VITAMIN A COLORIMETRIC AND BIOLOGICAL ASSAY.

BY EDWIN C. WISE AND FREDERICK W. HEYL.

The great length of time required for an accurate estimate of the vitamin A content of a material by the biological method is a most serious detriment to its usefulness. For example, the purchase of oils in commercial quantities on the basis of numerous samples submitted, studies of deterioration rates or the fractionation of vitamin A bearing materials, all become impractical if biological methods must be depended upon as a guide. Since the proposal by Rosenheim and Drummond (1) of the antimony trichloride test as a method of measuring vitamin A, much contradictory evidence has appeared as to the value of this test. More recently, Smith and Hazley (2) have shown that the unsaponifiable fraction of cod liver oils gives with antimony trichloride in chloroform a blue color proportional to its concentration; and Coward, *et al.* (3) have shown that the blue value of the unsaponifiable fraction of cod liver oil agrees closely with its vitamin A content determined biologically. The questioned reliability of the antimony trichloride test has no doubt been due, at least in part, to the application of the color test to the oil itself rather than to the unsaponifiable fraction.

During the past two years we have used the colorimetric test in conjunction with biological assays on a number of cod liver oils and vitamin A concentrates prepared from various liver fats. Our experience with fresh oils of known history has been very satisfactory and we have found the closest agreement between the color value and the biological values of the various materials assayed.

EXPERIMENTAL.

Colorimetric Assay.—The antimony trichloride solution was prepared in pure, alcohol-free chloroform, saturated at about 2° by allowing the solution to come to equilibrium in a bath of ice and water, the reagent being maintained at this temperature during the period of use. This procedure serves the twofold purpose of controlling both the concentration of the antimony trichloride reagent and the initial temperature of the reaction mixture (4). This procedure not only avoids two troublesome variables but decreases the rate of fading of the blue color. The material to be assayed is dissolved in pure chloroform and the concentration adjusted so that 0.2 cc., when mixed with 2.0 cc. of the antimony trichloride solution and observed through a depth of 1 cm., gives a blue color of 5 to 15 Lovibond units.

Color Measurement.—All color determinations were carried out with a Lovibond Tintometer (Tintometer, Ltd., England) which makes possible the accurate matching of the color produced both as to shade and brightness. The color is read 30 seconds after mixing the reagents and usually several trials are necessary before an exact match between the Lovibond glasses and the unknown is obtained. The tintometer is illuminated with a daylite lamp.

Biological Assay.—The biological assay used is similar to that in general use. Young rats weighing 40 Gm. are placed in individual cages and fed a vitamin A-free diet of corn starch 68 per cent, extracted casein 18 per cent, dried yeast 10 per cent and salt mixture 4 per cent, and receive vitamin D in the form of irradiated ergosterol in olive oil. In 35 to 40 days the rats begin to decline in weight and after

seven days of constant or declining weight the supplemental feeding of the material to be assayed is dropped directly into the mouths of the rats. The unit dose is considered to be the minimum daily amount which causes a gain of 2 to 4 Gm. per week for five weeks. At least 10 rats are placed on each level of dosage.

ASSAY OF COD LIVER OILS.

The antimony trichloride test is most often applied directly to cod liver oils, the usual procedure being to mix 0.2 cc. of a 20 per cent solution of cod liver oil (40 mg.) with 2 cc. of antimony trichloride reagent. In Fig. 1 are shown the dilution curves for two medicinal cod liver oils (Oil A—Oil B) from 0.0 to 40 mg. plotted from the data in Table I. At very low color values, one to three blue units, the curves are approximately linear, but it is these low color values which are most difficult to read even after considerable experience. At higher concentrations of oil, 40 mg., the dilution curves tend to flatten out so that a relatively large change in concentration of the oil makes little difference in the color value. The ratio of the blue values of the two oils differs with every change in the concentration of cod liver oil in the test solution which makes it necessary to select arbitrarily some level at which comparisons are made. These various difficulties detract considerably from the usefulness of the antimony trichloride test when applied directly to cod liver oils.

Fig. 1.

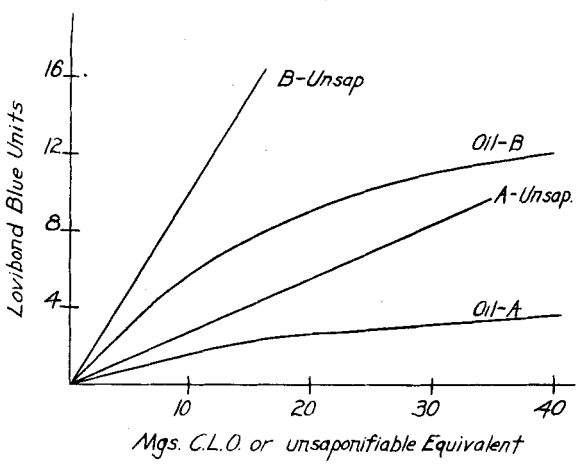

Fig. 2

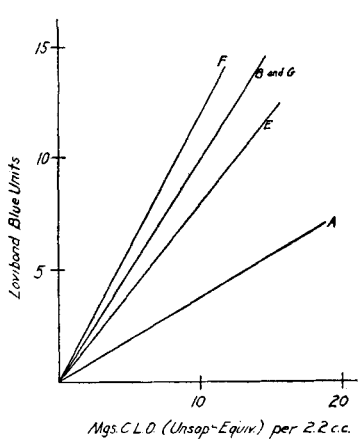

TABLE I.—BLUE VALUE.

