod-liver oil in chloroform contained in a 10 cc. graduated stoppered cylinder was added sufficient trichloroacetic acid solution (12 % in light petroleum) to make 5 cc. A few crystals of pyrogallol were added and the mixture well shaken at intervals during 30 minutes. Another similar cylinder was prepared, containing in addition to the above mixture 3 drops (delivered from a standard dropper and each weighing 0·02 g.) of 1 % solution of purified benzoyl peroxide. (Commercial benzoyl peroxide was dissolved in chloroform and precipitated by methyl alcohol.) The rose pink colour produced was measured, using diffused daylight in a Lovibond tintometer, the mixture being contained in a cell measuring 1 cm. in the direction of observation. Any difference between the tints with and without added peroxide may give some indication of the peroxide content of the oil. The reagent was prepared by diluting with three parts of light petroleum a saturated solution of trichloroacetic acid (A.R.) in light petroleum, the solvent being previously dried over concentrated sulphuric acid as suggested by Fearon.

Results.

The values obtained by the above investigation are summarised in Table III. It may be seen that none of the determinations by the phosphomolybdate test, the trichloroacetic acid test or Fearon's reaction can be considered as a measure of the substance measured by the antimony chloride test.

In Table IV are the results obtained by four different laboratories for six of the oils examined by the antimony trichloride test under rigid conditions to ensure as close uniformity as possible. Although their individual results differ widely, the order of potency of the oils (Table IV), obtained by each of the four workers, is almost the same except for oils B and H. At another time by special request, one of these workers (N. E.) examined oil F and recorded a blue value between 70 and 90, which is in close agreement with the writer's finding of 86.

B. *Preparation and examination of the unsaponifiable fractions of these oils.*

The unsaponifiable fractions of the oils were prepared by the rapid method described by Smith and Hazley [1930], in which 2 g. of the oil are saponified

by 10*N* potassium hydroxide in an open tube, and the unsaponifiable residue is extracted by repeated shakings with chloroform until the chloroform washings from the soap solution no longer give a blue colour with arsenic trichloride.

The supply of oils D, E and F was not sufficient to admit of their unsaponifiable residues being prepared.

The technique was slightly modified by the addition of a few drops of alcohol to the second and third extractions to effect more rapid separation of the mixture into two layers.

(1) *Antimony trichloride blue value.* The residues obtained by evaporation of the mixed chloroform extracts in the above method were dissolved in chloroform to a volume 5, 10 or more cc. according to the known blue value of the oil under examination. The proportion of unsaponifiable matter was not determined, but the results given in Table V are expressed as values of unsaponifiable matter equivalent to the 2 g. of original oil used in its preparation. As a rule, the results show that when readings are taken at the 4 to 6 range of Lovibond glasses and converted to the 20 % concentration, the unsaponifiable matter gives a higher blue value than the amount of oil from which it was prepared. This is in accord with the results of Smith and Hazley.

It may be further observed that whereas for oils of lower antimony trichloride values the relationships to oil A of their blue values after saponification have not greatly altered from the same relationships before saponification, this does not hold true for oils such as F and L of higher blue values. Apparently the somewhat drastic method of saponification has caused considerably more loss of chromogen in these oils than in other oils.

(2) *Fearon's reaction.* Concentrates of some oils unexpectedly gave traces of the Fearon reaction, which appears to show that by this method, saponification is not always complete. No consistent parallelism between Fearon-values

Table III. *Constants and colour values of cod-liver oils used in the biological investigation.*

