THE MICRO DETERMINATION OF TISSUE LIPIDS

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Recent interest in the nature and content of the lipids of tissues is constantly demanding more accurate methods for their determination. The present work was initiated as a result of the need for improved methods of estimating certain of the lipid fractions of glandular lipids in collaborative studies now under way between the departments of Physiological Chemistry and Anatomy (1).

A literature survey of extraction methods led us to the conclusion that complete lipid extraction can be attained by various methods and solvents. Obermer and Milton (2) and Rose and Riegel (3) reported that the 3 : 1 alcohol-ether solvent of Bloor and 1 : 1 alcohol-acetone of Schoenheimer and Sperry (4) are equally effective. Sperry (5) showed that extraction with these solvents is complete at room temperature for glandular tissues. We have confirmed these findings for beef liver and guinea-pig adrenal tissue, the former being relatively low and the latter high in lipid content.

A saponification method was developed from that of Bloor, using potassium hydroxide in alcohol-ether as described by Kelsey (6). Our method gave complete saponification of pure methyl esters of fatty acids, mixed triglycerides and pure cholesterol esters. Previous micro methods for the determination of fatty acids have generally been non-specific in nature, as, for example, the methods employing nephelometry, chylomicron count and dichromate oxidation. The titrimetric method of Stoddard and Drury (7) is specific as it measures the total carboxyl content after saponification and isolation of the fatty acids. It is a direct measure of the fatty acids provided their neutralization equivalent (mean molecular weight) is known. A simplified modification of the titrimetric method was developed and was tested against pure fatty acids.

The determination of cholesterol (both free and combined) was critically studied. Of the numerous methods proposed by other investigators, those based on the Liebermann-Burchard color reaction or the Windaus digitonin precipitation are the most widely accepted at present. The combination of the two principles by Kelsey (6) and by Schoenheimer and Sperry (4) has improved the accuracy and specificity of the analysis. These two methods were subjected to careful study. The Kelsey method in our hands gave erratic results even after considerable experience had been acquired in the use of the method. We could not use the Schoenheimer and Sperry procedure in its last detail because it required a specially designed colorimeter which was not available. A modification of their method was developed which can be used in an ordinary colorimeter and in the Evelyn Photoelectrometer, which we have employed. The method was found to give accurate results on both ester and free cholesterol.

In our experience we have found the phospholipid method of Bloor (8) to give satisfactory results in the determination of this lipid fraction.

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EXPERIMENTAL

I. Extraction of Tissues

Beef liver tissue was used in one series of studies of extraction methods. The tissue was ground up thoroughly and mixed well before the samples were weighed. Fifty ml. of 3 : 1 alcohol-ether was added to each sample and the extraction accomplished as indicated in Table I. The extracts were filtered into Erlenmeyer flasks and the extraction flasks washed with two 25 ml. portions of the solvent. The total extract was evaporated almost to dryness under reduced pressure and the residue extracted with petroleum ether. Total fatty acid and total cholesterol were determined on the petroleum ether extract by the method described later, the results appearing in Table I.

TABLE I

Analyses of Lipid Extracts of Beef Liver

Method	Weight of Sample mg.	Fatty Acid		Cholesterol	
		%	Avg.	%	Avg.
Grind with sand, stand twelve hours with solvent	753.8	3.72	.....	0.278	.....
	552.8	3.50	3.61	0.244	0.261
Grind with sand, stand twelve hours at room temperature with solvent	661.2	3.77	....	0.270	.....
	567.0	3.40	3.58	0.240	0.255
Grind with sand, boil five minutes with solvent, stand twelve hours	721.0	3.56	.....	0.277	.....
	616.6	3.46	3.51	0.252	0.264
Grind with sand, reflux one hour, stand twelve hours	584.8	3.39	.....	0.261	.....
	549.4	3.39	3.39	0.238	0.250
Saponify tissue ninety minutes, ext. with solvent after acidification..	346.2	3.41	.....	0.240	.....
	166.1	3.73	3.55	0.277	0.258

The average results in Table I indicate that essentially complete extractions were attained on liver tissue by the five procedures as described. Results by the saponification method serve as a standard of comparison.