Mg. in 0.2 Cc. Test Solution.	C. L. O. A.	C. L. O. B.
2.50	...	1.5
5.00	...	2.8
10.00	1.5	5.3
16.00	...	...
20.00	2.4	9.0
32.00	...	...
40.00	3.7	12.0

By the method of Smith and Hazley (2), it is possible to extract the unsaponifiable portion of small quantities of cod liver oil with no more effort than is required

to determine a dilution curve for the oil itself. This unsaponifiable extract gives color values which are linear and can be determined at any concentration at which it is possible to read the colorimeter, or the color value determined at any given concentration may be readily calculated to any other level.

In Fig. 1 are shown the dilution curves of the unsaponifiable extracts of the two oils A and B. Besides having the advantage of being linear the depth of color produced by a given quantity of unsaponifiable material is about twice as great as that produced by an equivalent amount of oil. This contributes appreciably to the sensitivity of the color assay. For example, 10.0 mg. of oil B gives a color value of 5.3 while the unsaponifiable extract equivalent to 10.0 mg. of oil gives a color value of 10.0 units.

In Table II are given the colorimetric and biological data for seven samples of medicinal cod liver oil. The color values have been obtained by saponification of 2-cc. samples of the oil and subsequent extraction of the unsaponifiable portion according to the method of Smith and Hazley. The unsaponifiable extract in chloroform was concentrated to 20 or 25 cc. and 0.2 cc. of this solution mixed with 2.0 cc. of the antimony trichloride reagent in a 1.0-cm. glass cell. Readings were made 30 seconds after mixing and the color value in Lovibond blue units calculated for 1.0 Gm. of the oil. Dilution curves are shown in Fig. 2. The color value in blue units per Gm. correspond very closely to the biological units calculated from the daily dose necessary to give a gain in weight of 3.0 Gm. per week for five weeks.

TABLE II.

Sample.	Color Units per Gram.	Dose Fed in Biological Assay Mg.	Average Weight Gain in Grams per Rat per Week.	Biological Units per Gram Calculated from Daily Dose.
A	275	4.0	2.9	250
B	1000	1.0	3.0	1000
C	1160	0.8	2.0	1250
D	980	1.0	3.1	1000
E	800	1.2	3.8	833
F	1200	0.8	3.1	1250
G	1000	1.0	2.5	1000

In a large number of medicinal cod liver oils from various sources we have found that the color value, determined as described above, is a reliable index of biological activity.

Several cod liver oil concentrates as well as concentrates from other liver fats have been assayed both colorimetrically and biologically with good agreement between the two methods.

SUMMARY.

Seven cod liver oils have been assayed colorimetrically and biologically. The blue color obtained by treating the unsaponifiable fractions of cod liver oils with antimony trichloride is a reliable index of their biological activity. Four of the seven oils were from different sources but all were from fresh stocks of medicinal oil. The validity of the color to vitamin relationship can be established only by numerous assays conducted by both methods. The colorimetric test is most useful in facilitating animal assays by indicating the proper dosage level.

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THE VITAMIN POTENCY OF CERTAIN LOFOTEN (NORWEGIAN) COD LIVER OILS.*

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The geographical source of cod liver oil does not unfailingly determine the vitamin potency or other important characteristics of cod liver oil, since variations in fishing, refining, storage, handling, etc., also contribute to the character of the finished product. It is as necessary to exercise discrimination in the selection of cod liver oil as in selecting any other commodity. This is particularly true in the case of cod liver oil in view of the decided variation in potency and chemical and physical characteristics of commercial supplies of this oil.

The following table is a contribution to the literature upon the subject of the vitamin potency of certain cod liver oils produced in the Lofoten Islands area of Norway:

Cod Liver Oil, Lot.	Vitamin "A" (Units per Gram) Not Less Than	Vitamin "D" (Units per Gram) Not Less Than
1	1000	108
2	1000	181
3	1000	111
4	1000	111
5	800	108
6	1000	156
7	1000	108
8	1000	108
9	1000	181
10	1000	181
11	1000	155
12	1000	155
13	1000	271
14	1000	181
15	800	155
16	1000	155
17	1000	155
18	1200	108
19	1200	155
20	1000	155
21	1000	155
22	1000	155
23	1200	155

* Scientific Section, A. PH. A., Toronto meeting, 1932.