Table 3. *Effect of national wheatmeal on the average calcium intakes and balances of six men*

(This is a summary of Table 2; the data on national wheatmeal are the average of 24 weeks, those on white flour of 23 weeks.)

	Average daily bread consumption (not counting approximately 80 g. flour eaten in cooked dishes) (g.)	Average daily Ca intake (g.)	Average daily Ca balance (g.)
Milling standard			
National wheatmeal (85 % extraction)	766	0.550	-0.110
White flour (75 % extraction)	707	0.552	-0.023

In these experiments the bread consumption was somewhat lower (about 530 g./day) than in ours (about 730 g./day). This explains why, in our subjects, the Ca loss was greater than in those of McCance & Widdowson, although we used 85 % extraction flour whilst the data of McCance & Widdowson refer to a 92 % extraction flour.

We wish to emphasize that the conditions under which the adverse effect of national wheatmeal on the Ca balance was observed were exceptional ones. They included an average daily intake of 1.7 lb. of bread (requiring an extra jam ration to make it palatable) as well as a deliberate and severe curtailment of the Ca supply. In a preliminary experiment where the average wheatmeal bread intake was about 1 lb./day and the average daily Ca supply 0.94 g., the Ca balance was in equilibrium (Table 4).

Table 4. *Ca balance at higher level of Ca intake and lower level of wheatmeal bread consumption*

(Average of 14 weekly periods; 5 subjects.)

	g.
Average wheatmeal bread intake	456
Average daily Ca intake	0.941
Average daily Ca excretion	0.911
Average daily Ca balance	+0.030

SUMMARY

1. Six adult male volunteers were kept on a diet containing a large proportion of bread (on average 766 g. wheatmeal bread of 85 % extraction or 707 g. 'white' bread of 75 % extraction) and about 0.55 g. Ca/day. Ca was determined in food, faeces and urine.

2. In every case national wheatmeal had an adverse effect on the Ca balance. On average 0.110 g. Ca was lost daily during the wheatmeal regime whilst 0.023 g. was lost during the white flour regime.

3. The results are in accordance with those obtained by McCance & Widdowson on 69 and 92 % extraction flours, and support the present policy of adding Ca salts to national wheatmeal.

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Colorimetric Determination of Total, Free and Ester Cholesterol in Tissue Extracts

By G. POPJÁK, *Department of Pathology, St Thomas's Hospital Medical School, London, S.E. 1*

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Kelsey's method [1939] for the determination of total, free and ester cholesterol was tested in the course of analyses of lipid extracts of various tissues (liver, kidney, heart, adrenals and blood). This method seemed to be particularly useful, because not only cholesterol, but also total fatty acids, and fatty acids derived from neutral fats and cholesteryl esters, could be determined on one sample. Kelsey claimed that the cholesterol obtained by his method, and estimated colorimetrically, is pure. However, in my experience, the cholesterol thus obtained

is contaminated by substances which often considerably influence the colour developed with the Liebermann-Burchard reagents, and make accurate colorimetric reading impossible. Apart from this disturbing factor, the method gave very low results: cholesterol added to lipid mixtures could not be satisfactorily recovered.

After some experiments a technique has been evolved which retains the advantages of Kelsey's method and is essentially a combination of the method of Schoenheimer & Sperry [1934], as modi-

fied by Sturges & Knudson [1938], with that of Kelsey. The cholesterol is precipitated with alcoholic digitonin in acidified medium at room temperature, and the digitonide separated by centrifuging. The decomposition of digitonide and the isolation of cholesterol are done by Kelsey's method, i.e. by boiling with benzene and precipitating the digitonin by light petroleum. There was no interference with the colour developed by the Liebermann-Burchard reaction with cholesterol samples obtained in this way; the colour was indistinguishable from that of the standard when compared in a colorimeter.