Two of the procedures, described in Table I, were applied to a specimen of adrenal tissue of an old guinea pig. The two adrenals of this animal weighed 619 mg. The tissue was ground and mixed and four samples treated as described in Table II. This time, however, free and total cholesterol, phospholipid and total fatty acid were determined, the results serving to compare the efficiency of extraction by the two procedures.

Since the previous data showed complete extraction by standing for twelve hours at room temperature, this procedure was adopted. The details we have adopted for preparing a lipid extract follow.

Extraction Procedure

The fresh tissue sample was weighed by difference from a ground-glass stoppered weighing bottle into a mortar, mixed with sand, and ground up as described by Bloor (8). The mixture was transferred quantitatively to a 125 ml. Erlenmeyer flask. The mortar and pestle were washed with 1 ml. distilled water, and then with portions of the solvent. A total of 40 ml. 3 : 1 alcohol-ether (redistilled solvents) was added to the tissue. The flask was stoppered and allowed to stand

at room temperature overnight. The extract was then filtered through a fat-free filter paper into a 125 ml. Erlenmeyer flask. The residue on the filter was washed with two 15 ml. portions of fresh solvent and the washings added to the main extract. The combined extracts were evaporated almost to dryness under reduced pressure. A trap of the type used in the Van Slyke determination of nitrogen was convenient for this process. The flask was heated in a water bath placed on the steam bath. When a small aqueous residue remained in the flask, the trap and flask were disengaged from the suction tubing. From 7 to 9 ml. petroleum ether in three small portions was washed through the trap into the flask. The flask was then removed from the trap and stoppered. When a series of samples had reached this point, they were all extracted three times with petroleum ether. The petroleum ether was decanted into 15 ml. centrifuge tubes. The solvent was evaporated off before the second and third extractions of the residue were added to the tube. The substance thus extracted into the centrifuge tubes represented the total lipid and was used for the differential anlaysis of the lipid fractions.

TABLE II

LIPID ANALYSES OF GUINEA-PIG ADRENAL, FOLLOWING TWO EXTRACTION METHODS

Method	Weight of Sample mg.	Phospholipid		Free Cholesterol		Total Cholesterol		Fatty Acid	
		%	Avg.	%	Avg.	%	Avg.	%	Avg.
Grind with sand, reflux one hour, stand twelve hours, room temperature	116.7	3.07	.....	0.21	.....	1.18	.....	14.58	.....
	78.9	2.73	2.90	0.25	0.23	1.26	1.22	16.30	15.54
Grind with sand, stand twelve hours, room temperature	88.3	2.95	....	0.27	.....	1.27	.....	15.50	.....
	100.0	3.19	3.07	0.23	0.25	1.27	1.27	16.18	15.84

II. THE SAPONIFICATION PROCEDURE

The efficiency of saponification by the Bloor method as described by Kelsey (6) was first tested on a specimen of pure cholesteryl palmitate, especially prepared for the purpose. The palmitic acid recovered from the saponification was estimated by the titration method, described in the next section of this paper. The results were erratic, the recovery of palmitic acid varying from 77 to 93 per cent on five samples. The method of saponification was therefore modified (see description below) and tested on a specimen of pure cholesteryl oleate. Preliminary tests on employing the method with a reaction time of 30, 60, and 90 minutes showed that even with this cholesteryl ester a thirty minute saponification is complete, recovery of the oleic acid being over 99 per cent.

