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Determination of Small Amounts of Total Cholesterol by the Tschugaeff Reaction with a Note on the Determination of Lathosterol

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A simple sensitive method for the determination of cholesterol based on Tschugaeff's color reaction is described.

No color is obtained with dihydrocholesterol or epicholestanol. Coprosterol gave a faint reaction (absorbance about 1/10 of that of cholesterol).

Amounts of cholesterol as low as 0.004 mg per ml reaction mixture can be determined with an accuracy of about 3 %.

Δ-7-cholestenol (lathosterol) gives a yellow color reaction under the same conditions as cholesterol with absorbance maximum of 395 mμ and almost no absorbance at 528 mμ where cholesterol yields maximum absorbance.

The Tschugaeff reaction (with acetyl chloride and zinc chloride) [1] is the obvious means to use for the determination of small amounts of cholesterol in biological material, since it is much more sensitive than the Liebermann-Burchard reaction used by most investigators. The present method is a modification (and simplification) of the one originally described by Görtz [2,3] for use with blood.

EXPERIMENTAL

Reagents.

Aqueous KOH solution 33 %.
Petroleum ether, b. p. below 50° C.
Ethanol, absolute
Sodium sulfate, anhydrous, analytical grade.
Glacial acetic acid » » *
Acetyl chloride » » *
Chloroform » » *
Zinc chloride, anhydrous » » (sticks).

* These reagents must be miscible with each other without turbidity.

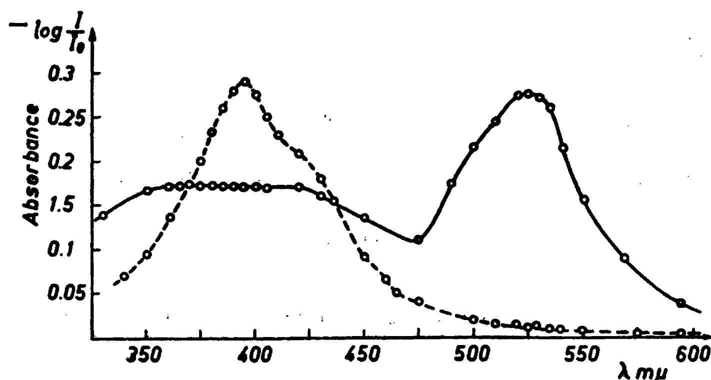

Fig. 1. Absorption curves. O——O *cholesterol, 0.0108 mg per ml reaction mixture.* O– – – –O *lathosterol, 0.0031 mg per ml reaction mixture.*

Zinc chloride solution in glacial acetic acid was prepared as follows.

40 g anhydrous $ZnCl_2$ (sticks, analytical grade) were quickly crushed and weighed in a dry 250 ml bottle and mixed with 153 ml glacial acetic acid. The bottle was shaken, stoppered and kept at 80° C for 2 ½ hours with occasional shaking. After cooling to room temperature the solution was filtered by suction through a glass filter.

The $ZnCl_2$-reagent was kept in a dark bottle with a tight glass stopper and could be used until it took up sufficient moisture to become turbid when shaken with chloroform. We were able to use the reagent at least 3 months.

Procedure. A. Extraction. The sample, *e. g.* 200—300 microliter bladder bile (or 2—3 ml fistula bile) or 0.5 g liver was placed in a 25 ml Erlenmeyer flask, then 33 % KOH and alcohol were added to make the mixture 17 % with respect to KOH, and 50 % with respect to C_2H_5OH. The mixture was heated on a steam bath either with the flask sealed with a glass ball, or, when the amount of cholesterol was particularly small, in a nitrogen atmosphere. In case of bile the time of heating was about ½ hour, tissue was heated 2 to 3 hours according to the fat content and time required for homogenization.

After cooling to room temperature each sample was extracted three times with 5 ml portions of petroleum ether (b. p. below 50° C). The extracts were combined and washed with water until neutrality to lithmus. The combined extract was now dried over anhydrous Na_2SO_4, and filtered by suction, the sodium sulfate being washed with petroleum ether. The anhydrous solution was evaporated *in vacuo* at 40—50° C, and the residue transferred with chloroform into a 10 ml test tube. The main part of the chloroform was then evaporated on the steam bath to give a final volume of about 2 ml.