Oil	Natural tint: Lovibond yellow	Natural tint: Lovibond red	Acid value % F.F.A.	Antimony trichloride test. Value calculated for 20 % concentration of oil. Lovibond blue	Phosphomolybdate test. Value calculated for 20 % concentration of oil. Lovibond blue	Trichloroacetic acid test. Value calculated for 20 % concentration of oil. Lovibond blue	Fearon's reaction: Without benzoyl peroxide	Fearon's reaction: With benzoyl peroxide
A	1·8	0	0·34	9·0	1·5	2·7	0·5	0·7
B	3·5	0·3	2·27	4·4	4·7	Nil	12·0	12·5
C	1·2	0·2	0·17	7·6	Trace	1·0	0·4	5·5
D	2·5	0·2	0·38	10·8	—	—	—	—
E	1·6	0·1	0·2	15·2	—	—	—	—
F	8·9	0·8	2·89	86·0	—	—	—	—
G	2·4	0·3	0·38	28·0	Trace	3·9	6·7	11·2
H	1·3	0·1	0·24	4·3	2·3	Trace	2·1	2·3
J	9·2	1·2	1·54	17·4	Trace	2·8	11·7	21·1
K	1·2	0	0·28	10·2	3·5	1·5	2·2	4·0
L	12·0	1·4	4·0	305	Nil	104	Nil	Nil

and blue values obtained by other tests, has been detected during this investigation.

(3) *Trichloroacetic acid test.* One feature of incidental interest is that the blue value given by trichloroacetic acid is greater for a concentrate than for the amount of oil from which it was prepared (Tables III and V). It is possible that an interfering substance has been removed during saponification and that thereby the chromogen is enabled to exert its full power. For concentrates A, B, C, G and H it will be seen that the blue values given by the trichloroacetic acid test run roughly parallel with values given by those concentrates with antimony trichloride.

Table IV. *Antimony trichloride Lovibond blue values of six of the cod-liver oils obtained by four different workers. (Calculated for 20 % dilutions of the oil.)*

Oil	H. A. J.	N. E.	T. T. C.	F. J. D.
A	11·2	11·1	12·4	9·0
B	4·4	4·4	4·7	4·4
C	8·1	8·0	10·0	7·6
G	24·0	24·0	30·6	28·0
H	5·7	5·1	4·1	4·3
J	16·0	13·8	24·0	17·4

It will be seen that although there is a wide variation in these figures, there is a fairly good agreement as to the ascending order of potency, which may be expressed thus:

Order of potency (Lovibond blue) of the six oils according to:

H. A. J.	B H C A J G
N. E.	B H C A J G
T. T. C.	H B C A J G
F. J. D.	H B C A J G

Table V. *Colour values given by unsaponifiable residues of cod-liver oils of Table III, and by two concentrates.*

Note. The unsaponifiable residues were prepared by the method of Smith and Hazley using 2 g. of oil.

Oil	Antimony trichloride test. Value calculated for 20 % concentration of original oil. Lovibond blue	Phosphomolybdate test. Value calculated for 20 % concentration of original oil. Lovibond blue	Trichloroacetic acid test. Value calculated for 20 % concentration of original oil. Lovibond blue	Fearon's test (with added benzoyl peroxide). *Note.* For unsaponifiable residues this should be negative (see text). Lovibond red
A	17·25	1·7	8·5	1·6
B	8·0	8·3	4·2	1·2
C	12·9	1·2	5·3	1·0
D	21·2	—	—	—
E	27·0	—	—	—
F	100·0	—	—	—
G	42·0	2·0	24·6	1·2
H	12·6	1·4	7·2	1·0
J	26·6	1·0	8·8	Nil
K	19·6	2·1	17·2	Trace
L	266·0	Nil	230·0	Nil
M	420·0	—	—	—
N	290·0	—	—	—

PHYSICAL EXAMINATION OF THE OILS.

By R. A. MORTON.

All the materials tested biologically have been examined as carefully as possible by the available physical methods. The spectroscopic measurements on the blue colours were carried out on the reagent as described by Carr and Price. The investigation of ultra-violet absorption followed the technique described by Morton and Heilbron [1928]. No corrections have been introduced. The notation $E^{1\%}_{1\,cm.}$ refers to the intensity of absorption given by a 1 % solution of the material to be tested using a 1 cm. cell, the concentration referring to the actual solution studied in the spectroscope. E is defined by $\log I_0/I = E$, I_0 being the intensity of the incident light and I that of the emergent light (entering and leaving the absorption cell).

