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Part I

The Preparation of Tissue Lipid Extracts

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IT IS obvious that the care, time, and effort devoted to the preparation of tissue extracts should be commensurate with the required accuracy and precision of the final analytical results desired. If the lipids are incompletely extracted or altered during the extraction, the results obtained may be less than desired regardless of the effort spent later or the precision of the apparatus and techniques used in the analysis.

General Conditions for Lipid Extraction. There are a number of conditions that must be met for proper extraction of tissue lipids. These vary with the tissue and the purpose for which the extract is desired. For the best extraction and minimum lipid alteration, all conditions listed below must be met to the fullest possible extent.

1. Carry out all procedures under an atmosphere of nitrogen.
2. Use purified solvents.
3. Rapidly remove the tissues after sacrifice.
4. Immediately finely subdivide the tissue.
5. Use the proper solvent and solvent-to-tissue ratio.
6. Use heat only when necessary.
7. Remove non-lipid impurities without loss of lipids.
8. Store lipids under conditions that minimize lipid alterations.

Nitrogen Atmosphere. A nitrogen atmosphere is used to prevent formation of oxidation products and alterations in the structure and solubility properties of the lipids. It is particularly needed during evaporation of a lipid extract. For some solvents it is desirable to remove oxygen by flushing with nitrogen.

Solvents. All solvents should be pure. Pure solvents can generally be obtained from commercial sources but redistillation may be necessary. It may be tedious to use only freshly redistilled solvents but it is a safe practice. Peroxide-free ethyl ether should be used. It can be prepared by distillation of the commercial product from hydroxylamine, ferrous sulfate, or sodium sulfite. To remove aldehydes from ethanol it is refluxed over KOH and redistilled. Pure chloroform rapidly breaks down; it should be freshly redistilled and immediately stabilized by addition of 1% methanol. It should be remembered that commercial reagent grade chloroform contains some methanol. Both chloroform and ethyl ether are more stable when stored in the refrigerator. Ether is stored over iron wire or sodium. Methanol (absolute) can usually be used after simple distillation. Petroleum ether may leave a residue upon evaporation that will interfere with some lipid measurements. Treatment of the petroleum ether with concentrated sulfuric acid followed by distillation removes these impurities. Merck's fat extraction petroleum ether does not require further purification other than simple distillation.

Screw-Cap Glassware. Test tubes, centrifuge tubes, Erlenmeyer flasks equipped with Teflon-lined screw-

Index to Part I

caps, are very useful when dealing with volatile organic solvents.

Tissue Sampling and Subdivision. Since the alteration in some lipids is extremely rapid, the tissues should be removed immediately upon sacrifice and quickly treated. If the tissue is not to be immediately treated with solvent, it can be quick-frozen with liquid air or dry ice. Storage of the tissue quick-frozen in physiological saline has been recommended for tissues on which unsaturated fatty acids are to be determined. Storage in a refrigerator does not prevent breakdown. Fine subdivision of a tissue may be accomplished by one of the following types of apparatus:

1. Mortar and pestle
2. Blendor
3. Disintegrator
4. Homogenizing tube
5. Tissue crusher

Mortar and Pestle. This technique is adequate for small tissues without the use of sand. With larger samples sand is required. The method is tedious and the resulting subdivided sample plus sand may be quite bulky. Solvent should be added during the grinding to prevent lipid breakdown. With a steel mortar and pestle the tissue may be frozen and then ground to a powder.

Blendor. Tissue subdivision by the use of a Waring Blendor is very common. Large samples of soft tissues can be subdivided by this apparatus, but tissues with appreciable connective tissue such as skin or skeletal muscle are difficult to finely subdivide by this means. The connective tissue tends to become entwined about the blades. Other disadvantages of the blendor are that it may not sufficiently divide the tissue, and that quantitative transfer from the extraction vessel is required, and this may be a bit difficult with the limited amount of solvent to be used. When chloroform and methanol are used during the blending operation, a more satisfactory subdivision frequently is obtained if the tissue is first blended with MeOH or $CHCl_3$-MeOH (1:1). After adding $CHCl_3$ to obtain the desired $CHCl_3$-MeOH ratio, the mixture is again briefly blended.

