A COMPARISON BETWEEN THE SPECTROPHOTOMETRIC AND BIOLOGICAL ASSAY FOR THE VITAMIN A CONTENT OF FISH-LIVER OILS.

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A standardized method of color analysis for Vitamin A (1) by the antimony trichloride reaction has been applied to commercial samples of fish-liver oils. A comparison between the spectrophotometric values and those obtained by biological assay would indicate that there is a considerable error in the antimony trichloride test.

The fish-liver oils which were used in this series of experiments were furnished by the Parke Davis and Company, the Abbott Laboratories, the Eli Lilly Company, the E. R. Squibb and Sons, the Mead Johnson Company and the Upjohn Laboratories. Each of these companies gave feeding tests on their oils so that data could be obtained on the relative values and agreement between the chemical and biological assays. The Parke Davis and Company, through the courtesy of Dr. A. D. Emmett, furnished in addition, colormetric data on their oils as obtained by the Carr Price method, which were made over a period of several years. The data furnished a check on the relation between the colormetric and spectrophotometric methods, as well as the influence of time on these oils. All of the samples used in the tests described in this paper were kept in a refrigerator at 5° C.

The oils have been divided into two sets, (a) those giving with the antimony trichloride reagent an absorption band at 608 mμ (Table I) and (b) those giving an absorption band at 622 mμ (Table II). Ordinary "cod-liver" oil sold in pharmaceutical trade is made from the genus of fish known as the *gadus morrhua*, and generally not exclusively from the cod fish. This group includes the cod, haddock, hake and pallock. All of the oils in Table I were designated as cod-liver oils. They included both domestic and foreign oils. Records on some of the oils would indicate that they had been in storage for some time, while others were quite fresh. There was no apparent relationship between the variations in the color test—biological assay values and the age or source of the oil.

In certain cases where a marked difference was shown between the values obtained from the two methods of assay, both types of assays were repeated and confirmed this difference.

TABLE I.

Comparison Between the Spectrophotometric and Biological Assay Data on Cod-Liver Oils (Based on the Absorption Band at 608 mμ).

Company	Sample No.	Biological Assay	Spectrophotometric Assay (Referred to Sample No. 11 as a Standard)	
I	1	573	590	
	2	500	780	
	3	1000	640	
	4	500	850	
	5	625	800	
II	6	625–500	590	
	7	400–500	690	
	8	450–550	750	
	9	600–666	770	
	10	625+	920	
III	11	1000	1000	
	12	3200	2790	
	13	800	820	
	14	700	670	
	15	600	670	
	16	1900	1060	
	17	800	590	
	18	1350	1730	
	19	1800	1930	
	20	1900	1500	
	21	1000	1190	
IV	22	1000	1170	
	23	1000	850	
	24	4000	5300	
	25	4500	5500	
	26	1300	1300	
				(½ Spectrophotometric Values)
V	27	650	1220*	610*
	28	700	1520	760
	29	500	1100	550
	30	650	1500	750
VI	31	1200	2600	1300
	32	500	1100	550
	33	850	1700	850

*Note.—In Groups V and VI it would seem necessary to multiply the spectrophotometric values by a factor of ½ to make the values obtained by this method agree with those obtained by the biological assay.

In Table II are recorded the results obtained from halibut-liver oils. Here, again, there was found to be a wide variation between the two methods of analysis. These oils were for the most part concentrates of the unsaponifiable fraction obtained from the crude oil. In certain cases (samples 34 and 35) the concentrated oils were diluted with sesame oil.

No explanation is offered for the necessity of this factor, of ½, as applied in groups V and VI in Table I, though a justification for its use can be seen in certain groups of oils, where all the members had to be corrected by the same factor. Both the Spectrophotometric and biological assays were repeated on certain of these oils, the results of which only confirmed the previously obtained values. Undoubtedly these variations, since they are so consistent, are due to some cause other than errors of observation, and may be ascribed to some inhibitor, activator or to alteration in the structural nature of the chromophore.

TABLE II.