It must be emphasised that, since the absorption at 328 $m\mu$ is not wholly due to one substance, the values for $E^{1\%}_{1\,cm.}$ are all likely to be high compared with the intrinsic absorption of vitamin A (if it proves to be such). The correction to be applied is, however, very small in some fresh cod-liver oils but must differ from oil to oil. In concentrates the magnitude of the correction remains equally uncertain. Instead, therefore, of introducing any corrections we have used the gross intensity of absorption at 328 $m\mu$. The instrumental error is about $\pm$ 3 % in this work, but the uncertainty as to the magnitude of the correction may raise the gross experimental error in extreme cases to $\pm$ 10 %.

With regard to the spectroscopic measurement of the absorption at 572 and 606 $m\mu$ in the blue solutions, it must be emphasised that none of the oils was very fresh when received for spectroscopic examination. The 606 $m\mu$ chromogen (arising in part from the spontaneous oxidation of a substance occurring in many oils [Heilbron, Gillam and Morton, 1931]) was always present in reasonable quantity, so that the 572 $m\mu$ absorption could not be seen visually in the spectroscope as a definite band. The readings were made by setting the spectroscope to 572 $m\mu$ and determining the intensity of absorption irrespective of whether a clear absorption band could be seen or not. There is almost certainly some degree of overlapping between the two absorption bands with their maxima at 572 $m\mu$ and 606 $m\mu$ respectively. The values for $E^{1\%}_{1\,cm.}$ 572 $m\mu$, therefore, may require some correction, but as we have no exact basis for this the values have not been adjusted. The gross value for $E^{1\%}_{1\,cm.}$ 572 $m\mu$, does not, however, appear to be very inaccurate; the experimental error may be $\pm$ 10 %.

From other investigations [Gillam and Morton, 1931; Heilbron, Morton and Gillam, 1931] we are quite satisfied that, in extreme cases, the agreement between the 606 $m\mu$ band and either the 572 $m\mu$ or 328 $m\mu$ bands may be very much worse than is indicated by the data published in this paper.

Results.

The results of the physical examination of the oils are summarised in Table VI.

Table VI.

Oil	Date of examination	$E^{1\%}_{1\,cm.}$ 328 $m\mu$ ultra-violet	$E^{1\%}_{1\,cm.}$ 572 $m\mu$ spectroscopic	$E^{1\%}_{1\,cm.}$ 606 $m\mu$ colour test
A	December, 1930	0·78	0·70	0·92
A	April, 1931	0·80	0·75	1·04
B	December, 1930	0·60	0·37	0·47
C	December, 1930	0·67	0·50	0·61
D	December, 1930	0·935	0·92	1·14
D	April, 1931	0·90	0·74	0·95
E	December, 1930	1·03	1·02	1·35
E	April, 1931	1·0	0·80	1·09
F	December, 1930	4·3	3·81	4·76
G	December, 1930	1·68	1·45	1·92
H	December, 1930	0·46	0·33	0·33
J	December, 1930	1·30	1·28	1·50
K	December, 1930	0·85	0·68	0·85
L	April, 1931	8·0	7·5	12·15
M	April, 1931	13·0	15·3 (583 $m\mu$)	21·2 (620 $m\mu$)
N	April, 1931	10·0	9·14 (583 $m\mu$)	11·4 (620 $m\mu$)
Estimated experimental error (gross)		±10 %	±10 %	±10 %

It is clear that a very close agreement occurs between the 328 $m\mu$ absorption and the 572 $m\mu$ absorption. A similar agreement occurs in these samples between the 328 $m\mu$ and the 606 $m\mu$ absorption bands, though the small deviations which exist between the 328 and 606 $m\mu$ bands on the one hand and between the 328 and 572 $m\mu$ bands on the other lie in opposite directions. This agreement between the 328 and 606 $m\mu$ bands is probably misleading, for it has been shown [Gillam and Morton, 1931] that, in many samples of cod-liver oil, there is close agreement between the 328 and 572 $m\mu$ bands but wide disagreement between the 328 and 606 $m\mu$ bands.

STATISTICAL EXAMINATION OF RESULTS.

By J. H. GADDUM.