Tissue Disintegrator. The term tissue disintegrator is used to describe the apparatus in which the cutting blades are inserted into a vessel from the top. This type of apparatus, which allows the blades to be removed from the subdivided tissue, is especially advantageous if the blendor vessel is a tube which can be centrifuged. Such apparatus is manufactured by the Lourdes Instrument Corp., Brooklyn 32, N.Y. The tissue may be disintegrated in the tube using rotar speeds up to 20,000 rpm. Although the sample size possible with this apparatus is less than with the blendor, the subdivision is finer. The tissue plus solvent can then be heated, if desired, centrifuged, and the solvent decanted. If a graduated tube is used the volume can be adjusted to a known value and aliquots removed directly for analysis.

Homogenizing Tube. The finest degree of tissue subdivision is achieved with a homogenizing tube although the sample size is somewhat limited. It is convenient to mechanically turn the pestle. For this a threaded male fitting is inserted into the chuck of a high-torque motor, and a female threaded fitting is attached to the pestle of the homogenizing tube by way of a piece of rubber tubing. The pestle may thus be rapidly removed and a clean one attached. A modified Tenbroeck tube is a most satisfactory homogenizing tube. The Tenbroeck tube has an enlargement at the top to serve as a solvent reservoir during the grinding process. The entire portion of the tube below the bulb and most of the pestle has a ground glass surface for efficient grinding. This tube is satisfactory for most tissues, but if 5 or 6 short (1 mm.) glass projections are fused to the tip of the pestle and ground to fit the curvature of the tube bottom, the modified tube is suitable for homogenizing tough tissues such as aorta and skin. After fine subdivision of the tissue, the volume may be adjusted and the tube centrifuged, or the contents may be transferred to another vessel for lipid extraction.

Tissue Crusher. With some tissues such as adult aorta or skin, the most efficient way to subdivide them is to freeze them solid and crush them. A tissue crusher such as that designed by Graeser *et al.* (1) serves very well. The apparatus consists of a stainless steel well and plunger. The apparatus and tissue are chilled with liquid air. The frozen tissue is placed in the well, covered with the plunger and crushed by a blow with a heavy hammer. Generally a single blow is sufficient to finely subdivide even the hardest tissue. Usually samples of the subdivided tissue are weighed out and then extracted.

Extraction of Lipids with Ethanol-Ether (2:1) (Bloor's Solvent). One of the most widely used solvent mixtures for lipid extraction has been ethanol-ether in the ratio of 3:1 as devised by Bloor. With most finely subdivided tissues Bloor's solvent will extract all the lipids. For some tissues, especially fatty tissues, heat is required. For other tissues, such as brain, the lipid extraction is incomplete. With all tissues several extractions with a solvent-to-tissue ratio of 10:1 may be more satisfactory in removing lipids but at the same time more non-lipid materials may be removed.

The ethanol-ether extract can be used directly for the analysis of lipid classes such as phosphatides and cholesterol. For other determinations, however, further purification of the extract is usually required (Fig. 1). It is also convenient to get the lipids into a more volatile solvent. This can be accomplished by one of the several ways described below. It should be appreciated, however, that less nonlipid material (chlorides, organic matter) is extracted by petroleum ether if the alcohol-ether extract is evaporated to complete dryness rather than to a very small aqueous volume. The final petroleum ether extract may be dried with anhydrous sodium sulfate, but addition of salt to the extract prior to the final extract is not recommended.

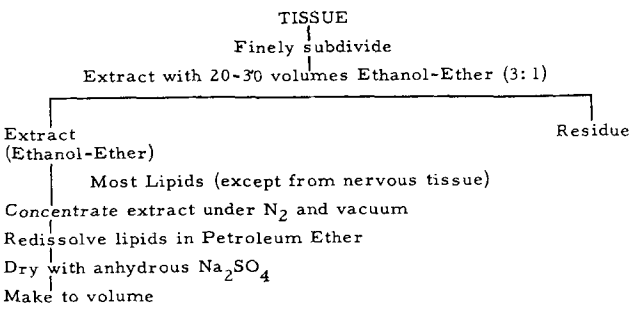

FIG. 1. Extraction of lipids with ethanol: ether (3:1).