B. Color reaction. To each of the above tubes, containing about 2 ml chloroform solution, was added 1 ml of the $ZnCl_2$-reagent and 1 ml of acetyl chloride. The addition of acetyl chloride was most conveniently done with a burette. The contents of the tubes were then mixed with a glass rod, and the tubes were placed in a water bath at precisely 65° C for exactly 15 minutes. Thereafter the tubes were cooled in ice water and the reaction mixture was transferred to a 5 ml volumetric flask; the tubes were washed several times with small amounts of chloroform to bring the volume in the flask up to the 5 ml mark. The eosin-red solution was then placed in a 10 mm cuvette and the light absorbance (density, $d = -\log \frac{I}{I_0}$) was measured within 30 minutes at 528 mμ in a Beckman spectrophotometer. The same reaction was carried out with 2 ml of a standard solution of cholesterol in chloroform (*e. g.* 0.02 mg/ml) and with 2 ml chloroform as a blank.

Because of the sensitivity of the reaction to variations in the permissible low concentration of water, it is advisable to use a standard with cholesterol for each series of determinations.

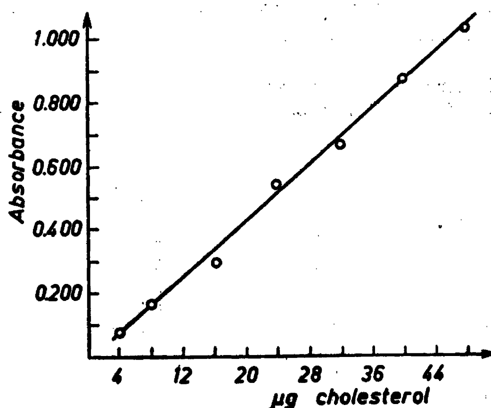

Fig. 2. Absorbance of cholesterol at 528 mµ after reaction with $ZnCl_2$-AcCl.

DISCUSSION OF THE METHOD

Development of the color at 65° C in 15 minutes was found more satisfactory than at 70° C in 12 minutes (as by Görtz [3]) or at 50° C in 20 minutes.

The maximal light absorbance was at 528 mµ. Fig. 1 shows the light absorbance curve found for 0.0108 mg cholesterol per ml reaction mixture and the corresponding curve for 0.0031 mg lathosterol. The developed color follows Beer's law in the concentration range from 0.004 to 0.048 mg cholesterol per ml reaction mixture as shown in Fig. 2.

We found that the proportion of the reagents mentioned gave the greatest color stability. The stability of the color is shown in Fig. 3 *.

Table 1 shows the results obtained by the present method and by the method of Sperry and Webb [4] as applied to hamster liver. The extraction of samples was carried out as described. The same extracts were used for both

Table 1. Comparison of the Sperry-Webb method with the $ZnCl_2$-AcCl-method.

mg cholesterol/ml extract	
Method of Sperry and Webb	Our method
0.0172	0.0188
0.0387	0.0382
0.0767	0.0758
0.0956	0.0963

* The authors thank Dr. S. Hartmann for making this curve by means of an automatically recording spectrophotometer.

Table 2. A series of routine cholesterol determinations with the $ZnCl_2$-AcCl-method.

Sample No.	Cholesterol mg per 5 ml	Light absorbance	Light absorbance per mg	Deviation from mean of single determination %
1	0.060	0.263	4.383	− 2.72
2	0.060	0.265	4.417	− 1.93
3	0.060	0.258	4.750	+ 5.22
4	0.024	0.107	4.458	− 0.99
5	0.024	0.110	4.583	+ 1.76
6	0.024	0.103	4.292	− 4.89
7	0.022	0.097	4.409	− 1.98
8	0.022	0.098	4.455	− 1.05
9	0.022	0.105	4.773	+ 5.68
10	0.018	0.078	4.333	− 3.89
11	0.018	0.083	4.611	+ 2.37
12	0.018	0.082	4.556	+ 1.18
		average	4.502	

methods, but with our procedure we used aliquots only one-tenths as large as those used for the Sperry-Webb determinations.

