

points in the molecule. To settle this question, one sample of periodate-oxidized wheat starch (1 C=O per 18 A.G.U.s) and one of periodate-oxidized waxy maize starch (1 C=O per 22 A.G.U.s) were pasted in hot water, cooled, an equal volume of carbonate-bicarbonate buffer (pH=9.5) added, making the final concentrations 1 and 2% starch, respectively. Both solutions were then put in a thermostatic bath at 27° C. for 2 weeks, and their intrinsic viscosities periodically measured; the starch concentrations were measured at the same time to correct for any retrogradation effects. Parallel experiments in the absence of buffer (pH = 6.5) were also run. No attempt was made to exclude atmospheric oxygen. The corrected intrinsic viscosities of all starch solutions, both buffered and unbuffered, remained unchanged throughout the entire 2 weeks' period. It may thus be presumed that no degradation had taken place.

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Sugar	Introduced Mg. (Fehling)	Recovered Mg.	Recovery, Per Cent	No. of Days Required to Reach Equilibrium
Glucose	38.5	38.9	101.0	7
Fructose	40.0	40.3	100.7	2
Mannose ^a	40.0	41.8	104.5	3
Arabinose	40.1	40.5	101.0	2
Xylose	39.8	41.1	103.3	8
Cellobiose	76.1	77.3	101.6	11
Rhamnose ^a	40.2	39.0	97.0	3

^a Amounts of all sugars introduced were triple checked by comparing amounts weighed out with corresponding values obtained by Fehling titration and hypiodite titration for aldoses (?). All three values were in agreement to within 1 to 2% except for mannose and rhamnose, where for some reason the hypiodite results alone were low.

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A Stable Reagent for the Liebermann-Burchard Reaction

Application to Rapid Serum Cholesterol Determination

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► Measurement of total serum cholesterol is a valuable test in the study of lipide metabolism. It requires a stable reagent for cholesterol color development and a simple procedure for the routine determination. A reagent for the Liebermann-Burchard reaction is described, which is stable for 2 weeks at room temperature and 4 weeks or longer under refrigeration, in contrast to 24 hours' stability of the ordinary acetic anhydride-sulfuric acid reagent. This reagent is also useful for rapid serum cholesterol determination. Only one reagent and one step are required.

THE current methods of choice for the determination of serum cholesterol are based upon the Liebermann-

Burchard reaction (3, 8). However, the acetic anhydride-sulfuric acid mixture for the final color development is not stable. Most methods, such as those of Schoenheimer and Sperry (10) and Bloor (2), require many steps for precipitation, washing, and dissolving. Pearson, Stern, and McGavaek (9) simplified the serum cholesterol assay by using excess heat generated during the addition of sulfuric acid to dissolve the precipitate from the serum. That method still required *p*-toluenesulfonic acid and many steps for an ordinary assay. Currently there are reports of use of two reagents for rapid serum cholesterol determination. However, the final color remains stable for only a minute or two, and the results average 10% or more higher than the conventional method (4, 6). Our experi-

ment was directed to finding a single reagent which would be stable for the Liebermann-Burchard reaction and also useful for routine serum cholesterol determination.

METHOD

Reagents. Acetic Anhydride-Sulfuric Acid Mixture. A mixture containing 30% glacial acetic acid, 60% acetic anhydride, and 10% sulfuric acid by volume was prepared in ice water according to the directions of Schoenheimer and Sperry (10). Anhydrous sodium sulfate was added to the mixture to a concentration of 2%. Cholesterol Standard (2 mg. per ml.), 200 mg. of cholesterol (alcohol) dissolved in 100 ml. of acetic acid.

Procedure for Color Development. To each of three 25-ml. Erlenmeyer flasks (or 15 × 125 mm. screw-cap

test tubes) labeled as blank, unknown, and standard, add 5 ml. of the acetic anhydride-sulfuric acid mixture. Then add 0.2 ml. of distilled water, 0.2 ml. of untreated unknown serum, and 0.2 ml. of standard slowly (3 to 5 seconds) along the edges of the corresponding flasks. Mix by gentle swirling and let stand at room temperature or in a water bath (21° to 25° C.) for 20 minutes. The precipitate undissolved after mixing will be dissolved on standing. Read at 550 or 610 m μ on a Beckman or Coleman spectrophotometer and calculate the unknown according to Beer's law.