Procedure

Total cholesterol. Samples of ethanol-ether extracts of tissues containing about 0.5 mg. of cholesterol are evaporated almost to dryness in a conical flask on a hot plate, then 10 ml. of ethanol-ether (1:1, v/v) and 0.2 ml. of a solution of KOH in H₂O containing 40 g./100 ml. of solution are added. The mixture is brought to boiling, the flask loosely stoppered and placed in an oven at 75° for 1.5–2 hr. After this time a few drops of phenol red are added and the mixture acidified with HCl of such a strength that 1 ml. makes the mixture definitely acid. After acidification the contents of the flask are evaporated almost to dryness on a hot plate, the last traces of ethanol being blown out by a current of air. The salt in the residue is dissolved in 0.5–1 ml. of water. If more water is added, it is likely that the extraction will be incomplete. The flask is replaced on the hot plate and, when the mixture is hot, 10 ml. of light petroleum (L.P.) (b.p. 40–60°) are poured into the flask, which is vigorously rotated. After a few minutes' standing, the L.P. extract is decanted from the coloured fluid into a 15 ml. conical centrifuge tube and concentrated, with the aid of a boiling rod,* by placing the tube in a beaker of hot water (about 50°), in order to allow space for the following extracts. The contents of the conical flask are extracted three times more with boiling L.P. After the last extraction the mouth of the flask is also rinsed with a few drops of L.P. All the extracts are combined in the centrifuge tube, and finally concentrated to approximately 0.5 ml. [Kelsey, 1939].

Cholesterol is precipitated by addition of 4 drops of 5% (w/w) HCl and of 4 ml. of a 0.25% (w/v) solution of digitonin in ethanol. The boiling rods are left in the tubes and used for thorough mixing of the contents. The tubes are left standing overnight in a sealed jar at room temperature. The boiling rods are removed to a numbered rack and the cholesterol digitonide separated by centrifuging for 5 min. The supernatant fluid (fluid A) is decanted and can be saved for the determination of total fatty acids. The cholesterol digitonide and the sides of the tube are washed with 3 ml. of acetone-ether (1:2, v/v), the digitonide well stirred with the boiling rod and again centrifuged for 5 min. The washing is decanted and the digitonide washed twice more with 3 ml. portions of ether [Schoenheimer & Sperry, 1934; Sturges & Knudson, 1938].

To the digitonide thus purified 7 ml. of benzene are added and the boiling rod replaced. The tube is put into a beaker of hot water (80–82°) and the benzene kept boiling for 45 min.; the amount of benzene is kept above 5 ml. by

adding small volumes of benzene to the tube from time to time. After 45 min. the benzene is concentrated to 2 ml., and while still hot, 9 ml. of L.P. are rapidly added to precipitate digitonin. Thorough mixing is essential, and about 10 min. are allowed for the precipitation of digitonin. After centrifuging for 5 min. the supernatant fluid, which contains the cholesterol, together with three successive L.P. washings of the digitonin, is poured into a small glass-stoppered conical flask and evaporated to dryness on a hot plate, the last traces of L.P. being blown out [Kelsey, 1939].

When the flasks are cool, the cholesterol is dissolved in 5 ml. of CHCl₃, and the flasks placed in a dish containing water kept at 18°. After a few minutes, 2 ml. of a freshly prepared mixture of acetic anhydride and conc. H₂SO₄ (20:1, v/v), also cooled to 18°, are added and well mixed with the CHCl₃. The flasks are kept in H₂O and placed in a dark place. Standards are prepared at the same time, and after 60 min. the colour comparison is made in a colorimeter [cf. Ireland, 1941]. Only the samples which are actually to be compared are removed from the darkness.

If the amount of cholesterol in the sample is over 1 mg., more digitonin can be used than described. In this case 14 ml. of benzene are used for the decomposition of digitonide, and at the stage of colour development the cholesterol is dissolved in 10 or 15 ml. of CHCl₃ and 4 or 6 ml. of the acetic anhydride-H₂SO₄ mixture are added.

If total fatty acids are to be determined, the supernatant fluid (fluid A) from the cholesterol digitonide is decanted into another centrifuge tube and concentrated to about 1 ml.; the acetone-ether washing of the digitonide is added and the whole again concentrated to 1 ml. The ether washings are also added; excess digitonin will be precipitated and is centrifuged off. The supernatant fluid contains the fatty acids. This fluid is quantitatively decanted and evaporated to dryness and the fatty acids dissolved in a small volume of L.P. Their amount can be determined by the dichromate oxidation method of Bloor [1928]. The fatty acids thus isolated cannot be used for the determination of their iodine values because, apparently during the treatment with digitonin and HCl, they become more saturated.

