A SIMPLE METHOD FOR THE ISOLATION AND PURIFICATION OF TOTAL LIPIDES FROM ANIMAL TISSUES*

By JORDI FOLCH, M. LEES,† AND G. H. SLOANE STANLEY‡

(*From the McLean Hospital Research Laboratories, Waverley, and the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts*)

(Received for publication, August 23, 1956)

Work from this laboratory resulted in the development of a method for the preparation and purification of brain lipides (1) which involved two successive operations. In the first step, the lipides were extracted by homogenizing the tissue with 2:1 chloroform-methanol (v/v), and filtering the homogenate. In the second step, the filtrate, which contained the tissue lipides accompanied by non-lipide substances, was freed from these substances by being placed in contact with at least 5-fold its volume of water. This water washing entailed the loss of about 1 per cent of the brain lipides.

This paper describes a simplified version of the method and reports the results of a study of its application to different tissues, including the efficiency of the washing procedure in terms of the removal from tissue lipides of some non-lipide substances of special biochemical interest. It also reports some pertinent ancillary findings. The modifications introduced into the method pertain only to the washing procedure. A chloroform-methanol extract of the tissue, prepared as described in the original version of the method, is mixed with 0.2 its volume of water to which, for certain purposes, different mineral salts may be added. A biphasic system without any interfacial fluff is obtained (2). The upper phase contains all of the non-lipide substances, most of the strandin, and only negligible amounts of the other lipides. The lower phase contains essentially all the tissue lipides other than strandin. In comparison with the original method, the present version has the advantage of being simpler, of being applicable to any scale desired, of substantially decreasing the losses of lipides incidental to the washing process, and, finally, of yielding a washed extract which can be taken to dryness without foaming and without splitting of the proteolipides (3).

* This work has been aided by grant No. B-130 from the United States Public Health Service.

† Fellow of the American Cancer Society, 1951–53.

‡ Eli Lilly Traveling Fellow in Medicine, 1953–55.

Procedure

Reagents—

Chloroform. Reagent grade.

Methanol. Reagent grade. For use with tissues relatively poor in lipides, such as muscle or blood plasma, both the chloroform and methanol must be redistilled.

Chloroform-methanol mixture. 2:1 by volume.

Pure solvents upper phase and pure solvents lower phase. Chloroform, methanol, and water are mixed in a separatory funnel in the proportions 8:4:3 by volume. When the mixture is allowed to stand, a biphasic system is obtained. The two phases are collected separately and stored in glass bottles. It has been found that the approximate proportions of chloroform, methanol, and water in the upper phase are 3:48:47 by volume. In the lower phase, the respective proportions are 86:14:1. Either of the phases may be prepared directly by making use of the above proportions.

Pure solvents upper phase containing 0.02 per cent $CaCl_2$, 0.017 per cent $MgCl_2$, 0.29 per cent NaCl, or 0.37 per cent KCl. These solutions can be prepared in one of two ways. One is to shake the appropriate amount of salt with pure solvents upper phase in a glass-stoppered vessel until solution is complete. The other is to proceed as for the preparation of pure solvents upper and lower phases except that, instead of water, 0.04 per cent aqueous $CaCl_2$, 0.034 per cent aqueous $MgCl_2$, 0.58 per cent aqueous NaCl, or 0.74 per cent aqueous KCl is used.

Extraction of Lipides—For the purposes of this description, the volume of a tissue sample will be computed on the assumption that the tissue has the specific gravity of water; *i.e.*, the volume of 1 gm. of tissue is 1 ml. The tissue or tissue fraction is homogenized with 2:1 chloroform-methanol mixture (v/v) to a final dilution 20-fold the volume of the tissue sample; *i.e.*, the homogenate from 1 gm. of tissue should be diluted to a volume of 20 ml. For amounts of tissue up to 1 gm., the homogenization is carried out in a Potter-Elvehjem type of homogenizer, the tube of which has been weighed, and calibrated at the volume of the final dilution of the particular tissue homogenate. Thus, the tissue sample can be weighed and the homogenate diluted to volume without a transfer. For brain or tissues of similar consistency, 3 minutes suffice for complete homogenization. Tougher tissues will require lengthier homogenization, and some organs rich in connective tissue, *e.g.* peripheral nerve, may require special handling such as grinding with a mortar and pestle at the temperature of dry ice before homogenization with the solvent mixture. For amounts greater than 1 gm., the tissue is homogenized in an adequate blendor with about a 17-fold volume of solvent mixture; the balance of solvent mixture required to dilute the homogenate to final volume is used to insure the quantitative transfer of the homogenate into a volumetric flask. After tem-

