V. STUDIES IN THE CHOLESTEROL CONTENT OF NORMAL HUMAN PLASMA

VII. AN IMPROVED MACROMETHOD FOR THE ESTIMATION OF CHOLESTEROL BY DIGITONIN

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THE methods in use for the estimation of cholesterol and its esters in the tissues are liable to many quite distinct sources of error. These errors may arise through faulty sampling, unsuitable methods of preparing the tissues for extraction, inadequate extraction owing to the adsorption of sterols on the proteins [Gardner & Gainsborough, 1927], incomplete hydrolysis of the esters [Gardner & Fox, 1924], incomplete extraction of the alkaline fluids after hydrolysis and lastly through defects inherent in the methods of estimation of the cholesterol in the extracts, for example, the slight but definite solubility of cholesterol digitonide in the solvents used.

Sperry & Schoenheimer [1935] claimed that oxalated plasma contained considerably smaller amounts of both total and free cholesterol per 100 ml. than either heparinized plasma or serum from the same sample of blood. The average difference between the amounts of total cholesterol in the oxalated and heparinized plasmas was 15.3%. We were much surprised at these results and made a series of duplicate analyses of blood plasma by our usual method [Gardner & Gainsborough, 1927], to determine the magnitude of the probable experimental error, and also to test the above findings of Sperry and Schoenheimer. Our investigations led to some modifications of the method we had previously described, and in this paper we give an account of these experiments and the reasons for the change.

Though many duplicate results obtained by our original method were satisfactory, there were cases in which estimations of the ester showed divergences. We think that these were due to cholesterol being washed away in the soapy alkaline solution obtained after saponification, in which it is known to be slightly soluble. Some illustrative results are shown in Table I.

Table I. *Duplicate analyses of plasma cholesterol.*

Sample	Plasma cholesterol, mg. per 100 g. (old method)			% total as ester
	Free	Ester	Total	
A.A. 1	57	164	221	74.2
	59	173	232	74.6
2	49	152	201	75.6
	49	134	183	73.2
3	51	136	187	72.7
	49	150	199	75.5

It will be noted that whereas the duplicate estimations of the free cholesterol agree to within 4 %, the duplicate estimations of the ester cholesterol differ by as much as 10 %, and that the latter difference is masked in the calculation of the percentage of the total as ester.

The saponification mixture is now rendered acid, and we extract the fatty acids and cholesterol immediately; it is important that no delay occur at this stage in order to avoid the re-esterification of the cholesterol which will take place in the presence of strong acid. We now generally obtain higher values for the ester fraction and a more constant relation between the free and ester fractions in normal subjects, as found by Sperry [1936].

This constant relation is, however, by no means a general rule; occasional cases are encountered in which the free cholesterol content of the plasma is extremely low. Such results are probably not due to error in the analyses as they have been confirmed in many cases by duplicate estimations. They have occurred in pathological conditions which, however, throw no light on their origin. The following might be quoted as examples:

Table II

Case	Disease	Plasma cholesterol, mg. per 100 g.			% total as ester	Method
		Free	Ester	Total		
E. C.	Gastric ulcer	6	155	161	96.3	New
		5	152	157	96.8	New
E. F.	Hyperpiesia	3	129	132	97.6	Old
		6	121	127	95.7	Old
A. P.	Carcinomatosis	6	185	191	96.9	New
		7	186	193	96.4	New

In one case an effort was made to trace the cause by observing the effect of dietetic variations, with the following uninformative results:

Table III. *Effect of dietetic variations on the plasma cholesterol of case B.C.*

Period days	Diet	Plasma cholesterol, mg. per 100 g. (old method)			% total as ester
		Free	Ester	Total	
	Normal hospital diet	8	125	133	94.0
		10	144	154	93.5
3	Very high carbo- hydrate, low fat	45	124	169	73.4
		48	123	171	71.9
5	Very high fat, low carbohydrate	45	160	205	78.0
		43	145	188	77.1
7	Normal hospital diet	26	156	182	85.7
		23	151	174	86.8
16	Normal hospital diet	30	98	128	76.6
		28	88	116	75.9

We note that Sperry [1937] found some marked deviations from normal, but apparently did not trust his findings (three analyses) and omitted them from his tables.

A further source of error was noted in the old procedure, for when the original alcohol-ether extract of the plasma was evaporated to dryness, the fatty residue was not stable, and further esterification of the free cholesterol occurred slowly on standing. In one case in which the residue stood at room temperature

it was found that 10 % of the free sterol was esterified in the first three days, and 28 % at the end of a week. We now take the obvious precaution of not allowing the extract to stand.