Description of Procedure

The lipid to be saponified was dissolved in 5 ml. of 3 : 1 alcohol ether in a 15 ml. centrifuge tube, and 0.2 ml. of 30 per cent aqueous KOH added. If complete solution did not occur after vigorous stirring, alcohol up to 2 ml. was added. The tube was warmed in a beaker of hot water on a steam bath, the solution being allowed to boil gently for 30 minutes. Bumping was prevented by a boiling rod. The beaker was then placed on a hot plate and the solvent boiled off rapidly and completely. The tube was removed from the beaker and allowed to cool. To the aqueous residue was added just enough 1.8 N HCl to make the mixture slightly acid. The mixture was stirred vigorously and then 7 ml. petroleum ether added

and the mixture again stirred. The tube was allowed to stand about fifteen minutes, and then the petroleum ether solution was decanted from the water layer with the suction apparatus described by Bloor (8). The fatty acid solution was decanted into the 25 ml. Erlenmeyer flasks in which the titration was to be performed. The aqueous residue was re-extracted with two portions of petroleum ether and the extracts combined. The ether was evaporated off and the residue dissolved in alcohol and titrated.

In addition to the cholesteryl palmitate and oleate, previously mentioned, the procedure was tested on a specimen of mixed glycerides of olive oil (mean molecular weight of fatty acids 282), a specimen of brain lecithins, and a known synthetic mixture of all four types of lipids. The results are given in Table III.

TABLE III

EFFICIENCY OF FATTY ACID RECOVERY BY MODIFIED SAPONIFICATION PROCEDURE

Fatty Acid in Sample mg.	Number of Determinations	Sample	Fatty Acid Recovered mg.	Recovery Avg. Per Cent
5.39	3	Methyl palmitate	5.32	98.0
6.18	3	Mixed triglycerides	6.16	100.2
8.40	3	Lecithins	8.31	99.0*
4.02	3	Cholesterol oleate	4.00	99.4
10.00	4	Mixed esters (above four)	10.05	100.4†

*Range of recoveries 98.2–100.4%.
†Range 100.1–101.2%.

III. A TITRIMETRIC METHOD FOR THE DETERMINATION OF FATTY ACIDS

The fatty acids employed in this work were highly purified specimens, prepared especially for solubility determinations by H. F. Foreman (9) in this laboratory.

In preliminary work the oxidative method of Bloor (10), applied to oleic acid, was found to give satisfactory results, recoveries on 5 mg. samples being 96 to 102 per cent. The titration method of Stoddard and Drury (7) also proved satisfactory. It was our experience, however, that phenolphthalein possessed some advantages over thymolphthalein. This latter indicator goes through a series of color changes from colorless to yellow to green to blue in alcoholic solution, thus making the end-point difficult to determine. Our titrations with 0.035 N alkali were carried out with a special burette. The details of the method and results follow.

Description of Titrimetric Method

An automatic 5 ml. Exax burette, graduated to hundredths, was fitted with a special tip which delivers 150 drops per ml. solution. The standard alkali was kept in a paraffin coated bottle protected from the carbon dioxide of the air by Ascarite and concentrated alkali traps. The sample of fatty acid, usually in petroleum ether solution, was measured into a 25 ml. Erlenmeyer flask and the solvent evaporated off. The residue was dissolved in 10 ml. 95 per cent alcohol, measured by a pipette. From 5 to 7 drops of 1 per cent phenolphthalein was added, and the sample titrated to the first faint pink color. A series of blank titrations on 10 ml. samples of the alcohol was made and the titrations corrected accordingly. The blank amounted to 0.063 ml. The alkali used in the titration was standardized against Bureau of Standards potassium acid phthalate. Results on pure oleic, palmitic and stearic acids are shown in Table IV.

