PREPARATION OF LIPIDE EXTRACTS FROM BRAIN TISSUE*

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For the preparation of lipide extracts from tissues, the method of Bloor (1), either in its original form or with slight modifications, has been a standard procedure. This method consists in extracting the tissue with a mixture of ethyl alcohol and ether. Since the extract obtained is known to contain non-lipide contaminants, it is usually taken to dryness and the residue extracted with a solvent, such as chloroform or petroleum ether, which exhibits a highly specific solvent power for lipides. However, the secondary extracts obtained have been shown to contain substances other than lipides (2, 3).

In the case of nervous tissue, it has been common experience that all of the lipides present in tissue are not extracted by Bloor's procedure (4, 5). Thus, different workers have found it necessary to introduce a subsequent extraction of the tissue with another solvent of higher solvent power for lipides than Bloor's mixture. This second solvent has usually been chloroform (4, 5). The methods thus developed are time-consuming, complicated, and, owing to the fact that they involve protracted treatment of the tissue with boiling solvents, they are open to the general objection that the procedure followed results in changing the chemical nature of some of the lipides. Furthermore, the extracts thus obtained are known to contain non-lipide contaminants (4, 5).

This paper describes a simple method for the preparation of extracts of total pure lipides from brain tissue. The method consists of homogenizing the tissue with a chloroform-methanol mixture. The clear lipide extract thus obtained is then washed free of non-lipide contaminants by being placed in contact with a large amount of water. The whole procedure can be run at 0° and thus any danger of chemical changes in lipides is reasonably excluded.

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The usefulness and limitations of the method can be outlined as follows: (*a*) by this method all of the proteolipides (6), all of the strandin (7), and all of the lipides, with the exception of a constant amount of brain diphosphoinositide, which is not extracted because it is bound to a protein fraction by a bond that is resistant to solvent action, are extracted from brain tissue; (*b*) the washing procedure frees the extract of all non-lipide contaminants. It also removes most of the strandin and about 1 per cent of lipides (other than strandin) present in the extract. The lipides removed in the washing appear to consist mainly of brain diphosphoinositide.

The high efficiency of the washing procedure described requires some explanation. It does not depend for its mechanism of action on simple diffusion across a stable interphase, but on a complex sequence of events that can be outlined as follows: When the extract and water come in contact, methanol diffuses from the extract into the water. This leaves chloroform in the extract side of the interphase and this chloroform, being heavier than the rest of the extract, flows downward into the mass of the extract, a new layer of fresh extract replacing it at the interphase. Parallel to that, the methanol-water mixture that has resulted on the water side of the interphase flows upward into the mass of water because it is lighter than water and is replaced at the interphase by a new layer of water. With new extract and new water coming in contact at the interphase, the above cycle of events is repeated. Thus, there is a continuous flow of fresh extract and of water coming into contact at the interphase for as long as methanol diffuses from the extract into the water. The result obtained is similar to having placed a very thin layer of extract in contact with an ever changing layer of water. When equilibration has been reached, the system shows the following aspect. There is an upper transparent water-methanol phase, a lower chloroform phase, and an accumulation of material at the level of the interphase which floats on top of the chloroform and which can be described as a "fluff." The significance and composition of this fluff are discussed in the accompanying paper on proteolipides (6).

Results obtained by this method have been compared with those by two other methods that have been recently described; namely, that of McKibbin and Taylor (4) and that of Brante (5). It has been found that by our method substantially more substances are extracted than by that of Brante, by which substantially more substances are extracted than by the method of McKibbin and Taylor. The observed differences in amount of substances extracted by the different methods can be accounted for in terms of the amounts of proteolipides and the amounts of strandin that are extracted respectively by each one of the three methods that have been compared.

The method as described has been in use in our laboratory for almost

3 years. It has been applied successfully to practically all animal tissues. This paper is limited to a detailed study of the method as applied to the nervous system because it is only for this tissue that the usefulness and the shortcomings of the procedure have been thoroughly established.

Procedure

Reagents and Apparatus—Methanol, absolute, analytical reagent; chloroform, analytical reagent; chloroform-methanol mixture, 2:1 by volume; Waring blendor with macro and micro containers; Potter-Elvehjem homogenizers of different capacities; fat-free filter paper (8) and alundum (9).