Comparison Between the Spectrophotometric and Biological Assay Data on Halibut-Liver Oils (Based on the Absorption Band at 622 mμ).

Company	Sample No.	Biological Assay	Spectrophotometric Assay (Referred to the Height of the Extinction Coefficient of No. 11, Table I, as a Standard)
VII	34	500	650
	35	2500	2310
	36	30000	37800
VIII	37	37,500–50,000	64700
	38	37,500–50,000	113800
	39	25,000–37,500	78400
	40	13000	29300
IX	41	5000	17000
	42	32500	94800
	43	5000	3800
	44	17500	73200
X	45	62500	84000

In Table II are presented data on the halibut-liver oils, which gave a band at 622 mμ, or some 14 mμ further towards the red as compared with the color obtained from the cod-liver oils with antimony trichloride. This shift has been reported by Morton (2). Heilbron, Gillman and Morton (3) suggest that the substance giving the 572 mμ (our 578 mμ) band is an ester and the substance producing the 583 mμ band (which appears at the same time as the 622 mμ band) is an alcohol derived from the former substance by saponification. None of the oils in Table II gave the 608 mμ band as far as could be observed by inspection of the absorption bands, and no amount of dilution with $CHCl_3$ shifted the position of the 622 mμ band. There was a weaker band at 705 mμ which apparently accompanies the 622 mμ band.

The existence of vitamin-containing oils giving markedly different absorption bands (608 mμ and 622 mμ) leads one to postulate the theory that Vitamin A is not a definite chemical compound, but that the physiological effect produced by the so-called "A" vitamin is due to any one of a series of closely related compounds, which must have certain structures in common. As already stated Morton suggests that this difference may be as simple as that between an alcohol and an ester. However, the fact that carotene fades in a manner similar to the fading of fish oils suggests a more complicated mechanism.

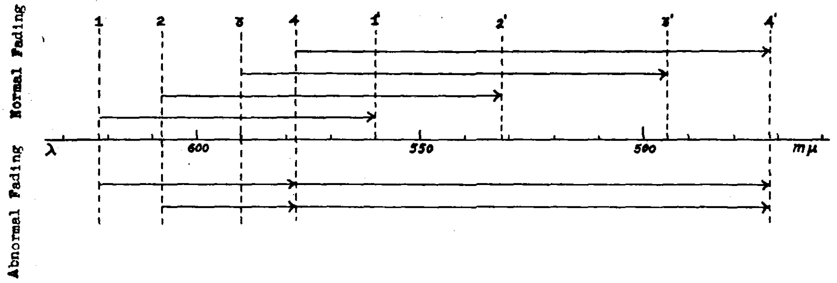

FIGURE 1. Graphical representation of the changes in absorption bands on fading. 1 to 1′ representing the change from 622 mμ to 560 mμ; 2 to 2′ from 608 to 532 mμ; 3 to 3′ from 590 to 495 mμ; and 4 to 4′ from 578 to 472 mμ. The abnormal fading represented below the wave length scale indicates an apparent change from the original chromogen to the 578 form and then fading of the 578 to the final 472 form. This abnormal fading is slight in the case of the 608 mμ chromogen but is the principal reaction in the case of the 622 mμ chromogen.

Following the diagrammatic outline (Figure 1) for the band shifts:

(a) It can be seen that the cod-liver oils producing a band at 608 mμ fade to give a band at 532 mμ (2 to 2′) and those giving a band at 578 mμ fade to 472 mμ (4 to 4′). In case a rather concentrated solution was used so as to give only the 608 mμ and no 578 mμ band, faded bands were observed at both 532 mμ and 472 mμ (2 to 4′). This would indicate that there is a shift from 608 to 578 together with the shift from 608 to 532 mμ, the latter being more rapid than the former. If the amount of chromogen is unusually large, some 608 changes to 578 which undergoes its normal fading reaction to 472 mμ. In this case just considered, no 578 mμ band appears because the change from 578 to 472 mμ is more rapid than that from 608 to 578 mμ.

