LIMITATIONS OF THE ANTIMONY TRICHLORIDE TEST FOR QUANTITATIVE ESTIMATION OF VITAMINE A.*

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An accurate chemical test for the vitamine content of cod liver oil would be of tremendous value; it would result in the saving of much time and money to those who must at present assay the oil biologically and it would provide a means of investigating cod-liver oil to those who are interested in the potency of this oil but unable to perform the biological tests. Moreover such tests would greatly facilitate the correct development of the industry which produces the crude oil from the livers. Although practically no progress has been made with respect to a chemical test for the vitamine D present in cod liver oil, the studies of the vitamine A content of the oil have resulted in the development of certain color tests which have been of sufficient promise to warrant intensive study. The antimony trichloride test (1,2) which has been applied to cod liver oil produces a blue color which could be used as a measure of the vitamine A potency of the oil provided that in all cases the intensity of the blue color is proportional to the vitamine A content of the oil.

In order to measure and express the intensity of the blue color developed by the antimony trichloride test two methods were studied.

(*a*) USE OF COPPER SULPHATE STANDARD.

For the determination of vitamine A potency by this method a standard blue color is first prepared with a copper sulphate solution of definite strength. A 30% chloroform solution of an oil of known vitamine A content—biologically assayed—is made up and several drops of this solution are then added to 2.0 cc. of the saturated antimony trichloride solution in chloroform, until a known number of these drops has been found to produce a color which matches the previously prepared copper sulphate standard color. The unknown oil is then tested in the same manner and the potency of the unknown is calculated by assuming that potency is inversely proportional to the number of drops found necessary for a match of the standard color.

One of the chief objections to this method is that it does not permit an accurate reading of the blue color inasmuch as variable amounts of red and yellow also develop in the reaction mixture. Though this defect can be partly overcome by the use of a few drops of cobalt and ferric chloride solutions in addition to copper sulphate, the use of Lovibond standard glasses, as described below, is much to be preferred.

(*b*) USE OF LOVIBOND COLOR GLASSES.

The method used was essentially as suggested by Wokes and Willimott (2) and all our results were in complete agreement with their findings as to the best procedure to be followed. In this method, a saturated solution of antimony trichloride in chloroform is prepared and 2.0 cc. are placed in a Lovibond color test-tube. To this is then added a definite amount of a 30% chloroform solution of the oil so that a reading preferably between 5.0 and 12.0 Lovibond blue units is obtained—

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0.1 to 0.4 cc. of the oil solution will generally suffice. The color produced is immediately read in a Wesson tintometer with Lovibond glasses and expressed in blue units, though red and yellow are also used in the actual reading, if found necessary, to facilitate an accurate matching of the blue color. The test is strongly influenced by such factors as temperature, time and concentration of the reagents, and the recommendations of Wokes and Willimott (2) in respect to these factors must be closely adhered to.

RELATIONSHIP BETWEEN COLOR TEST AND BIOLOGICAL ASSAY.

In order to be able to draw conclusions regarding the vitamine A potency of an oil from the intensity of the blue color, it is necessary to know exactly how the color produced by a biologically assayed sample varies when this oil is diluted with known quantities of a vitamine A free oil so as to produce samples which would have 75%, 50%, etc., of the potency of the original oil. To accomplish this object a standardized sample of cod liver oil was diluted with deodorized peanut oil to definite concentrations and therefore to known vitamine A potencies; the peanut oil produced no blue color when tested by the antimony trichloride method. The resulting oils were then tested colorimetrically.

TABLE I.—AMERICAN COD LIVER OIL.

Oil mixture. % Cod liver oil.	% Peanut oil.	Vit. A potency U. S. P. Units.	Color reading Lovibond blue units, 0.2 cc. of 30% $CHCl_3$ vol. of oil.
100	0	715 (biological assay)	12.4
66.7	33.3	477	8.3
50	50	358	6.3
33.3	66.7	238	4.3
16.7	83.3	119	3.0

The above work was repeated on European oil also biologically assayed and the following results were obtained, using first 0.2 cc. and then 0.4 cc. of the 30% solution of oil in chloroform.

TABLE II.—EUROPEAN COD LIVER OIL.

Oil mixture. % Cod liver oil.	% Peanut oil.	Vit. A potency U. S. P. units.	Color reading Lovibond blue units. 0.2 cc.	0.4 cc.
100	0	425 (biological assay)	7.6	15.2
66.7	33.3	283	5.8	11.5
50	50	213	4.0	8.0
33.3	66.7	142	3.4	6.7
16.7	83.3	71	1.9	3.7

When the above results were plotted (Fig. 1), two straight lines were obtained within the experimental limits of error, showing that the intensity of the reaction color is a linear function of the amount of oil present and its vitamine A potency.

It is evident that these two lines, which were obtained by using biologically assayed samples of cod liver oil, lie very close to each other and it might be that if a sufficiently great number of oils of both types were used a single line would be obtained. However, this fact must not be considered to indicate that American and European cod liver oils are of equal potency; Tables I and II indicate that the

particular samples of American and European oils which were used possessed vitamine A potencies of 715 and 425 U. S. P. units, respectively, as determined biologically.