EXPERIMENTAL RESULTS AND DISCUSSIONS

Conditions for Reagent Preparation. An attempt to preserve the reagent under partial vacuum was not effective. A mixture of lower than 10% sulfuric acid stayed colorless for months but lost its activity as the 3.3% level in the ordinary method. Concentrations of sulfuric acid over 10% discolored quickly and resulted in a violent reaction during color development. A high level of 10% but without sodium sulfate was less stable and resulted in an unstable final color which was obvious to the naked eye. Two per cent anhydrous sodium sulfate in the reagent was the optimum amount. This reagent was stable more than 2 weeks at room temperature. A brownish color in the solution indicated reagent deterioration and it is advisable to refrigerate the solution. We removed the reagent from cool storage an

Table I. Comparison of Absorbances by Schoenheimer and Sperry's and Our Method

(Average of 5 determinations)

Cholesterol, Mg. %	Wave Length			
	550 M μ		610 M μ	
	Schoenheimer's method	Present method	Schoenheimer's method	Present method
0	0	0	0	0
80 ^a	0.06	0.07	0.11	0.12
160 ^a	0.12	0.14	0.22	0.23
240 ^a	0.18	0.20	0.33	0.35
320	0.24	0.25	0.45	0.45

^a Significant difference between two methods at these levels according to group comparison of Snedecor (11).

Table II. Comparison of Cholesterol Determinations by Pearson's and Our Method in Routine Laboratory Work

Sample	Number Comparison	Pearson's Method, Mg. %	Present Method, Mg. %
Control	34	215.2 \pm 2	214.8 \pm 0.8
Unknown	58	238.6 \pm 9.4	246.9 \pm 9.4 ^a

^a Serum blank used in Pearson's work, but not in ours.

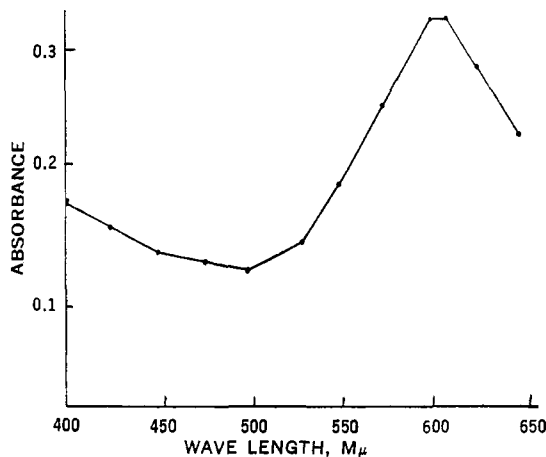


Figure 1. Cholesterol absorbance at different wave lengths

0.4 mg. of cholesterol
0.2-ml. standard

hour before using and refrigerated afterward. No indication of decreasing activity in the reagent was observed after more than 4 weeks. Acetic acid was a better solvent than chloroform in our method.

Choice of Wave Length. According to Hawk, Oser, and Summerson (5), the maximum sensitivity is not the chief consideration in the selection of the proper wave length for analytical work; hence a wave length which gave the widest range between unknowns was chosen. The present method had the widest absorbance range at 550 and 610 m μ (Figure 1). We preferred 550 rather than 610 m μ for routine work because 610 required a wider slit width and there was more interference.

Interferences. Because free hemoglobin and bilirubin are the common color substances in serum, they were tested for their interference in cholesterol determination. Free hemoglobin was prepared according to Block, Durrum, and Zweig (1). C.P. Bilirubin (Nutritional Biochemical Corp., Cleveland 28, Ohio) was used for the test. In our trial we observed that 50 mg. % hemoglobin gave no reading at 550 m μ and less than 0.01 at 610 m μ . The 50 mg. % level of hemoglobin is 5 and 10 times higher than the normal serum content. Therefore, hemoglobin caused little interference under ordinary conditions. Nevertheless, precaution should be used with hemolyzed blood.

Bilirubin interfered at 550 and 610 m μ . However, no reading was obtained at the normal level of 0.05 mg. %. A reading of 0.01 was obtained if the level was raised to 5.0 mg., 10 times higher than the normal value. This could be corrected easily by subtracting a serum blank prepared by diluting 0.2 ml. of unknown serum with 5 ml. of acetic acid (95%). The requirement for such a serum blank correction is not clinically significant in routine work, because a reading of 0.01 will give 5 to 8 mg. % of cholesterol in the normal cholesterol curve between 150 and 250 mg. of cholesterol.