perature equilibration and final volume adjustment, the homogenate is filtered through a fat-free paper into a glass-stoppered vessel. For the purposes of computation, this extract corresponds to 0.05 its volume of tissue; *i.e.*, 1 ml. of extract corresponds to 0.05 gm. of tissue.

Washing of Crude Extract—The crude extract is mixed thoroughly with 0.2 its volume of either water or an adequate salt solution (see "Experimental"), and the mixture is allowed to separate into two phases, without interfacial fluff, either by standing or by centrifugation. The volumes of the upper and lower phases are, respectively, 40 and 60 per cent of the total volume of the system. As much of the upper phase as possible is removed by siphoning, and removal of its solutes is completed by rinsing the interface three times with small amounts of pure solvents upper phase in such a way as not to disturb the lower phase. Finally, the lower phase and remaining rinsing fluid are made into one phase by the addition of methanol, and the resulting solution is diluted to any desired final volume by the addition of 2:1 chloroform-methanol mixture.

The procedure can be run on any scale that is otherwise technically feasible, and the actual details of operation will vary according to the amount of extract being washed. For instance, if 10 ml. of crude extract are to be washed, the extract is placed in a 15 ml. centrifuge tube. To it are added 2 ml. of either water or salt solution, the two liquids are mixed with a stirring rod, the rod is then rinsed into the tube with a minimal amount of pure solvents lower phase, and the tube is capped with aluminum foil and centrifuged until complete separation of the system into two phases without any interfacial fluff is obtained. The duration of centrifugation varies from about 20 minutes at 2400 r.p.m. for white matter extracts to a very short time for blood plasma. The volumes of the upper and lower phases are 4.8 and 7.2 ml., respectively. The upper phase is removed as completely as possible with a pipette or with a suction arrangement such as the one described by Van Slyke and Rieben (4). Next, the inside wall of the tube is rinsed with about 1.5 ml. of pure solvents upper phase, which are allowed to flow gently from a pipette so that the washing fluid collects on top of the lower phase without any mixing of the two phases. The tube is rotated gently to insure mixing of the rinsing fluid with the remaining original upper phase, and the mixture is removed. This rinsing of the tube wall and interphase with pure solvents upper phase is repeated twice. Finally, the lower phase is diluted to a volume of 10 ml. as outlined above. With tissues poor in proteolipides, *e.g.* muscle, plasma, and liver, or if time is no object, centrifugation may be omitted from the washing procedure. Instead, the extract plus water mixture can be allowed to separate into two phases by prolonged standing. In that case, it is more convenient to carry out the washing in glass-stoppered cylinders.

Permissible Departures from Procedure—The technique described can be

changed in many details if so indicated by the size and nature of the tissue sample or by the particular problem under study. Thus, if necessary, in the preparation of the crude extract, the tissue homogenate can be diluted to more than 20-fold the volume of tissue. Also, centrifugation can be used in preference to filtration as a means of obtaining a clear extract. Centrifugation of the homogenate itself is unsatisfactory because the specific gravity of the solvent mixture is too close to the density of the suspended material. Therefore, if centrifugation is to be used, it is necessary to lower the specific gravity of the homogenate by the addition of methanol. Usually, the addition of 0.2 its volume of methanol suffices for the purpose. The amount of methanol added must be noted.