Extraction of Lipides from Tissue—The tissue sample is homogenized with chloroform-methanol mixture, 2:1 by volume, in the proportion of at least 20 cc. of mixture per gm. of tissue. Potter-Elvehjem tissue grinders are used for amounts up to 1 gm., and a Waring blendor for larger amounts. Usually 3 minutes suffice for complete homogenization. The homogenate is transferred to an adequate volumetric flask, the homogenizer being rinsed with three successive portions of solvent mixture which are combined with the homogenate. After mixing and making up to volume, the flask contents are filtered through a fat-free filter paper into a glass-stoppered vessel.

Washing of Crude Extract—A beaker of slightly larger capacity than the volume of extract to be washed is submerged in a larger beaker nine-tenths full of water and of a capacity at least 10-fold the volume of extract to be washed. The aliquot of extract to be washed is then delivered into the small beaker from a volumetric pipette. Back-flow of water into the pipette must be avoided and delivery of the extract must be slow enough to prevent unnecessary turbulence. The system is covered and allowed to stand overnight. The next day the system has the following appearance. There is a clear chloroform phase in the small beaker which occupies about three-fifths of the original volume of the extract. There is an overlying clear water-methanol phase. At the interphase, there is an accumulation which can be described as a "fluff." This fluff consists of a mixture of proteolipides and free lipides.

Redissolution of Fluff—By the use of suction and then of a pipette, the water phase is removed as completely as possible without disturbing the fluff. A layer of water only 3 or 4 mm. thick should remain. To the chloroform solution, fluff, and water in the beaker is added a volume of methanol about one-fourth of the original volume of the aliquot of extract that has been washed. If, on stirring, a single phase is not formed, as shown by a milky appearance of the mixture, more methanol is added to it dropwise with constant stirring until one phase results. The clear solution obtained is the washed extract.

In order to store it or to take aliquots for analysis, the washed extract

is transferred through a funnel into a volumetric flask. The beaker and funnel are rinsed with three successive aliquots of chloroform-methanol mixture, the rinsings being combined with the main body of the solution. The flask contents are mixed and made up to volume with chloroform-methanol mixture.

Removal of Protein Moiety from Proteolipides—In this case the extract is transferred quantitatively from the beaker into a suction flask and taken to dryness on the water pump at a bath temperature of 60°. If needed, ethanol is added to control foaming. The residue is extracted twice with one-fourth the original volume of hot solvent mixture, and the extracts are filtered through a fat-free filter paper into a volumetric flask of appropriate size and made up to volume with solvent mixture.

EXPERIMENTAL

Analytical Methods—Some of the methods used have been described elsewhere (10). Total and free cholesterol have been estimated by the method of Schoenheimer and Sperry (11) as described by Sperry (12); proteolipide protein and strandin by methods described in accompanying papers (6, 7); total and free inositol by bioassay with yeast as a test organism (13, 14). The composition of the nutrient media is due to Novelli.[1]

Total solutes have been estimated by placing an aliquot of solution in a weighing bottle and allowing it to evaporate at room temperature (with or without a vacuum). The residue is dried at 105° for 2 hours and weighed. With proper care the over-all error of the method is 0.1 mg.

Degree of Completeness of Extraction of Lipides—To study this point, the tissue suspension in chloroform-methanol was filtered through a Büchner funnel, filtration being stopped before the residue had a chance to dry. The residue was then reextracted with a new portion of solvent mixture by boiling under a reflux for 24 hours. The second extract thus obtained was filtered and concentrated to dryness. This procedure was carried out routinely in the first 50 odd tissue extractions that were performed. The amount of solids in the second extract amounted in no case to more than 1/200 of the amount of solids in the first extract. Three typical results were as follows: 188.5 gm. of white matter were extracted with 3600 cc. of solvent mixture. The tissue residue was reextracted with 2 liters of solvent mixture. The first extract contained 34.7 gm. of solids. The second extract contained 0.176 gm. From 58.7 gm. of gray matter, the first extract contained 3.97 gm., and the second extract 22 mg. From 897 mg. of astrocytoma tissue, the first extract contained 50.2 mg. of solids, and the second extract <0.3 mg.