TABLE IV

THE TITRIMETRIC DETERMINATION OF PURE FATTY ACIDS

Specimen	No. of Det'ns	Alkali ml. (Corrected)	Fatty Acid		Recovery Per Cent
			Taken mg.	Found mg.	
Alcohol Blank	5	0.063	.....	.....	...
Stearic Acid	5	1.173	11.30	11.31	100
" "	3	0.581	5.65	5.60	99
" "	2	0.459	4.52	4.43	98
" "	3	0.351	3.39	3.35	99
" "	2	0.236	2.26	2.27	100
" "	3	0.118	1.13	1.14	101
Palmitic "	2	1.240	10.76	10.76	100
" "	3	0.613	5.38	5.31	99
" "	3	0.381	3.23	3.30	102
Oleic "	1	1.171	11.30	11.19	99
" "	2	0.353	3.39	3.38	100

IV. AN INVESTIGATION OF FREE AND COMBINED CHOLESTEROL PROCEDURES

Pure samples of cholesterol were prepared from beef brain tissue. Standard stock solutions of this cholesterol were made up in petroleum ether and kept tightly stoppered at 0° C. Samples were measured from these stock solutions for all subsequent analyses on pure cholesterol.

The Evelyn photoelectrometer (11) was used throughout the investigation for the measurement of the color intensity of the cholesterol samples. The galvanometer reading, G, when calculated to L, or (2-log G), should give a straight line of slope K when plotted against the weight in mg. of cholesterol in the samples.

The accuracy of the color determination as recommended by Kelsey was first studied. The samples were measured from the stock solutions into 25 x 200 mm. soft glass test tubes which had been optically tested for use in the photoelectrometer. The solvent was evaporated off, and 10 ml. chloroform, 1.0 ml. acetic anhydride, and 0.1 ml. concentrated sulfuric acid were added to each tube. The samples were well stoppered, mixed, kept at a temperature of 23° C. for twenty minutes and read in the instrument. The average values for K found for two series of twenty samples from 0.1 to 1.0 mg. in size, were 0.68 ± 0.054 and 0.71 ± 0.061. The variation is over 8 per cent.

Kelsey's method of separating free and ester cholesterol by digitonin precipitation was next studied. It involves the evaporation to dryness of a mixture of the lipid and a solution of alcoholic digitonin. Extraction of the residue with petroleum ether removes the cholesterol ester and other lipids. The cholesterol digitonide is decomposed by refluxing it in benzene. The addition of petroleum ether to the hot benzene dissolves the cholesterol and the digitonin is precipitated. The variable results by this method are shown in Table V.

A further study of the method was made on samples of pure cholesterol, in which the amount of cholesterol not precipitated by the digitonin, the amount not decomposed by the benzene, and the amount normally determined by the Kelsey method were determined. These three fractions were determined by our modified method described below. The comparatively large samples were used in order to establish the limits of the method when 5 ml. 0.2 per cent digitonin solution was used. The results are shown in Table VI.

In the Schoenheimer and Sperry method (4) the cholesterol is quantitatively precipitated as the digitonide in a mixture of equal parts of absolute alcohol,

acetone, and water. The washed and dried precipitate is then dissolved in 1 ml. of hot glacial acetic acid and the solution is cooled. Two ml. of acetic anhydride and 0.1 ml. of concentrated sulfuric acid are added and the color intensity of the solution determined after twenty-five minutes at 25° C. In order to use the

TABLE V

THE DETERMINATION OF FREE AND ESTER CHOLESTEROL IN KNOWN SYNTHETIC MIXTURES BY THE KELSEY METHOD

Composition of Sample	Recovery of Free Cholesterol		Recovery of Ester Cholesterol	
	mg.	Per Cent	mg.	Per Cent
0.491 mg. free, 0.491 mg. ester	0.478	97	0.540	110
0.491 mg. free, 0.0 mg. ester	0.468	96	0.064	...
0.0 mg. free, 0.491 mg. ester	0.087	...	0.469	96
0.261 mg. free, 0.0 mg. ester	0.228	88	...	...
0.261 mg. free, 0.491 mg. ester	0.235	90	...	...
0.261 mg. free, 0.786 mg. ester	0.269	103	...	...
0.261 mg. free, 0.982 mg. ester	0.284	109	...	...
0.417 mg. free, 0.0 mg. ester	0.383	92	...	...
0.823 mg. free, 0.0 mg. ester	0.711	86	...	...
0.0 mg. free, 0.491 mg. ester	0.019	...	...	...
0.0 mg. free, 0.786 mg. ester	0.019	...	...	...