The extract is evaporated under vacuum in a rotary Teflon-lined screw-cap tube and a 40–60°C. water bath until most of the ethanol has been removed and little aqueous phase remains. A measured volume of petroleum ether is added to the tube which is capped tightly, shaken, and allowed to stand or centrifuged. Practically all of the lipids will be in the upper petroleum ether phase.

The extract is evaporated just to dryness under nitrogen in a beaker and the lipids extracted with 3–4 portions of petroleum ether, bringing the solution just to a boil each time. The extract should be filtered or centrifuged to remove suspended materials.

The extract is evaporated almost to dryness under nitrogen in a sidearm Erlenmeyer flask (Fig. 2) or a

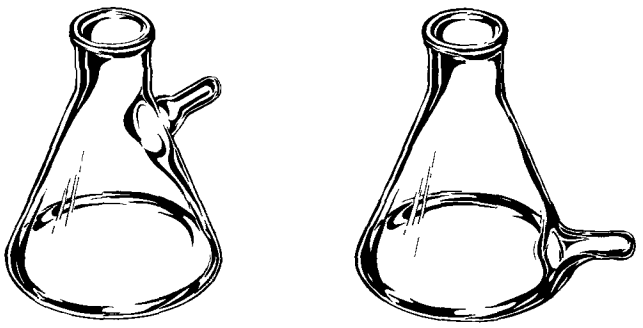

FIG. 2. Sidearm Erlenmeyer flasks for extracting lipids from small volumes of aqueous phase.

sidearm Kjeldahl flask using a 40–60°C. water bath and vacuum. The aqueous phase is extracted with 3–4 portions of petroleum ether.

The extract is evaporated under vacuum in a rotary evaporator designed by Craig *et al.* (2) (Fig. 3).

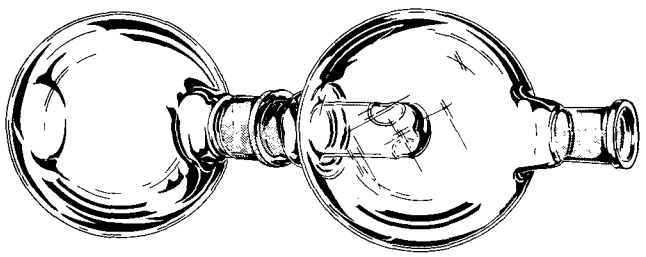

FIG. 3. Rotary evaporation flask designed by Craig, Gregory and Hausmann (2).

During the evaporation the lipids tend to be protected by the solvent vapors, but when the flask is finally vented, the flask should be flooded with nitrogen to prevent oxidative changes in the lipids. This can be accomplished if the flask is connected via a T tube both to a nitrogen tank and a vacuum outlet. Venting is performed by shutting off the suction and turning on the nitrogen.

Extraction with Chloroform-Methanol. A mixture of chloroform-methanol (2:1) is the most commonly used and satisfactory solvent mixture for the extraction of lipids from tissue, including brain (Fig. 4). Not only can most lipids be efficiently removed by this solvent, but the extract obtained can be conveniently and thoroughly purified.

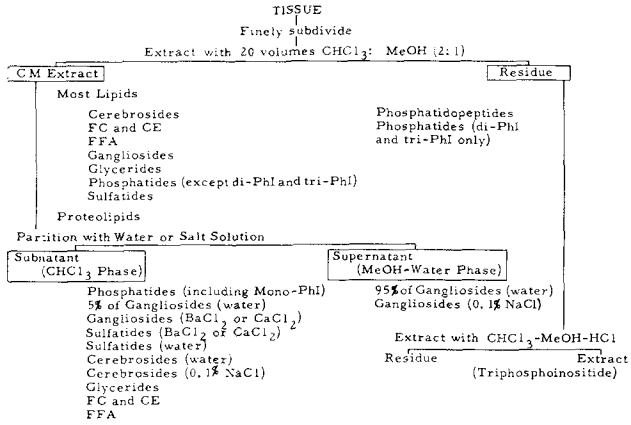

FIG. 4. Extraction of lipids with chloroform: methanol (2:1); partitioning to remove non-lipid contaminants.

The finely subdivided tissue is usually treated with 20 volumes of chloroform-methanol (2:1) at room temperature or in the cold. Most of the lipids and the proteolipids are extracted into the solvent under these conditions. There is some evidence that hot solvent is required to completely extract the gangliosides. The residue remaining after the extraction contains phosphatido-peptides, diphosphoinositides, and triphosphoinositides.