At the same occasion it was shown that the specific light absorbance at our method was 8 times that of the Liebermann-Burchard reaction used in the Sperry-Webb method.

ACCURACY OF THE METHOD

From a standard solution of cholesterol in chloroform different known aliquots were taken and the described procedure was carried out. The results are shown in Table 2.

The mean square error of the single measurement is $\sqrt{\frac{\Delta^2}{n-1}} = \pm 0.155$.

The mean square error of the average of the measurements is $\sqrt{\frac{\Delta^2}{n(n-1)}} = \pm 0.044$.

SPECIFICITY OF THE REACTION

In Table 3 are given the absorbances obtained with cholesterol, some cholesterol esters and some other sterols.

Dihydrocholesterol and epi-cholestanol gave no color.

Mixtures of cholesterol and dihydrocholesterol in varying proportions gave absorbances proportional to the amount of cholesterol.

Two samples of coprosterol prepared from different materials gave a faint reaction with absorption maximum at the same wave length as cholesterol but with absorbance of about 1/10 of that of cholesterol.

Mixtures of cholesterol and coprosterol in varying proportions gave absorbances lying on a straight line between the absorbances of the two substances.

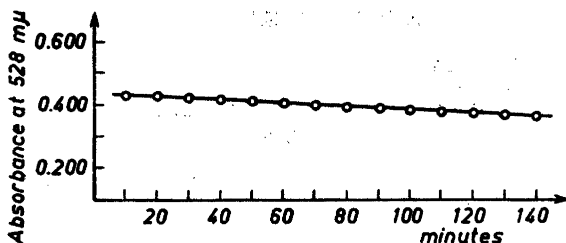

Fig. 3. The effect of time on the color stability of the reaction at 20° C. (Determined by means of a recording spectrophotometer).

The following bile acids: cholic, desoxycholic, chenodesoxycholic, glycocholic and taurocholic acid gave a yellowish-brown color reaction; the maximal absorbance of these acids was in the interval 375—450 mμ.

Δ-7-cholestenol, "lathosterol", gave a yellow color with a maximal light absorbance at 395 mμ (Fig. 1) and almost no absorbance at 528 mμ. The absorbance at 395 mμ follows Beer's law at least in the concentration interval 0.003—0.028 mg lathosterol/ml of the final mixture. Mixtures of cholesterol and lathosterol gave absorbances lying on a straight line. The present method may be utilized for simultaneous determination of cholesterol and lathosterol.

Table 3. Absorbance obtained with cholesterol, cholesterol esters, and some other sterols.

Compound	Melting point °C found	Melting point °C in literature	mg/ml	Light absorbance at 528 mμ	Absorbance mmole/ml
Dihydrocholesterol *	141.5	140.5	0.0197	0.000	0
Epi-cholesterol *	182	183	0.0190	0.000	0
Coprostanol **	101—102	100—102	0.0197	0.054	1 066
Coprostanol ***	100—101	100.5	0.0197	0.047	930
Cholesterol ****	148.0	147	0.0197	0.462	9 593
Cholesterylacetate	115.3	114.5	0.0208	0.308	8 349
Cholesterylpalmitate	88.3	90	0.0179	0.254	8 868
Cholesterylbenzoate	145—146	145.5	0.0227	0.455	9 837
"Phytosterol" (Merck)	94—97	—	0.0237	0.315	—
Stigmasterol (Merck)	168.8	170	0.0100	0.338	13 949
Stigmasterol (La Roche)	167.9	170	0.0214	0.537	10 356
Δ-7-Cholestenol (lathosterol) *****	123—125	123—124.5	0.0288	(0.020)	(268)

* Gives no color reaction with $ZnCl_2$-AcCl in chloroform.
** Isolated from human feces, purified through the digitonide after treatment with bromine.
*** Isolated from dog feces.
**** Purified through the dibromide.
***** The authors thank Professor L. Fieser for furnishing the sample of lathosterol.

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