(b) The above hypothesis is supported by the fact that if more chromogen is present, both 608 and 578 mμ bands are produced in the blue solution, and both 532 and 472 mμ bands appear in the faded solution. In this case, the 578 mμ band is entirely extinct before the 608 mμ band is extinct, indicating that the amount of chromogen has been reduced to the amount present in case (a) above. These considerations also explain why solutions of oil dilute enough to produce no 578 mμ bands must be used to obtain spectrophotometric values of the 608 mμ bands which are directly proportional to the amount of oil used.

(c) In the case of the halibut-liver oils, a band is produced at 622 mμ, which corresponds to the 608 mμ band produced in ordinary oils. Since the predominate band in the faded solution is at 472 mμ, most of the 622 chromogen must change to the 578 chromogen, which then undergoes its normal fading reaction. Other bands appear in the faded solutions of these oil concentrates at 545, 560, and 580 mμ, only the one at 472 being strong and well-defined.

(d) If the solution of cod-liver oil is made up so that only the 578 mμ (and no 608 mμ) band is produced, the only absorption band in the faded solution is at 472 mμ (4 to 4′).

(e) Carotene gives a band with the $SbCl_3$ reagent at 590 mμ, which fades to give a band at 488 mμ. (Fig. 1, 3 to 3′).

The linear equation ($y = mx + b$) can be used to predict the position of the absorption bands in the faded solutions of these substances.

Let y = band shift expressed in cm^{-1};
x = original position of the band expressed in cm^{-1}; and
m and b are constants to be determined.

Using the band shifts, 578 to 472 mμ and 608 to 532 mμ, the constants m and b can be found, and their values are 1.797 and −27,215 respectively. These values can be substituted in the equation, and the value of "x" determined for each substance, from which the position of the faded band can be calulated:

Original band ("x")	Value of "y"	Faded Band Calculated	Faded Band Observed
(a) Cod-liver oil			
608 mμ	2,348 cm^{-1}	532 mμ	532 mμ
578 mμ	3,879 cm^{-1}	472 mμ	472 mμ
(b) Halibut-liver oil			
622 mμ	1,683 cm^{-1}	563 mμ	560 mμ
(c) Carotene			
590 mμ	5,249 cm^{-1}	495 mμ	488 mμ

The values obtained are all within the limits of experimental accuracy.

It is to be noted that if the "chromogen" in the oil concentrates reacts in what we might term normal fading we would expect to obtain a band at about 560 mμ, where a weak band was observed.

The mechanism of the fading of the 622 mμ band to a band at 560 mμ appears to be perfectly normal and subject to the same explanations that might be applied to the fading of the other solutions. As indicated by the strength of the 560 mμ band as compared with that of the 472 mμ band formed in the same solution only a small part of the chromogen undergoes normal fading, while the principal reaction is the conversion of the chromogen giving a band at 622 mμ into one with a band at 580 mμ which in turn fades to the substance with a band at 472 mμ. Due to the similarity of the bands there is very little doubt but that the 580 mμ band, which was not very well defined, is the same as the 578 mμ band. If, as is apparently the case, the speed of the fading reaction of the chromogen with a band at 578 mμ to one with a band at 472 is equal to, or greater than the speed of the reaction converting the chromogen with a band at 622 mμ to one with a band at 578 mμ, little, if any, indication of the presence of the 578 mμ band would be apparent in the fading solution. It follows then that the chromogen with a band at 622 mμ must either be more stable than the chromogen with a band at 608 mμ, with regard to what we have termed the normal fading (see Fig. 1) or less stable than the chromogen with the 608 mμ band with regard to conversion to the form with a band at 578 mμ, since the principal reaction on the fading of the chromogen with a band at 622 mμ is the formation of a substance with a band at 472 mμ rather than at 560 mμ. Observations on the rate of fading of the halibut-liver oil and the cod-liver oil colors showed that the colors formed by the antimony trichloride reaction with the halibut-liver oils were much more permanent than those colors formed with the cod-liver oils.