Nonlipid impurities can be removed from the chloroform-methanol by partitioning with water or salt solutions using the techniques of Folch, Lees, and Sloane-Stanley (3). For this the chloroform-methanol extract is mixed with 0.2 its volume of water or salt solution. A biphasic system without any interfacial fluff is obtained after standing overnight or following the centrifugation. The upper phase is removed as completely as possible by means of a pipette and suction, and the inside wall of the tube is rinsed several times with pure solvents upper phase, and removed as before. (Pure solvents upper phase is made by shaking pure chloroform-methanol (2:1) with 0.2 volume of water or salt solution).

The partitioning depends upon the aqueous solution used. If water is used for partitioning, the upper phase contains all of the nonlipid substances, about 95% of the gangliosides and negligible amounts of the other lipids. To get all of the gangliosides in the MeOH-water upper phase and the cerebrosides in the $CHCL_3$ phase, partitioning with 0.1% NaCl solution is used. If $BaCl_2$ or $CaCl_2$ solution is used, most of the gangliosides are found in the lower chloroform phase. To get the free fatty acids into the chloroform phase, partitioning with $CaCl_2$ solution should be used. Successive washing of the lower phase with pure solvents upper phase pull phosphatidyl serine and sulfatides from the lower phase, apparently because of the effect of mineral salts which tend to keep the acidic lipids in the $CHCl_3$ phase. Most of this salt in the chloroform-methanol extract is removed in the first washing.

Recently a method was reported by Bligh and Dyer (4) for the simple extraction of lipids from cod flesh using small volumes of solvent. The method was said to extract the lipids completely and to remove nonlipid impurities as well as is accomplished by the Folch *et al.* procedure (3). Whether the method can be applied to preparation of lipid extracts from various mammalian tissues is not known. The tissue

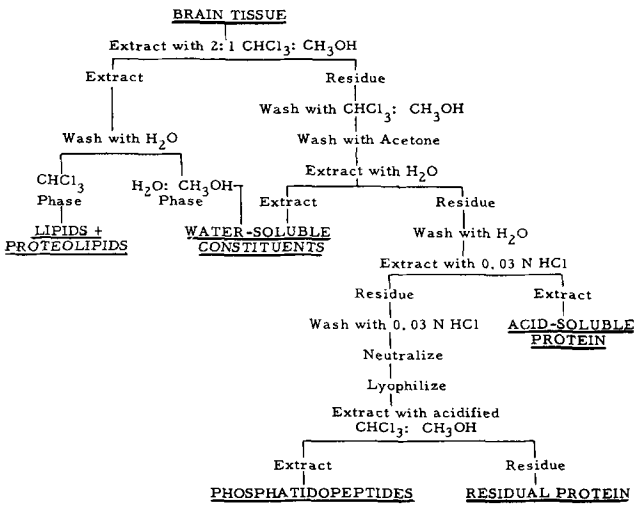

FIG. 5. Separation of phosphatidopeptides.

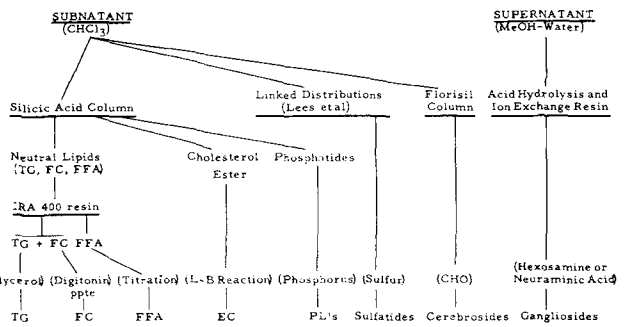

FIG. 6. Determination of lipids in the extracts.

(100 g.) is blended with 100 ml. of chloroform and 200 ml. MeOH for 2 min. (the tissue must contain 80% water or else water is added to make up the difference). Then 100 ml. of $CHCl_3$ are added and blended for 30 sec. Finally, 100 ml. of water are added and blended for 30 sec. The blended material is placed on a Büchner funnel and the filtrate collected under suction. The residue plus filter paper are blended with 100 ml. $CHCl_3$, filtered as before, and the mass washed with 50 ml. $CHCl_3$. The filtrate is allowed to separate into two phases in a graduate cylinder. After noting the volume of the lower $CHCl_3$ phase (containing the lipids), the upper MeOH-water phase is removed by suction and discarded.