TABLE VI

CHOLESTEROL DETERMINATION BY KELSEY METHOD

Cholesterol in Sample	Cholesterol Not Precipitated		Cholesterol In Residue		Cholesterol Determined	
mg.	mg.	Per Cent	mg.	Per Cent	mg.	Per Cent
0.166	0.005	3.0	0.005	3.0	0.141	84.8
0.333	0.027	8.2	0.012	3.0	0.288	86.4
0.498	0.065	13.2	0.012	2.5	0.414	83.0
0.834	0.000	0.0	0.034	4.1	0.789	94.6
1.668	0.108	6.5	0.311	18.6	1.227	73.5
2.502	0.006	0.2	0.852	34.0	1.643	65.7
3.336	0.539	16.2	1.051	31.4	1.762	52.8
4.270	1.386	32.5	1.106	25.9	1.864	43.7
5.002	2.080	41.6	0.754	15.1	2.160	43.2

Evelyn photoelectrometer in our work, all the reagents were doubled and the color intensity read through the 6 ml. aperture. On samples of cholesterol from 0.1 to 0.4 mg., the value of K was found to be 1.04 ± 0.04. Corrected to a volume of 11 ml. for comparison with the value of $0.70 \pm .06$ obtained by the Kelsey method, K becomes 0.57 ± 0.02. This lower value for K explains why small samples of cholesterol are more difficultly determined using acetic acid as the solvent.

Investigation revealed that the cholesterol digitonide could be dissolved in a relatively small volume of hot glacial acetic acid. To this solution, chloroform could be added without causing the precipitation of either the cholesterol digitonide or free digitonin. The color intensity of this solution approached that of a chloroform solution of cholesterol and could easily be read in the photoelectrometer even in 12 ml. volumes.

Experiments were conducted to ascertain the most effective concentration of each reagent, glacial acetic acid, chloroform, acetic anhydride, and sulfuric acid, as well as the most desirable temperature for the color reaction. The results are shown in Table VII.

TABLE VII

THE LIEBERMANN-BURCHARD COLOR REACTION: EFFECT OF TEMPERATURE AND CONCENTRATION OF REAGENTS

Sample	No. of Samples	Light Filter	ml.				Temp.	K	Time
			Acetic Acid	Chloroform	Anhydride	Sulfuric Acid			
Cholesterol	40	6600	0	10	1	0.1	23	0.70	20
Cholesterol	5	6600	0	10	1	0.1	25	0.73	25
Cholesterol	5	6600	0	10	1	0.1	17	0.59	65
Cholesterol	5	6200	4	0	8	0.4	26	0.65	30
Cholesterol	5	6200	4	0	8	0.4	27	0.65	35
Cholesterol	5	6200	4	0	8	0.4	18	0.57	65
Cholesterol	5	6200	2	8	2	0.5	32	0.83	12
Cholesterol	6	6200	2	8	2	0.5	16	0.81	50
Digitonide	5	6200	4	0	8	0.4	18	0.52	75
Digitonide	1	6200	2	0	10	0.5	18	0.66	..
Digitonide	1	6200	6	0	6	0.5	18	0.53	..
Digitonide	1	6200	2	3	7	0.5	18	0.70	..
Digitonide	1	6200	2	4	6	0.5	18	0.68	..
Digitonide	1	6200	2	5	5	0.5	18	0.74	..
Digitonide	1	6200	2	6	4	0.5	18	0.75	..
Digitonide	1	6200	2	8	2	0.5	18	0.73	..
Digitonide	1	6200	2	9	1	0.5	18	0.66	..
Digitonide	1	6200	2	8	2	0.2	18	0.45	110
Digitonide	1	6200	2	8	2	0.4	18	0.73	65
Digitonide	2	6200	2	8	2	0.5	18	0.72	60
Digitonide	1	6200	2	8	2	0.7	18	0.70	45

The precipitation of the cholesterol as the digitonide by the Schoenheimer and Sperry method was incomplete for samples of 0.1 to 0.5 mg. This is indicated in Table III, where the digitonide gives consistently lower values for K than the pure cholesterol samples after comparable treatment. Increasing the volume of 0.2 per cent aqueous digitonin solution from one to four ml. per sample did not result in the quantitative precipitation of the cholesterol. However, by using 2 ml. of 0.4 per cent aqueous digitonin, 2 ml. acetone, and 2 ml. alcohol and carrying out the precipitation at a lower temperature, quantitative results were obtained as shown in Table VIII.