Comparison with Other Methods. SCHOENHEIMER AND SPERRY. C.P. Cholesterol was used for this comparison. The absorbances of the various concentrations of cholesterol by the Schoenheimer and Sperry and the present method are shown in Table I. The absorbance from the present mixture gave a slightly higher reading than that of Schoenheimer and Sperry. This means that the present reagent resulted in a larger difference between the unknowns and would be a better solution.

The color development from the present method reached its maximum within 20 minutes and was stable 20 minutes thereafter, while the conventional method needs at least 30 minutes to develop and was stable only 10 minutes. Another advantage of the method was the less critical temperature fluctuation. There was no difference in readings between 21° and 25° C. in our method.

PEARSON *et al.* The two methods are compared in Table II. The total serum cholesterol of the control from the two methods was similar. The unknown readings in Pearson's method were slightly lower than those of our method.

However, Fisher's test did not give any significant difference (11).

LAWRY *et al.* (7). The results of total serum cholesterol by the present method were also compared with those of Lawry *et al.* Venous blood from 300 normal persons of different ages and sexes was studied. To avoid personal deviation, one technician was responsible for all determinations. The results obtained by the present method were similar to those of Lawry *et al.* (Table III). The data for men between 50 and 59 and women between 60 and 69 were higher than those of Lawry *et al.*, probably because of the sampling difference. Males consistently had higher values than females until the sixth decade. The level rose with age in both sexes. The male reached the maximum at 50 to 60, but the female continued to rise.

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Age, Years	No. of Subjects	(Mg. %)		Lawry's Data	
		Our Results		M	F
		M	F		
Day old	13	91 ± 10			
1-9	13		73 ± 10		
	4	180 ± 23			
	4		120 ± 27		
10-19	6	150 ± 49			
	5		147 ± 33		
20-29	13	211 ± 49		212	
	19		201 ± 41		198
30-39	14	236 ± 62		230	
	30		222 ± 51		290
40-49	15	236 ± 50		240	
	28		230 ± 44		225
50-59	11	273 ± 55		244	
	22		247 ± 19		252
60-69	17	237 ± 59		235	
	10		335 ± 22		263
83	1	135			

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Spectrophotometric Determination of Several Bile Acids as Conjugates

Extraction with Ethyl Acetate

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► Because of the recent evidence indicating a radical change in the state of conjugation of bile acids in liver disease as well as the continued interest in these compounds because they are the main cholesterol metabolites, the determination of the bile acids has assumed great importance. Most analytical methods currently used are not suitable because they require a hydrolysis step. The process causes gross losses and makes it impossible to obtain information concerning the state of conjugation. This paper presents a procedure for the rapid separation of the glycine and taurine conjugates of cholic and deoxycholic acids, and their spectrophotometric determination. Representative specimens of animal and human gall bladder bile have been analyzed and values for the four conjugates are presented.

THE bile acids, 24-carbon carboxylic acids of the cholane series, are found conjugated with glycine and taurine in the bile of most vertebrates and all mammals (6). The commonly occurring bile acids are the conjugates of cholic (3,7,12-trihydroxy-), deoxycholic (3,12-dihydroxy-), and chenodeoxycholic acids (3,7-dihydroxycholanic acid). Recent studies have shown a drastic change in the proportions of conjugates in bile from patients suffering from liver and biliary disease (15).

Most procedures for the estimation of these compounds in bile have in common a hydrolysis step. This has been necessary because of the difficulties involved in the extraction and subsequent separation of the conjugates owing to the great hydrophilic properties of these compounds. It has been repeatedly recog-

nized that hydrolysis caused a structural alteration of the bile acid molecule, which results in large errors in the subsequent spectrophotometric results (1, 9). The hydrolytic step, however, does obviate the exacting and tedious countercurrent (1) and chromatographic (13) methods, which are unsuitable for rapid clinical procedures.

This paper describes a relatively rapid process for the separation and subsequent spectrophotometric estimation of the conjugates of cholic and deoxycholic acids in bile.

METHODS AND MATERIALS

Purification and Separation of Conjugates. REAGENTS. Reagent grade absolute ethyl alcohol, ether, hexane,

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