Proteolipids. The proteolipids can be extracted by means of chloroform-methanol (2:1) as described above. If the chloroform-methanol extract is partitioned with a large volume of water an upper phase, an interfacial "fluff" and a lower phase are obtained as described by Folch *et al.* (5). The fluff contains the proteolipids. By addition of MeOH (after removing the upper phase) the fluff plus the lower $CHCl_3$ phase become a single clear phase. When this clear phase is evaporated to dryness (with considerable foaming) the proteolipids are denatured and cannot be redissolved in $CHCl_3$ or chloroform-methanol.

Extraction with 20 volumes of $CHCl_3$-MeOH and partitioning with 0.2 volumes of water or salt solution as described above (Fig. 4), however, gives upper and lower phases but no interfacial fluff. The $CHCl_3$ lower phase, containing the proteolipids, can be evaporated to dryness without foaming and without denaturation of the proteolipids.

Gangliosides. Gangliosides in the $CHCl_3$-MeOH extract can be eliminated completely from the lower $CHCl_3$ phase (after partitioning) by 3 washings with pure solvents upper phase containing 0.05% NaCl. The gangliosides may be isolated by combining the washings and dialyzing to remove the salts. The gangliosides are nondialyzable.

If the $CHCl_3$-MeOH extract is partitioned against a solution of $BaCl_2$ or $CaCl_2$, the gangliosides go predominantly to the lower chloroform phase.

Sulfatides. An extract containing most of the sulfatides free from other lipids can be prepared from brain tissue by the method of Lees *et al.* (6) using the procedure termed "linked distributions". Linked distributions consist of distributing the lipids between two phases of a solvent system in which the sulfatides have a distribution coefficient markedly in favor of one of the phases, collecting that phase, adding to it a solvent or combination of solvents to make a second two-phase system, and continuing this type of procedure until the sulfatides are adequately concentrated. The solvents used are added in succession and mixed thoroughly after each addition.

The sulfatides can be purified by precipitation with methanol or by chromatography on a Florosil column.

Phosphatidopeptides. Phosphatidopeptides containing inositol are not extracted (from brain tissue) with neutral solvents such as $CHCl_3$-MeOH (2:1). However, the phosphatidopeptides are extracted with $CHCl_3$-MeOH-HCl (200:100:1). Because the acid, whether present in aqueous solution or in organic solvent, causes the release of acid-soluble protein which contaminates the phosphatidopeptides, LeBaron and Rothleder (7) treated the $CHCl_3$-MeOH insoluble residue with dilute aqueous acid to remove this protein before extraction of the phosphatidopeptide with $CHCl_3$-MeOH-HCl (Fig. 5).

All steps were carried out at 4°C. The fresh brain tissue was extracted with 20 volumes of $CHCl_3$-MeOH (2:1) and the residue obtained extracted 4 times with 10 volumes of $CHCl_3$-MeOH (2:1). The $CHCl_3$-MeOH was removed from the residue by treatment with 10 volumes of acetone, then the water soluble materials were removed from the residue with 10 volumes of distilled water and washed 3 times with distilled water. The viscous water-soluble protein from the tissue residue was extracted with 10 volumes of 0.03N aqueous HCl and washed 3 times with 10 volumes of 0.03N HCl. The material was centrifuged after each wash. The water was removed from the remaining residue by lyophilization, then the phosphatidopeptides were extracted with 10 volumes of $CHCl_3$-MeOH-12N HCl (200:100:1). About 0.7 mg. of phosphatidopeptides were obtained from 1 g. of fresh beef white matter. The extraction is not quantitative.

Triphosphoinositides. According to Dittmer and Dawson (8) when fresh brain tissue is extracted with neutral solvent (e.g., $CHCl_3$-MeOH) the monophosphoinositides are removed, but triphosphoinositide and possibly diphosphoinositide remain in the tissue residue. Pretreatment of brain tissue with acetone, however, altered it such that all inositol phosphatides were extractable with neutral $CHCl_3$-MeOH. Treatment with acidified $CHCl_3$MeOH removed all inositol phosphatides. The method of Dittmer and Dawson (8) described below make use of this latter property.