TABLE VIII

THE PRECIPITATION OF CHOLESTEROL DIGITONIDE

Volume of Digitonin Solution ml.	Temperature ° C.	L	Cholesterol Calculated mg.	Cholesterol Recovery Per Cent
0	26	0.260	0.321	98
6	26	0.254	0.314	96
6	16	0.260	0.321	98
6	8	0.260	0.321	98

Description of Cholesterol Procedure

To the lipid sample in a 15 ml. centrifuge tube was added 2 ml. each of acetone, alcohol and 0.4 per cent digitonin solution (prepared by evaporating a solution of one gram digitonin in one liter distilled water to 250 ml.). The mixture was stirred thoroughly and the tube was stoppered and placed in the refrigerator overnight. The tube was then centrifuged at a high rate of speed for ten minutes and the supernatant liquid decanted. The precipitate was washed with acetone-ether and then with ether as described by Schoenheimer and Sperry. The precipitate was dried by placing the tube in a beaker of warm water. The tube was then placed in a pan containing about two inches of sand and kept in an oven at about 120° for about twenty minutes. Then the pan and its contents were removed. Two ml. of glacial acetic was pipetted into the tube and the contents of the tube well mixed. The tube was removed from the sand bath after a few minutes and allowed to come to room temperature. Ten ml. of the color reagent was pipetted into the sample. The reagent was prepared by mixing 20 ml. acetic anhydride (C. P. 99 per cent), 80 ml. chloroform, C. P., and 5 ml. concentrated sulfuric acid. The chloroform and anhydride may be measured in a graduated cylinder and the sulfuric acid measured in an ordinary pipette. The reagent is kept at 16° C. and is usually prepared fresh for each set of ten samples. After the addition of the reagent to the sample, the solution was mixed thoroughly and transferred to the colorimeter tubes. The transfer is not necessarily quantitative, since the color intensity of the sample is to be measured. The colorimeter tube was stoppered immediately and placed in a beaker containing water at 16° C., and covered with a heavy towel. The beaker was placed in the constant temperature refrigerator at 16° C. The color intensity of the solution was read in the Evelyn photoelectrometer after about fifty minutes standing, using the 6200 Å light filter. The readings were checked at intervals of five minutes until a constant maximum reading was obtained. The blank reading was checked at 100 between each sample reading.

The standardization curve obtained by the above method on both pure cholesterol and cholesterol digitonide gave a value for K of 0.81 ± 0.01 for the former, and 0.82 ± 0.01 for the latter. The variation is ± 1.5 per cent.

SUMMARY

Procedures are described for the extraction of tissue lipids and for the saponification of these lipids. The lipids of liver and adrenal tissues are completely extracted by 3 : 1 alcohol-ether in five hours at room temperature. Glycerides, cholesterol esters and methyl esters were shown to be completely saponified by heating for thirty minutes with a solution of potassium hydroxide in alcohol-ether. An improved titrimetric method for the estimation of fatty acids is described. Amounts of fatty acids from 1–10 mg. can be determined with an accuracy of ± 2.0 per cent. A modification of the Schoenheimer and Sperry method for determination of free and combined cholesterol has been devised, after critical study of the best conditions for precipitation of cholesterol digitonide and the development of color in the Liebermann-Burchard reaction. The modified method is adapted for use in the Evelyn Photoelectrometer, and may be used in other colorimeters of this type.

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