In the washing procedure described, chloroform, methanol, and water are present in the system tissue extract plus water in the proportions 8:4:3 by volume, as can be computed if account is taken of the fact that the extract contains all the water from the tissue. These proportions are critical and must be kept constant. Therefore, in cases in which the tissue extraction has been substantially changed, it is necessary to modify the washing procedure in a way that will restore the required proportions of solvents. For instance, if the homogenate has been diluted to 40-fold the volume of tissue, the water contributed to the extract by the latter will be half as much as in the standard 20-fold dilution; *i.e.*, it will be 2 per cent of the extract as compared to the usual 4 per cent. Therefore, the amount of water added to the extract for washing should be 22 per cent instead of the usual 20 per cent. If methanol has been added to the extract, twice as much chloroform must also be added and the amount of water adjusted accordingly.

EXPERIMENTAL

Analytical Methods—Most of the methods used in this study have been described elsewhere (3, 5).

Degree of Completeness of Extraction of Tissue Lipides—Earlier work had shown that the extraction procedure removes all lipides from brain (1) and blood plasma (6), with the exception of a specific fraction of lipides which is combined to tissue proteins by a linkage which withstands the action of neutral solvents. In the present study, the completeness of extraction of lipides from liver and muscle was studied by reextracting the residue with hot solvent and determining the amount of lipides in the second extract. The original extraction can be considered complete if the second extract contains no more lipides than can be accounted for by the aliquot of first extract left wetting the residue. The experiment was carried out as follows: The tissue was homogenized with chloroform-methanol as described, and the homogenate filtered through a previously weighed Büchner funnel,

filtration being stopped before the insoluble residue became dry. The filter was weighed again and the weight of the wet residue it contained was computed by difference. Next, the residue was reextracted with a new portion of solvent mixture by boiling under reflux for 24 hours, the suspension was filtered, and the twice extracted residue collected and dried to constant weight. The amount of first extract left wetting the tissue residue could then be computed from the equation, ml. of extract in residue = (weight of wet residue after first extraction minus weight of dried residue)/-(specific gravity of first extract). In the case of liver, 40 gm. of tissue were extracted as outlined above in succession with 760 ml. and 400 ml. of solvent mixture. The first extract contained 2.46 mg. of lipides per ml., while 28.8 ml. of extract with a computed total lipide content of 71 mg. were left in the residue. The second extract contained a total of 69 mg. of lipides; *i.e.*, the amount to be expected from the aliquot of the first extract in the residue. In an identical experiment with muscle tissue, the first extract contained 0.743 mg. of lipides per ml., while 24.3 ml. of extract with a total lipide content of 18.1 mg. were left wetting the residue. The second extract contained a total of 21.6 mg. of lipides; *i.e.*, 3.5 mg. more than were to be expected from the aliquot of the first extract remaining in the residue. This difference, which amounts to <0.5 per cent of total tissue lipides, cannot be considered significant.

Study of Washing Procedure—The washing procedure has been studied by (*a*) determining the amount of lipides lost during the washing, (*b*) determining the amount of non-lipide substances remaining in the lower phase, (*c*) investigating an effect of certain non-lipide substances upon the distribution of lipides between the two phases formed during the washing procedure, (*d*) determining the effect of mineral salts on the distribution of lipides in this particular biphasic system, and finally (*e*) ascertaining the efficiency of the washing procedure in relation to some substances of importance in metabolic studies by the use of radioisotopes.

Loss of Lipides Incidental to Washing Procedure and Degree of Removal of Non-Lipide Contaminants—Since lipides are undialyzable, the amount of undialyzable substances in the upper phase would represent the maximal amount of lipides lost, and the dialyzable substances would, of necessity, represent non-lipide contaminants. In a typical experiment, 175 ml. of brain white matter extract were washed with 35 ml. of water. The upper phase, which had a volume of 84 ml., was collected quantitatively. The lower phase was equilibrated with 84 ml. of pure solvents upper phase, and the resulting second upper phase was collected. Both upper phases were concentrated to dryness by vacuum distillation of the solvents, the residues were each dissolved in 10 ml. of water, and the solutions were dialyzed exhaustively. The dialyzable and undialyzable fractions thus obtained were

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.