Ox brain (200 g.) was extracted with 2 l. of $CHCl_3$-MeOH (1:1) and the residue washed with 1 l. of $CHCl_3$-MeOH (2:1). The residue was then washed 3 times with 400 ml. volumes of $CHCl_3$-MeOH (2:1) containing 2 ml. of 12*N* HCl (37°C. for 20 min.). The combined extracts were shaken with 0.2 volume of 0.9% NaCl, centrifuged, and the interfacial material retained. The lower $CHCl_3$ phase was shaken with 0.2 volume of 0.9% NaCl, centrifuged, the interfacial material combined with that obtained above, and the combined material was shaken with 400 ml. of $CHCl_3$: MeOH (2:1) and 80 ml. of 0.9% NaCl, centrifuged, and the interfacial material collected. The interfacial material was heated with 80 ml. of acetone at the boiling point for 2 min. and taken to dryness *in vacuo*. The residue was treated with 80 ml. of ethanol after which the ethanol was evaporated on a boiling water bath and finally *in vacuo*. The residue was extracted twice with 200 ml. of $CHCl_3$-MeOH (2:1) containing 0.05 ml. of 12N HCl (5 min. at 37°C.), and filtered through glass wool. The combined extracts were shaken vigorously with 0.2 volume of *N* HCl, centrifuged, and the lower layer collected. To remove the protein from the phosphatidopeptide, 0.5 volume of methanol was added to the $CHCl_3$ lower layer and shaken with 0.2 volume of *N* HCl. The lower layer was collected, shaken with 0.2 volume of water, centrifuged, and the lower layer containing the crude triphosphoinositide was collected. The triphosphoinositide was purified via its sodium or ammonium salt.

The extracts obtained as described above may be used for the direct determination of some lipids by estimation of phosphorus, sulfur, carbohydrate, fatty acids, etc. (Fig. 6). For the analysis and characterization of other lipids, however, further fractionation is necessary.

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Column Chromatography—Introduction and General Considerations

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THE INVOLVEMENT of lipids, either as actual or potential factors, in heart disease has given impetus to the search for new and better methods for investigation of the lipid classes in biological samples. The advent of gas-liquid chromatography for precise analysis of mixtures of fatty acids has created a specific need for better class separation methods. To a great extent this need has been met by a number of chromatographic techniques, all of which are applicable to particular types of investigations. Among those presently available are cellulose paper, glass paper, thin layer, and column chromatography. In studies where moderate amounts of material must be isolated for further characterization and analysis, techniques of column chromatography are particularly helpful. Specific procedures have been developed which allow collection of milligram quantities which is usually quite adequate for further investigation.

General Considerations

Before introduction of a specialized technique to any group, it is advisable to consider some general facts relative to theory and to develop a mutual understanding with respect to the terminology to be employed.

While ion exchange chromatography has been used to some extent for highly specialized separations column chromatography, as usually applied, depends upon general adsorption phenomena rather than a specific one of ion exchange. In this instance the materials to be separated (solute or adsorbate) are adsorbed on columns of alumina, silica, or other inorganic material (adsorbents), and then desorbed (eluted) with appropriate solvents (eluants). With these factors in mind, let us consider the materials to be selected for a column chromatographic application and the factors involved in the selection of each component.

Adsorbent

In most cases the selection of the adsorbent is the first problem encountered, and is usually the simplest to solve initially since it is somewhat empirical. An obvious requirement is that it be able to adsorb the solutes strongly but not so much that they are difficult to displace. Preferably, it should have a differential affinity for the solutes. The initial choice is usually made on the basis of existing information with final selection based on trial.

Of equal importance in the selection of the adsorbent are some very practical considerations. Particle size should be given special attention. It should be small enough to give a maximum of adsorbtive surface, yet not so small that unrealistic flow rates are obtained from the standpoint of time and other factors. A final problem to consider is the availability and reproducibility of the material. This is particularly true for silicic acid, which is an excellent absorbent for lipid separations, provided duplication of preparation and activation can be achieved from one batch to the other. This difficulty has been a primary objec-