Tissue residues extracted twice as described were in certain cases ana-

[1] We are indebted to Dr. G. D. Novelli for this information.

lyzed for total fatty acids. The residue was saponified with boiling 20 per cent NaOH under a reflux overnight, and the product cooled, acidified to pH 2 with HCl, and extracted twice with an equal volume of ether. The ether extracts were combined, washed twice with an equal volume of water, and dried. Total solids in the ether extract usually amounted to 0.5 per cent of the weight of tissue residue. This corresponds to 0.3 per cent of the amount of lipides originally extracted from the tissue. This was interpreted at the time as evidence of complete extraction of the tissue lipides.

TABLE I

Effect of Washing on Amounts of Different Solutes Present in Chloroform-Methanol Extracts from Gray and White Matter of Brain

All results are expressed as mg. per gm. of wet tissue.

Solutes	Extracts from gray matter			Extracts from white matter		
	Before washing	After washing	Per cent of solutes in crude extract removed by washing	Before washing	After washing	Per cent of solutes in crude extract removed by washing
	mg.	*mg.*		*mg.*	*mg.*	
Total solids	66.8	58.2	13.00	183.1	176.5	3.8
Cholesterol	10.5	10.5	None	38.1	38.3	None
P	1.53	1.49	2.6	3.07	2.95	3.9
N	1.95	1.17	39.9	4.67	4.22	9.7
Carbohydrate (as galactose)	1.34	0.73*	45.2	10.00	9.8	2.0

* The washing procedure removes from crude extracts of gray matter an amount of strandin corresponding to between 3.0 and 3.5 mg. per gm. of wet tissue. This represents a removal of strandin carbohydrate amounting to between 0.6 and 0.7 mg. per gm. of wet tissue. Therefore, the loss of carbohydrate from the extract during washing can be entirely accounted for in terms of strandin removed by washing.

Actually, as already stated, these residual fatty acids are constituents of a constant amount of brain diphosphoinositide that is not extracted by this procedure. Proof of this statement will be given in a future publication.

Study of Washing Procedure—The effect of the washing procedure on the crude lipide extracts has been studied (*a*) by comparing the composition of extracts before and after washing, and (*b*) by washing the same aliquot of extract several times in succession, collecting the successive washings separately, and studying the solutes they contain.

Table I gives results of analyses of extracts from gray and white matter before and after washing. It can be seen from these results that no significant amount of cholesterol, phosphatides, or cerebrosides is removed from either extract by the washing procedure.

The results of study of solutes in successive washings can be summarized as follows: In extracts of gray matter, the first washing removes 13 to 14 per cent of solutes in the extract. Two-fifths of the material washed away is strandin. The second and the third washings remove 3 and 2 per cent, respectively, of the solutes in the extract, about half of the fraction washed away being strandin. Solids from the second and successive washings are completely soluble in chloroform; *i.e.*, they are lipides. It appears then that the second and third washings each remove from the extract about 1 per cent of lipides present other than strandin. In the case of white matter, the first washing removes 4 to 5 per cent of solutes in the extract. Successive washings remove a further 1 per cent each. Solids in washings after the first are completely soluble in chloroform; *i.e.*, they are lipides.

A study has been made of substances removed from an extract from whole brain which throws some light on the nature of lipides, other than strandin, that are removed during washing. Two 100 cc. aliquots of a whole brain crude extract, which contained 5.65 mg. of total solutes per cc., were washed three times in succession, and the washings collected separately and analyzed for total solids, strandin, P, free inositol, and total inositol. Total solids were 40.2, 16.8, and 13.2 mg., respectively, for the first, second, and third washings; *i.e.*, 7.1, 3.0, and 2.3 per cent of the solids in the crude extract. Other results (in mg.) are presented, the figures in parentheses indicating in each case the amount of that component as per cent of total solids in the particular washing analyzed.

Washing	Strandin	P	Total inositol	Free inositol
1st	14.1 (35)	0.5 (1.24)	2.9 (7.3)	2.9 (7.3)
2nd	8.8 (52)	0.195 (1.15)	0.81 (4.9)	(<0.05)
3rd	4.65 (36)	0.295 (2.25)	0.67 (5.0)	(<0.05)

It can be seen from these data that the first washing removes a large amount of free inositol from the crude extract, while the second and third extracts remove only combined inositol. The ratio of inositol to P in the second and third extracts is, within the experimental error of the method, close enough to the theoretical ratio for brain diphosphoinositide to warrant the conclusion that most of the lipide P removed from the extracts in the course of washing is brain diphosphoinositide. The fact that for the first washing the values for total inositol and free inositol are the same does not show that no combined inositol is removed during the first washing, because, assuming that the first washing contains as much combined inositol as the second and third do, this amount would be only a

small fraction of the total inositol present, which would fall within the margin of error of the method of bioassay.