Table III is presented to show the agreement over a period of years of the spectrophotometric and colorimetric values. There is very little aging effect over the period indicated.

One of the cod-liver oils was found to be highly flavored with peppermint oil. The spectrophotometric readings on this sample were not proportional to the amount of oil used unless the oil was comparatively dilute, (about a 3% solution in $CHCl_3$). The values obtained by this procedure agreed with the animal assay. It was subsequently found that peppermint oil in the presence of certain reagents such as sulfuric acid, acetic anhydride, etc., and to a lesser extent with antimony trichloride, gives an absorption band similar to that produced by the fish-liver oils. Perhaps of even more significance is the fact that in the standard color reaction for peppermint oil (5) a blue color is produced with absorption bands at 622, 580, 505 and a weak band at 705 mμ. It was the presence of this latter weak band in the more stable and concentrated color solutions of the peppermint oils that prompted a reexamination of the blue color produced with the halibut-liver oils, with the discovery of a similar band at 705 mμ. (4) In some peppermint oil solutions, particularly those to which mineral acids were added, a band was produced at 605 mμ instead of at 622 mμ. The conditions under which either of these two bands (605 mμ or 622 mμ) might be produced were not determined.

TABLE III.

Comparison Between the Colorimetric and Spectrophotometric Assay of Cod-Liver Oils.

Sample No.	Date of Observation			
	Colorimetric			Spectrophotometric
	1928	1928	1931	1931
11	10	9	12	10
12	19	22	27	27.9
13	10	9	10	8.2
14	5.9	4.7	7.5	6.7
15	6.5	6.5	7.5	6.7
16	10	9	10	10.6
17	5.9	5.9	3.9	5.9
18	15	15	16	17.3
19	....	15	16	19.3
20	....	13	13	15.0
21	....	12	12	11.9
27	10	7.5	10	12.2
28	....	....	13	15.2
29	....	9	10	11.0
30	....	12	13	15.0

The nature of this blue material in the peppermint oil test is a matter of uncertainty. It has been reported to be azulene, which in turn is said to be produced by the oxidation of cadinene (a member of the sesquiterpene family). An investigation of the green color produced by cadinene, obtained by distillation of cade oil, showed bands at 605 and 670 mμ, while a sample of German Chamomile oil in chloroform (obtained from Fritschie Bros. who obtained it from Schimmel and Co.) (the source of the material used by Ruczika in his study of azulene (6)) which was bright bule in color gave sharp bands at 608 and 660 mμ. Careful distillation of oil of peppermint produced a fraction, B. P. 60-65° (2-3 mm.) in which practically all of the color forming material is to be found. The blue solutions give a most brilliant red fluorescence.

SUMMARY.

A method for the testing of oils for vitamin A by the use of the $SbCl_3$ reagent has been applied to a number of commercial samples with some agreement between the colorimetric and biological assay values within certain groups of samples.

Differences which did occur were consistent in the samples from any one laboratory or for a a given set of samples.

The nature of the absorption bands produced by $SbCl_3$ on cod-liver oils, halibut-liver oils, and carotene and the resulting faded bands would indicate that several closely allied compounds may be responsible for both the color reactions and the vitamin properties.

Oil of peppermint contains a color-forming compound which has to a rather marked degree the same type of chromogen as is to be found in certain oils known to contain vitamin A.

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Geology.

This book, gotten out in the usual McGraw-Hill style, may well fill a place in the text-book field that has been neglected, i. e., "one-semester beginning course for college students," as the authors say in the preface. The general arrangement and order of presentation would seem to be that which would do very well in such a short course. The text is not over-burdened with needless detail, although some of the words are the less common ones of geologic literature. The illustrations are from many sources; numerous of them are new to textbooks. While I cannot recommend it for longer courses, it should find use in those places where Geology only receives one semester or one quarter of the time of a student—WILLARD BERRY.

Geology, by W. H. Emmons, G. A. Thiel, C. R. Staufer, and I. S. Allison. xii + 514 pp. New York, The McGraw-Hill Book Co., 1932.