The results obtained have been examined statistically in order to extract information as to the relative accuracy of the different methods of measuring the concentration of vitamin A, and in order to discover whether the discrepancies shown by some of the oils could be attributed to the known error of sampling of the biological assay.

When the error to be expected in the measurement of any given quantity is proportional to the quantity itself, this error is usually expressed as a percentage of the result. The percentage standard deviation, which is sometimes called the coefficient of variation, is a convenient index of accuracy so long as it is small compared with 100, but when it is large its exact interpretation becomes ambiguous. An error of + 100 % or more may occur in a biological assay, but the expression "an error of − 100 %" has no useful meaning. When

the error is not small compared with 100 % it is clearly not desirable to express it as a percentage at all. Another difficulty arises from the doubt as to whether the error should be calculated as a percentage of the observed value or of the true value, and from the difficulty of carrying out the calculation in the latter case.

These difficulties disappear when the distribution of the logarithms of the readings is studied instead of that of the readings themselves. The error of the test can then be directly expressed as the error of the logarithm of the reading. In the present case the standard deviation of the logarithms (to base 10) of the results has been calculated and the square of this quantity is referred to as the "logarithmic variance." The meaning of the result expressed in this way is not immediately intelligible to those who are not accustomed to this form of expression, but when the calculations are otherwise complete, the result can easily be put in a more readily intelligible form. This method of calculation has been adopted here because, in cases like this, it is simpler, less ambiguous, and probably more rational than the calculation of coefficients of variation. For this purpose it is assumed that the logarithms of the readings are normally distributed. Arguments were put forward by Galton [1879] in favour of the view that, especially in the case of vital phenomena, this assumption was more rational than the usual assumption that the readings themselves were normally distributed. When the error is small compared with the actual readings, the two assumptions give almost identical results, and the chief reason that the former assumption is not generally adopted is probably that, in many cases, it would increase the complexity of the calculations without appreciably affecting the final result [Yule, 1924, Chap. VII, Par. 26].

The sampling error of the biological test.

The sampling error is due to the fact that, even when the conditions are so carefully controlled that the average response of the rats to the vitamin is constant, different individual rats will give different responses. The error is diminished by taking the average response of a number of rats, and its size is dependent on the number of rats used.

The standard deviation (σ) of the response of individual rats (increase of weight) has already been worked out from the results obtained with oil A and published [Coward *et al.* 1930]. These results appear to justify the assumption that this standard deviation is independent of the dose of cod-liver oil used. In order to obtain the best possible estimate of σ the published figures have been recalculated, using the equation $\sigma^2 = \frac{\Sigma d^2}{n-1}$ where $d =$ the deviation of each individual response from the mean response, and $n =$ the number of animals. This formula is likely to give a more accurate estimate than that previously used. The most probable value of σ^2 was found by taking the weighted mean of the different values of σ^2 thus obtained. The weight of each individual estimate of σ^2 was taken to be proportional to n. Separate estimates of the value of σ

for male rats and for female rats have also been obtained by applying the same method to the results with oils B–N. The estimates obtained by these calculations are shown in Table VIII.

It has been found that the response bears a linear relationship to the logarithm of the daily dose. If therefore the distribution of the response is normal, the distribution of the logarithm of the result of the assay will also be normal. A series of biological assays carried out in this way thus probably provides a good example of a series of readings of which the logarithms are normally distributed. This is the kind of distribution implied by the assumptions made in this paper and discussed above.

The standard deviation of the logarithm of the result (λ_1) is obtained by dividing σ by the slope of the line connecting the response with the logarithm (to base 10) of the dose. This is shown in the last column in Table VIII. Since the slope of the curve for male rats is greater than that for female rats, the error of the result is smaller when male rats are used than when female rats are used. It is therefore preferable to use male rats for this test when possible. The largest source of error in these estimates of λ_1 probably lies in our estimate of the slope of the curve connecting dose and effect. The data were considered insufficient for a satisfactory estimate of this error. The error of each test has therefore been calculated on the assumption that $\lambda_1 = 0{\cdot}4$ for all the rats used, and has been taken to be equal to $\frac{0{\cdot}4}{\sqrt{n}}$. It is reasonable to assume that the estimate obtained in this way is larger than the true sampling error of the test. Nevertheless it is found that the errors so calculated are not large enough to account for the discrepancies which occur between physical and biological estimates.

