I. CHOLESTEROL AND CHOLESTEROL ESTERS IN DOG BILE

Quantitative Methods

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(Received for publication, December 28, 1933)

In connection with other studies made in this laboratory on the constituents of the bile, with new methods available, it seemed wise to re-examine the whole question of cholesterol elimination in the bile. Method difficulties have impaired the value of many previous studies of biliary cholesterol. Recently, Elman and Taussig (6) and Andrews and Hrdina (1) have described methods of great similarity which are accurate for the quantitative analysis of biliary cholesterol.

The need of another method grew out of a series of experiments in which it was desired to determine the occurrence of *esters* of cholesterol in bile. In the methods (1, 6) mentioned, treatment with alkali is utilized to "fix" the bile pigments, and as such treatment would cause hydrolysis of any combined lipoids, these methods could not be used in the determination of esterified cholesterol. The occurrence of cholesterol esters in bile is a point about which there are differences of opinion. Thannhauser (9) states that all cholesterol in human bile occurs in a free state due to the action of an ester-splitting enzyme, "cholesterolesterase," which sets free the esterified cholesterol. Thomas (10) is quoted to the effect that cholesterol occurs in dog bile as an ester.

A large colony of closed, sterile bile fistula dogs of the type described by Rous and McMaster (8) furnished ample material for investigation.

Methods

1. The colorimetric method used for the determination of the total cholesterol in bile is that of Elman and Taussig (6) which is described in detail by them. At

the suggestion of Dr. W. R. Bloor we have used a red glass filter in the colorimeter when making these quantitations. This filter is of the type Corning No. 10, signal red (which transmits 96 per cent at 700 Å.u. and 0.0 per cent at 595 Å.u.). The filter affords the advantage of a much easier color match and consecutive readings on the same specimens check much more closely with the filter than they do without it. Also the filter eliminates the difficulty sometimes encountered when the extract is faintly brown. This method has served our need for determination of the total cholesterol in bile satisfactorily and duplicates checked within small limits.

2. For the determination of the *esters of cholesterol in bile* we have used a colorimetric method which is a modification of the well known Bloor (2) and Bloor and Knudson (5) methods for the determination of blood plasma cholesterol. This method as applied to bile, consists of running 5–10 cc. of bile (the volume of bile used depending upon the cholesterol content) slowly into 75 cc. of a mixture of 1 part ethyl ether and 3 parts of 95 per cent alcohol in a 100 cc. volumetric flask, shaking the flask vigorously at the same time to insure the formation of a fine precipitate. The mixture is then slowly brought to the boiling point, cooled, made up to 100 cc. with alcohol-ether mixture and filtered through a No. 2 Whatman filter paper. This extract keeps indefinitely if stoppered tightly and placed in the dark. Aliquots of the extract are taken, an excess of an alcoholic solution of digitonin added (2 cc. of a 0.50 per cent solution usually suffice) and the mixture is taken to dryness on the steam bath. The residue is extracted 3 times with petroleum ether (the fraction boiling off below 60°C.), using 20 cc. each time and boiling the solvent down to about 10 cc. The extract is then filtered with gentle suction through a sintered glass filter of the type marked "4G4 Schott and Gen. Jena." The filtrate is evaporated to dryness, extracted with chloroform and the color developed as in the method for total cholesterol. Pure cholesterol having a melting point of 145°C. is used as a standard. Two concentrations of standard are used, one containing 1.0 mg. per 5 cc., and the other 0.5 mg. per 5 cc. This method has been checked by adding esterified cholesterol in blood plasma to bile, with satisfactory recovery.

3. As a control over the above methods, the oxidative digitonin method of Okey (7) and Bloor (3) as modified by Yasuda (11) was used. We are grateful to Mr. P. L. MacLachlan of the Department of Biochemistry, who very kindly made the great majority of the digitonin determinations.

Comparison of the Colorimetric Method with the Oxidative Digitonin Method for Total Cholesterol in Bile

The results of these determinations are shown in Table 1. The colorimetric method gives results which run consistently 20 per cent higher than the digitonin method. These figures are in accord with those of Bloor (4) who has made a similar comparison of the colorimetric and digitonin methods as applied to blood plasma over a long

series of determinations and has expressed the opinion that the colorimetric values more closely approximate the true values. Our figures are at some variance with those of Elman and Taussig (6), who in a series of four determinations showed the colorimetric determinations to run sometimes greater and sometimes less than the digitonin. In our opinion, the variation in the digitonin results recorded by these workers can be largely, if not entirely, explained by the fact that the method of Okey (7) is not as uniformly accurate as it is in the form as modified by Yasuda (11).

TABLE 1

Comparison of Colorimetric and Oxidative Digitonin Methods Applied to Dog Bile

No.	Colorimetric	Digitonin	Colorimetric higher values than digitonin
	mg.	*mg.*	*per cent*
1	1.12	0.87	22
2	1.00	0.79	21
3	1.13	0.90	20
4	1.28	1.05	18
5	1.19	0.93	22
6	1.23	0.99	20
7	0.80	0.64	20
8	0.64	0.54	16
9	0.80	0.64	20
10	0.71	0.61	22
11	0.82	0.64	22
12	0.67	0.52	22
Average			20

Just why the colorimetric method should run so consistently 20 per cent higher than the digitonin method is rather perplexing. Determinations of pure cholesterol and cholesterol added to bile of known concentration yield by the colorimetric method about 99 per cent of the theoretical values, whereas the digitonin method yields about 7 per cent less than the theoretical. It is possible that sterols related to cholesterol occurring in bile exert an influence in the development of a color which is not evident in the oxidative determinations.

The Physical State of Cholesterol Occurring in Normal Dog Bile

A series of six duplicate determinations on samples of bile from several healthy closed bile fistula dogs were made by the colorimetric and oxidative digitonin methods. The total cholesterol content of these bile specimens ranged from 0.190 mg. to 0.895 mg. In no instance was there evidence of esterified cholesterol in amounts permitting quantitation. From these results it is evident that in dog bile, as in human bile, cholesterol occurs entirely in an uncombined form.

CONCLUSIONS

The colorimetric method for the determination of total cholesterol in dog bile is consistently accurate as checked by the oxidative digitonin method. This method has the further advantages of being simple, rapid and economical.

A method for the determination of esterified cholesterol in bile is described and it is shown that there are no esters of cholesterol in normal dog bile.

We are indebted to Professor W. R. Bloor and Mr. P. L. MacLachlan of the Department of Biochemistry for their helpful advice and assistance in this work.

BIBLIOGRAPHY

1. Andrews, E., and Hrdina, L., *Proc. Soc. Exp. Biol. and Med.*, 1932, **29**, 1102.
2. Bloor, W. R., *J. Biol. Chem.*, 1916, **24**, 227.
3. Bloor, W. R., *J. Biol. Chem.*, 1928, **77**, 53.
4. Bloor, W. R., personal communication to the author.
5. Bloor, W. R., and Knudson, A., *J. Biol. Chem.*, 1916, **27**, 107.
6. Elman, R., and Taussig, J. B., *J. Lab. and Clin. Med.*, 1933, **17**, 274.
7. Okey, R., *J. Biol. Chem.*, 1930, **88**, 367.
8. Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1923, **37**, 11.
9. Thannhauser, S. J., *Deutsch. Arch. klin. Med.*, 1922, **141**, 290.
10. Thomas, R., Inaugural dissertation, Strasbourg, J. H. E. Heitz, 1890, cited by McMaster, P. D., *J. Exp. Med.*, 1924, **40**, 33.
11. Yasuda, M., *J. Biol. Chem.*, 1931, **92**, 303.