Free cholesterol. To determine free cholesterol, it is necessary to separate first the phospholipids from a suitable sample of the ethanol-ether extract of the tissues, since digitonin will also precipitate the phospholipids [McEwen & MacLachlan, 1941]. A sample of the extract is evaporated to dryness on a hot plate and the residue thoroughly extracted with L.P.; the L.P. extract is concentrated to 2 ml. in a conical centrifuge tube and 7 ml. of acetone and 3 drops of a saturated solution of MgCl₂ in ethanol are added to precipitate phospholipids [Bloor, 1929]. After centrifuging, the phospholipid-free supernatant fluid, which contains chiefly free cholesterol, cholesteryl esters and neutral fats, is decanted into another centrifuge tube. The phospholipids are washed with two 2 ml. portions of acetone. These are combined with the decantate and the whole concentrated to 0.5 ml. by immersing the centrifuge tube in a beaker of hot water (about 60°) and by using a boiling rod. 4 ml. of the digitonin solution and 4 drops of 5% HCl are added and the tubes left overnight in a sealed jar. The cholesterol digitonide is separated by centrifuging. The supernatant fluid (fluid B) is decanted and can be saved for the determination of ester cholesterol, and of fatty acids derived from neutral fats and cholesteryl esters. The purification, decomposition of the digitonide and the colour development are done as described for total cholesterol.

* A 1 cm. long capillary tube fused on to a 2 mm. glass rod.

Ester cholesterol. Fluid B, supernatant after the free cholesterol has been separated as digitonide, contains cholesteryl esters, neutral fats and excess digitonin. After the excess digitonin has been precipitated as indicated for the determination of total fatty acids (treatment of fluid A), the supernatant fluid is decanted, evaporated and saponified, extracted with L.P., and the cholesterol determined by precipitation with digitonin as described for total cholesterol. If desired, the fatty acids derived from neutral fats and cholesteryl esters can also be separated in the same way as total fatty acids.

Table 1 shows the amounts of cholesterol recovered after it has been added, dissolved in CHCl_3 , to ethanol-ether extracts of known cholesterol content. The accuracy of the method is further illustrated by the results given in Table 2, which are examples of determinations of total, free and ester cholesterol on various tissue extracts.

Table 1. *Recovery of cholesterol added in CHCl_3 solution to ethanol-ether extracts of tissues*

Cholesterol added (mg.)	Cholesterol recovered (mg.)	Cholesterol added (mg.)	Cholesterol recovered (mg.)
0.200	0.199	0.500	0.496
0.200	0.199	0.500	0.502
0.200	0.198	0.500	0.498
0.200	0.200	0.500	0.500
0.400	0.400	1.000	1.000
0.400	0.400	1.000	1.000

Table 2. *Results of total, free and ester cholesterol determinations in extracts of various tissues*

Material	Cholesterol content mg./100 g. moist tissue		
	Total	Free	Ester
Human kidney cortex	220	202	22
	224	198	27
	381	358	23
	326	219	107
Human kidney medulla	193	137	45
	230	171	60
	255	194	64
Human liver	252	225	30
Human adrenal	2500	420	2100
Human plasma	152*	65*	85*
Human heart	88	74	12

* mg./100 ml. of plasma.

SUMMARY

A method is described for determination of total, free and ester cholesterol in tissue extracts by a combination of the method of Schoenheimer and Sperry (modified by Sturges and Knudson) with that of Kelsey.

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Adrenaline and Related Substances in Blood and Tissues

By W. RAAB, *Departments of Biochemistry and Medicine, University of Vermont, College of Medicine, Burlington, Vt., U.S.A.*

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The results obtained with the colorimetric method of Shaw [1938], for the assay of adrenaline in blood and tissues, cannot be attributed to adrenaline alone, since the denominator of the 'specific ratio', i.e. the ratio of the results with acid and with alkali [cf. Shaw, 1938], was found, with a few exceptions, to be considerably lower than that of adrenaline itself (Table 1). Quantitative adsorption of ascorbic acid by $\text{Al}(\text{OH})_3$ at a pH of about 8.5 has been regularly observed by the writer, and thus ascorbic acid must be regarded as participating in the colorimetric results, contrary to a statement by Shaw [1938]. Despite the low value of the denominator of the specific ratio (henceforth designated d.s.r.) for ascorbic acid (0.3), this substance alone cannot

be responsible for the low d.s.r.'s generally found in blood and most tissues, including the adrenal glands (Table 1). Its chromogenic intensity is too weak (Table 2) and its reported average concentrations in blood and tissues (Table 3) are too small.

Many chromogenic substances other than adrenaline and ascorbic acid are eliminated by the adsorption procedures of the method. Certain chromogenic hormonal steroids, which are by themselves not adsorbed on $\text{Al}(\text{OH})_3$, were originally believed [Raab, 1941 *a, b, c*] to be partially adsorbed at pH 8.5 in the presence of adrenaline, owing to the formation of a hypothetical 'adreno-cortical' compound. This conception is now abandoned since it was found that steroids pass quantitatively into