Inspection of the above results shows that the blue color obtained with the samples produced by dilution of a standard sample of cod liver oil is directly proportional to the vitamine content of the diluted samples. Therefore, if the color test may be implicitly relied upon, one should be able to take a series of unknown samples and using either a known sample as a control or calculating the results from the above graph determine the potency of the unknowns. A series of thirteen oils which had been biologically assayed was selected and colorimetrically assayed using a standard sample as a control (the person making the test did not know the biological assay of the unknowns). The results are tabulated below.

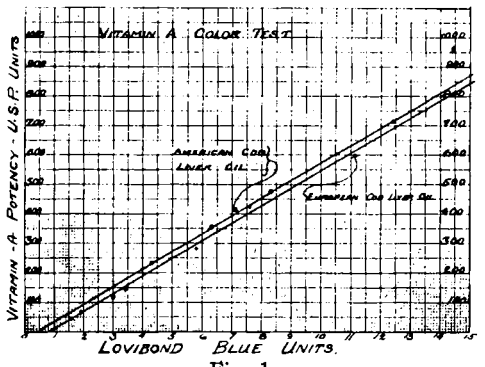

Fig. 1.

TABLE III.—BIOLOGICAL ASSAY VS. COLORIMETRIC ASSAY.

Sample no.	Source of oil.	Vit. A assay. Biological. U. S. P. units.	Vit. A assay. Colorimetric.
1	American (control)	667	667
2	American	715	750
3	American	1000	648
4	American	832	2335
5	American	770	935
6	American	715	365
7	American	770	1780
8	American	715	825
9	American	588	890
10	American	667	4475
11	American	832	1000
12	European	450	400
13	European	450	442
14	European	450	488

These results show that only five of the thirteen unknowns yielded colorimetric assays within 110 U. S. P. (2 to 15%) units of the biological assay; of the remaining eight, all oils except 2 (Nos. 3 and 6) gave colorimetric values which exceeded the biological assay by from 20 to 600%.

TABLE IV.

Sample no.	Source of oil.	Vit. A assay. Biological.	Vit. A assay. Colorimetric.
1	American	833	1700
2	American	770	842
3	American	562	750
4	Japanese	833	1000

A second set of four unknowns was selected and tested by an operator different from the one who tested those recorded in Table III. In this case, instead of using a control oil the results were read from the lines given in Fig. I using the experimental values found for the blue color as expressed in Lovibond Units. These results are given in Table IV.

In this case also it will be noted that the difference between the results of the two tests varies from a small difference (9%) in oil No. 2 to a large difference (100%) in oil No. 1. From the work reported on these two sets of unknowns one must conclude that the color test for vitamine A in cod liver oil has its limitations; until we have certain definite knowledge of the constituents of the oil which are related to the development of the blue color and the manner in which these substances are affected qualitatively and quantitatively by the conditions under which the oil has existed we will not be able to apply this test successfully.

In considering the results tabulated in Tables III and IV it must be stated that the biological and colorimetric assays were not made simultaneously but that a period of 6–9 months elapsed between the two tests; the biological assays being made first in all cases except those of Sample 11 in Table III and Sample 4 in Table IV. The oils varied considerably (2 months to several years) in age in so far as the time elapsing between the data of manufacture and that at which the biological assay was made is concerned. In all cases except one these oils had been under anaerobic conditions; oil No. 10 in Table III had been exposed to air during a portion of the time whereas the companion Sample No. 4 was under anaerobic conditions all the time.

Among the factors which influence the intensity of the blue color and the extent to which this color is indicative of vitamine A potency the following are possibly of importance: the source, age, manner of production and storage.

During our future studies we intend to give consideration to these factors.

REFERENCES.

(1) Carr & Price, *Biochem. J.*, 20 (1926), 497.
(2) Wokes & Willimott, *Analyst*, 52 (1927), 515.

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Irradiated Ergosterol. Effect on blood. Source of increase in serum calcium induced by Irradiated Ergosterol. A. F. Hess, M. Weinstock, and H. Rivkin—*Proc. Soc. Exptl. Biol. Med.*, 28 (1928), 199.—Through *Squibb Abstract Bulletin.*

The question was investigated whether the calcium in hypercalcemia produced in normal infants and animals by ingestion of irradiated ergosterol is taken from the bones and other tissues, or is the result of increased absorption from the intenstine. When large amounts of irradiated ergosterol, *i. e.*, 1 mg. daily, were fed to young rats in whom calcium depletion of the blood was effected by the ration including 0.8 mg. Ca and 400 mg. P a day (Ca:P 1:500), it was found that without exception the calcium could be increased rapidly 50 per cent or more. The high phosphorus content of the diet interfered markedly with the absorption of calcium. The results indicate that when the diet contains almost no calcium, the calcium which is supplied to the blood on giving irradiated ergosterol is derived from the tissues.—J. P.