The limits between which the biological result may be expected to vary owing to the error of sampling are shown in Table IX. These limits correspond to twice the standard error; the two values given for each oil were obtained by first dividing and then multiplying the biological estimate by the antilogarithm of $\frac{2 \times 0{\cdot}4}{\sqrt{n}}$, where n = the number of rats used. The potency corresponding to the standard curve has been taken as 100. This is probably equal to the potency of the oil A. The error in the estimate of the potency of the oil A from the curve has been calculated in the same way as the errors for the other oils.

The relative accuracy of the different methods of estimating vitamin A.

The results on which these calculations are based are shown in Table VII. The ordinary methods of calculating correlation or regression coefficients are not suitable in this case, because they give undue weight to the results obtained with the more potent oils. The method which has been used depends on calculating the distribution of the logarithms of the results. Some general arguments justifying the assumptions involved have been given.

Let x be any one of the figures in column I of Table VII, and y the corresponding figure in column II. If the two tests both give results directly pro-

Table VII. *Biological, chemical and physical measurements of 11 samples of cod-liver oil and 2 concentrates.*

Oil	Biol. value relative to oil A (=100) I	Blue value, $SbCl_3$ test. Lovibond measure. Oil II	Blue value, $SbCl_3$ test. Lovibond measure. unsap. fraction III	Blue value, $SbCl_3$ test. Spectrophotometric measure. 572 $m\mu$ IV	Blue value, $SbCl_3$ test. Spectrophotometric measure. 606 $m\mu$ V	Ultraviolet band 328 $m\mu$ oil itself VI
A	100	9·0	17·25	0·70	0·92	0·78
B	78·4	4·4	8·0	0·37	0·47	0·60
C	154·9	7·6	12·9	0·50	0·61	0·67
D	129·8	10·8	21·2	0·92	1·14	0·935
E	175·5	15·2	27·0	1·02	1·35	1·03
F	627·3	86·0	100·0	3·81	4·76	4·3
G	152·7	28·0	42·0	1·45	1·92	1·68
H	78·2	4·3	12·6	0·33	0·33	0·46
J	122·0	17·4	26·6	1·28	1·50	1·30
K	245·8	10·2	19·6	0·68	0·85	0·85
L	1562·3	305·0	266·0	7·5	12·15	8·0
M (Conc.)	1987·5	—	420·0	15·3 (583 $m\mu$)	21·2 (620 $m\mu$)	13·0
M (Conc.)	1551·5	—	290·0	9·14 (583 $m\mu$)	11·4 (620 $m\mu$)	10·0

portional to the vitamin A content of the oil, x/y should be constant for the different oils. In order to test how far this is so $(\log_{10}x - \log_{10}y)$ was calculated for each oil. The values obtained, which would be all equal if the error of both tests was zero, were found to vary considerably. The mean and standard deviation of $(\log_{10}x - \log_{10}y)$ were calculated.

Table VIII. *Showing estimates of the standard deviation* (σ) *of the response (increase of weight), and the standard deviation* (λ_1) *of the logarithm of an estimate of the daily dose based on an experiment with one rat.*

	Total number of rats used for the estimate	σ	λ_1
Male and female rats used in experiments with oil A	156	13·2	—
Male rats used in experiments with oils B–N	163	13·4	0·268
Female rats used in experiments with oils B–N	184	10·6	0·386

The mean provides an estimate of the factor by which the blue value of crude oil should be multiplied in order to find the biological activity. This factor has been calculated for all the chemical and physical tests and the results are given in Table IX. In this Table all the different determinations have been reduced to the units in which the biological results are stated by multiplying each figure by the appropriate factor determined in this way. It will be seen that many of the physical measurements lie outside the range of the known error of the biological test. In particular the physical methods appear to have over-estimated the potency of oils G and J and under-estimated those of oils C and K.

