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A Fixed Color Standard for Cholesterol Determinations.

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Schoenheimer and Sperry[1] described a micro method for free and ester cholesterol in serum, which. with the use of a photometer, gives highly accurate results. For clinical and many physiological purposes the use of a colorimeter is to be preferred. The only difficulty in applying the ordinary micro-colorimeter to the Schoenheimer and Sperry method for serum-cholesterol determinations has been the necessity for developing the color on several standard solutions of cholesterol for each series of determinations. With the kind cooperation of Dr. Schoenheimer and Dr. Sperry the problem of eliminating this obstacle was undertaken.

No changes were made in the original procedure up to the point of color development. Since it proved difficult to measure the small quantities of concentrated sulfuric acid accurately, it was found convenient to mix the acetic anhydride and sulfuric acid together. Using acetic anhydride and sulfuric acid in proportion of 15:1, it was found that with a 2 cc. sample of mixed reagent, the color reached a maximum at 27 minutes and remained constant for 10 minutes. This mixed reagent is stable for one hour only.

In seeking a fixed color standard, several substances were tried. It was found that only India Ink and Carter's Midnight Black Ink gave a true visual match. Copper sulfate and naphthol Green B were found unsatisfactory because of inexact matching. India Ink was discarded because the diluted suspension settled out on standing.

The standard solution of Carter's Ink is prepared by diluting 1 cc. to a liter with 10% acetic acid. Setting solutions of known concentration of the ink at 20 mm. on one side of the colorimeter,* and moving the standard, it was found that the length of column of the standard required to match the other solution was proportional to the concentration, thus showing that Beer's law was obeyed for a wide range of concentrations. This ink solution is standard-

[1] Schoenheimer, R., and Sperry, W. M., *J. Biol. Chem.*, 1934, **106**, 745.

* A Bausch and Lomb, Dubosecq Colorimeter (60 mm.), with microcups, plungers, eye-cap diaphragm, and an Eastman Kodak No. 71A color filter in the eyepiece, was used.

ized daily against 1 cc. of a 0.0075% solution of cholesterol in glacial acetic acid.

The procedure followed is practically identical with the original one. The dried precipitate of digitonide is dissolved in 1 cc. of glacial acetic acid at 70°C. and then cooled in a water bath at 25°C. Two cc. of the mixture of anhydride and sulfuric acid is added, the solution stirred and placed in a water bath at 25° in the dark for 29 minutes. The solution is then pipetted into the cup of the micro colorimeter and set at 20 mm. The reading is obtained by matching the standard ink solution against this setting. This concentration of ink standard provides a sufficient range of intensities to cover values from less than 30 mg. free cholesterol per 100 cc. up to more than 500 mg. total cholesterol per 100 cc., by setting the unknown solution at appropriate levels.

With this procedure it has been found possible to determine known solutions of free and ester cholesterol in acetone alcohol within an average accuracy of about 4%. Only once an error of 11% was encountered when as little as 0.024 mg. of cholesterol was determined.

This method has also proved satisfactory in determining serum cholesterol. Analyses in Table I give an idea of the precision to be expected.

TABLE I.

Vol. of Serum cc.	Vol. Extract cc.	Free Chol. in 100 cc. Serum mg.	Deviations mg.	Total Chol. in 100 cc. Serum mg.	Deviations mg.
0.2	5	75	1.1	260	9
0.2	5	75.9	0.2	265	5
0.5	10	80.6	4.5	280	11
0.5	10	76.1	0	268	1
0.5	10	72.7	3.4	274	5
		Av. 76.1	1.8	Av. 269	6.2
			equivalent to 2.3%		equivalent to 2.2%

Summary. A fixed color standard for use with Schoenheimer's and Sperry's micro method for serum cholesterol is described. The use of a mixture of acetic anhydride and sulfuric acid is suggested. The method has been applied to the analysis of known solutions of cholesterol and its esters and to the determination of serum cholesterol and cholesterol esters with satisfactory results.