Furthermore, plasma for the estimation of cholesterol should not be allowed to stand for any period before analysis, as there may be an increase of the ester fraction at the expense of the free. Sperry [1935] finds a considerable degree of esterification to occur in incubation experiments at 37°, and though we can confirm this change, we found that only about 3 mg. of sterol per 100 g. of plasma are so changed, compared with the very much higher figures quoted by Sperry. We could detect this change in heparinized plasma and in serum, but not in oxalated plasma.

In order to avoid the above errors, we now use the following method, which has been in use in this laboratory with satisfactory results for nearly two years.

Method

5–10 g. of serum or plasma are run in a thin stream into a mixture of 180 ml. absolute alcohol and 60 ml. ether contained in a 500 ml. Erlenmeyer flask which is rotated briskly; the fluid is brought to the boil and after cooling is allowed to stand overnight. The supernatant liquid is filtered through an 11 cm. No. 41 Whatman filter paper and the proteins are transferred to the paper, pressed dry and returned to the flask; this protein is then extracted successively with about 50 ml. portions of boiling alcohol-ether mixture, ether, boiling alcohol and finally ether, and the combined filtrates are evaporated to dryness; the residue is immediately extracted with three successive portions of about 25 ml. absolute alcohol, the fluid being filtered through a small filter into a 150 ml. beaker. A solution of digitonin in absolute alcohol is then added in amount approximately 10 % in excess of the probable amount required; the solution is heated to boiling point and after cooling to room temperature water is added until a turbidity appears, as recommended by Fex [1920]. After standing overnight, the fluid is evaporated to dryness in the water bath and the nearly dry residue is extracted three times with 20 ml. portions of dry ether, each portion being filtered through a weighed Gooch crucible. These ether washings, combined with the ether washings of the small filter paper used above, are returned to the original extraction flask for the subsequent estimation of the ester. The residue remaining in the beaker is transferred to the crucible with boiling water and is washed with boiling water until the filtrate no longer froths on shaking. The crucible is dried in a water oven overnight and then in an air oven at 120° to constant weight; it is weighed in a closed weighing bottle (with another similar one on the opposite pan), as cholesterol digitonide is hygroscopic. The total ether washings are made up to about 100 ml., a considerable excess of sodium ethoxide in especially pure alcohol is added and the mixture is refluxed at about 40° on an electric heater overnight. Water is then added, the liquid is made acid to Congo red with strong HCl and saturated with NaCl in order to effect a good separation in the subsequent extraction. The fluid is then extracted with ether in a separating funnel, the acid liquor being transferred to a second funnel where two further extractions are made with ether, which is added to that in the first funnel. The acid layer is extracted once more with ether and is discarded. The main ether washings in the first funnel are shaken out with distilled water until free from chlorides and the aqueous layer is re-extracted with the ether in the second funnel. The combined ether extracts are evaporated to dryness, the residue is taken up in three successive portions of absolute alcohol and the cholesterol is estimated as above.

The digitonin used was the British Drug Houses product which is completely soluble in water; new specimens of digitonin, particularly those of unknown origin, should be tested for solubility and estimated against pure cholesterol.

The Gooch crucibles were packed with a layer about 3 mm. thick of powminco asbestos, which was well macerated with alcohol until all the fibres were broken down to a fine paste; the pad was washed with a jet of alcohol to remove all loose fibres and the crucible was dried in the air oven at 120°.

Using the above method, we obtained close agreement between duplicates, as indicated by some typical results in Table IV.

Table IV. *Duplicate analyses of plasma cholesterol.*

Sample	Plasma cholesterol, mg. per 100 g. (new method)			% total as ester
	Free	Ester	Total	
1. M. J.	54	164	218	75.2
	53	159	212	75.0
2. L. H.	66	217	283	76.7
	65	221	286	77.3
3. R. M.	55	142	197	72.1
	53	144	197	73.1
4. M. H.	67	177	244	72.5
	68	176	244	72.1

SUMMARY

1. The errors of estimation of cholesterol in blood plasma by the macroscopic technique are mainly due to incomplete extraction of cholesterol, esterification of free cholesterol in the extracted material and loss of sterol during the extraction following saponification.

2. A modified method is described which avoids these errors. Duplicates of free and ester cholesterol agree within 2 % in the majority of cases.

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