If $\lambda =$ the standard deviation of $(\log_{10}x - \log_{10}y)$, λ^2 gives an estimate of the combined logarithmic variances of the physical and biological methods of estimating vitamin A. If the logarithmic variance of sampling (λ_1^2) represents

Table IX. *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). Oils arranged in order of biological activity.*

	Biological value I		Blue value $SbCl_3$ test. Lovibond measure		Blue value $SbCl_3$ test. Spectrophotometric		Ultra-violet band 328 $m\mu$
	Most probable	Possible range ($\pm 2\lambda$)	Crude oil	Unsap. fraction	572 $m\mu$	606 $m\mu$	oil itself
Oil	I		II	III	IV	V	VI
H	78	51– 119	48	81	57	46	70
B	78	55– 111	49	52	64	65	92
A	100	86– 116	101	111	121	127	120
J	122	82– 181	195	171	221	207	199
D	130	97– 174	121	137	159	157	143
G	153	96– 242	314	271	250	265	257
C	155	125– 193	85	83	86	84	103
E	175	134– 230	170	174	176	186	158
K	246	137– 440	114	126	117	117	130
F	627	495– 794	964	644	656	657	659
N	1551	866–2778	—	1870	1575	1572	1533
L	1562	954–2557	3418	1714	1293	1676	1227
M	1987	1110–3560	—	2170	2637	2924	1993
Factors by which readings in Table VII were multiplied to obtain the above results			11·2	6·442	172·3	137·9	153·3

the whole error of the biological test, the logarithmic variance of the physical test will be equal to $(\lambda^2 - \lambda_1^2)$. This has been calculated for each of the physical tests and the results are shown in Table X. The value taken for λ_1^2 was the

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

			Blue value $SbCl_3$ test				
			Lovibond measure		Spectrophotometric measure		Ultra-violet absorption at 328 $m\mu$ of oil itself
		Biological value I	Crude oil II	Unsap. fraction III	572 $m\mu$ IV	606 $m\mu$ V	VI
Biological test	λ_1^2	0·0080	—	—	—	—	—
Ratio of biological to physical results	λ^2	—	0·0554	0·0252	0·0340	0·0328	0·0194
Physical results	$\lambda^2 - \lambda_1^2$	—	0·0474	0·0172	0·0260	0·0248	0·0114
Percentage range corresponding to $\pm 2\sqrt{\lambda^2 - \lambda_1^2}$ (within which the result may be expected to lie 21 times out of 22)		66–151	37–273	55–183	48–210	48–206	61–163

mean of the values for the tests on the different oils. These results have been put in a more familiar form by calculating the range (corresponding to twice the standard error) within which the result may be expected to lie 21 times out of 22, expressed as a percentage of the true value.

The fact that the apparent errors of the physical tests, measured in this

way, are much larger than the errors of the physical measurements themselves might be explained in more than one way. Some oils might contain substances which were biologically inactive and yet gave a blue colour with $SbCl_3$ and absorption in the ultra-violet, while other oils might contain substances which masked the vitamin A in both these physical tests. Both kinds of physical test might measure the concentration of some substance other than vitamin A which has a roughly similar distribution. It is also possible that the biological test is subject to unknown errors due, for example, to a variation in the sensitivity of the whole stock of rats, or to the presence in some oils of physiologically active substances other than vitamin A. It is hoped to test this latter possibility by means of further experiments.

The estimates of the logarithmic variance in Table X place the tests in order of their accuracy when applied to the particular oils used in these experiments, but these estimates are themselves subject to an error of sampling dependent on the number of oils tested. The significance of the difference between these estimates of the accuracy of the different tests has been studied by the methods given by Fisher [1930, Par. 41]. By comparing the estimates of λ^2 it has been found that the probability that the test in column VI is more accurate than that in column II is slightly greater than 0·95. Similar calculations, also based on the estimates of λ^2, show that the probability that the test in column III is more accurate than that in column II is about 0·8.