These data also show that strandin and presumably brain diphosphoinositide account for most of the solids removed from the extract by the second and third washings.

It is necessary to conclude that a certain amount of lipides (other than strandin) is removed from the extract by the first washing. The actual amount can only be approximately estimated. Assuming that at least as much lipide (other than strandin) is removed by the first washing as by the second, the amount would be of the order of 1.0 per cent of the

TABLE II

Data on Comparative Study of Extraction by Three Methods

All values are expressed as mg. per gm. of wet tissue.

Materials in extract or fractions therefrom	Present method	Method of Brante (5)	Method of McKibbin and Taylor (4)
	mg.	*mg.*	*mg.*
Total solids in crude extract	120.2	111.8	104.9
Free inositol in crude extract	0.6	0.6	0.6
Solids removed by washing	8.4	6.7	5.0
Strandin in washing	3.0	1.09	0.42
Solids in washing other than strandin	5.1	5.06	4.58
Proteolipide protein	10.8	2.6	1.9
Lipides (total solids in extract less solids removed by washing less proteolipide protein)	101.0	102.3	98.0

total solutes in the extract and it would consist mostly of brain diphosphoinositide.

Effect of Amount of Water Used in Washing on Amount of Solids Removed from Extract—Three 25 cc. aliquots of a total brain chloroform-methanol extract, which contained 5.65 mg. of solutes per cc. (141.25 mg. in 25 cc. aliquots), were placed in contact with 125, 250, and 600 cc., respectively, of water and let stand overnight. The three water washings were collected separately and dried. The residues weighed 10.2, 9.9, and 10.1 mg., respectively. Thus, it appears that the amount of solutes removed from the extract by water was the same between volumes of water 5-fold and 24-fold the volume of extract being washed.

Comparison of Results Obtained by Present Method with Those by Methods of Brante (5) and of McKibbin and Taylor (4)—Two brain hemispheres were homogenized in a Waring blendor for 5 minutes at 3°, and weighed samples of the homogenate were extracted in duplicate by each of the three methods. The crude extracts obtained were analyzed for total solids,

proteolipide protein, and free inositol. Duplicate values for total solids and proteolipide protein agreed within 3 per cent. Aliquots from each extract were washed with water by the washing procedure already described. Since this procedure depends on the presence of methanol for its mechanism of action, extracts obtained by the methods of Brante and of McKibbin and Taylor were diluted by addition of half their volume of methanol prior to washing. The washings were collected separately and analyzed for total solids and for strandin. The results obtained are given in Table II, from which it can be seen (*a*) that only small amounts of proteolipide protein and of strandin are extracted by the methods of Brante and of McKibbin and Taylor; (*b*) that the larger percentage of tissue solids extracted by the present method compared with the other two can be attributed entirely to the presence, in extracts prepared by this method, of larger amounts of proteolipide protein and of strandin; (*c*) that the amount of non-lipide contaminants in the extracts, as indicated by the amount of total solids removed by washing minus strandin present in the washing, is essentially identical in all three extracts. This is further shown by the fact that crude extracts prepared by the three methods contain identical amounts of free inositol, and that the same amount of tissue lipides, in the strictest sense, is extracted by all three methods; *i.e.*, lipides with the exclusion of strandin and proteolipide protein.

SUMMARY

1. A simple method for the preparation of lipide extracts from brain is described. The method consists in homogenizing the tissue with a 2:1 chloroform-methanol mixture. Insoluble substances are removed by filtration and the filtrate is washed with water to remove non-lipide contaminants.

2. Proteolipides (6), strandin (7), and lipides, with the exception of a constant amount of brain diphosphoinositide which is bound to a protein by a bond that is resistant to solvent action, are extracted by this method.

3. The washing procedure removes from the crude extract all non-lipide contaminants, most of the strandin, and about 1 per cent of lipides. The lipides removed appear to be mostly brain diphosphoinositide.

4. By this method more tissue solids are extracted than by those of Brante (5) and of McKibbin and Taylor (4). The solids extracted in excess of those extracted by the other two methods have been identified as proteolipides and strandin.

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