When allowance is made for the sampling error of the biological test by comparing the values of $(\lambda^2 - \lambda_1^2)$ both these probabilities are greatly increased. The value of λ_1^2 is not known with sufficient accuracy to warrant a precise estimate of the probability, but there is clearly a quite definite presumption in favour of the conclusion that the measurement of the ultra-violet absorption of the oil itself and the measurement of the Lovibond blue value for the unsaponifiable fraction both provide more accurate estimates of vitamin A than the Lovibond blue value for the crude oil.

Discussion.

For practical purposes the possibility of accepting any one of the chemical or physical measurements of the oil as a measure of its vitamin A potency may be determined from an examination of Table XI.

It is evident that the six oils and two concentrates collected in the upper part of Table XI show good agreement between the biological value, the blue value of the unsaponifiable fraction and the intensity of absorption at 328 $m\mu$. Three only of these oils show agreement between biological value and blue value of the oil itself. The five oils collected in the lower part of Table XI cannot be said to show any degree of agreement between biological and physical measurements that would serve any useful purpose, though one of these oils (B) shows better agreement between biological value and the intensity of its absorption at 328 $m\mu$ than between its biological value and any of its other

Table XI.

Oil	Biological value	Blue value of oil	Blue value of unsap. fraction	Intensity of absorption at 328 $m\mu$
H	78	48	81	70
A	100	101	111	120
D	130	121	137	143
E	175	170	174	158
F	627	964	644	659
N	1551	—	1870	1533
L	1562	3418	1714	1227
M	1987	—	2170	1993
B	78	49	52	92
J	122	195	171	199
G	153	314	271	257
C	155	85	83	103
K	246	114	126	130

physical values. It would, therefore, appear to be quite unsafe to accept the blue value of the oil itself as a measure of its vitamin A value. Both the blue value of the unsaponifiable fraction of an oil and the intensity of absorption at 328 $m\mu$ appear to be more often in agreement with the biological value, but these also may be widely divergent, a fact which would appear to indicate that certain oils may contain substances which increase the blue value and other oils may contain substances which decrease the blue value.

The statistical examination of the results has strengthened this opinion.

It may be significant that the oils for which we have found the best agreement between biological and physical measurements are those which we have received direct from manufacturers and which are known to be pure cod-liver oils of recent preparation.

Summary.

Eleven samples of cod-liver oil and two concentrates have been examined and the following values determined.

1. Biological value; vitamin A content as measured by resumption of growth in rats whose reserves of this factor had been exhausted by feeding on a diet deficient in vitamin A.

2. Blue value; obtained by the action of antimony trichloride on the oil itself and measured by the Lovibond tintometer.

3. Blue value; obtained by the action of antimony trichloride on the unsaponifiable fraction of the oil and measured by the Lovibond tintometer.

4. Intensity of absorption at 572 $m\mu$, using the blue solution obtained by the action of antimony trichloride on the oil itself, measured spectrophotometrically.

5. Intensity of absorption at 606 $m\mu$, using the blue solution obtained by the action of antimony trichloride on the oil itself, measured spectrophotometrically.

6. Intensity of absorption at 328 $m\mu$ for the oil itself, measured spectrophotometrically.

Statistical examination of the results shows:

(*a*) that the discrepancies between the physical and biological measurements are much larger than the known sampling error of the biological test;

(*b*) that, of the physical and chemical measurements, the best agreement with the figures determined by the biological method was given by the measurement of the 328 $m\mu$ absorption band, and nearly as good agreement was given by the Lovibond blue value of the unsaponifiable fraction of the oil;

(*c*) that both the 572 $m\mu$ and 606 $m\mu$ bands gave less good agreement;

(*d*) that the Lovibond blue values obtained from the oils themselves give the least good agreement and it would appear to be unwise to rely upon this method for the measure of vitamin A in an oil. It can only be used as a very crude indication of vitamin A potency;

The oils whose biological values most closely agree with the values of the 328 $m\mu$ band and the Lovibond blue values of the unsaponifiable fractions are those which were received direct from the manufacturers and examined within a few months of their preparation.

We have to thank Messrs Isaac Spencer and Messrs Allen and Hanbury for supplying samples of oil; Miss B. G. E. Morgan for help given in the biological part of this work and Mr H. W. Ling for help given in the chemical part.

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