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-

tion to the use of this material for general lipid separations.

Column

Selection of the column size is governed to a great extent by the amount of adsorbent to be used, but both problems should be considered simultaneously. The best separations are usually obtained with long narrow columns simply because there is more room for separation of components. However the more narrow the column the slower the flow will be with any given particle size. A good general practice is to keep the ratio of length to diameter within a range of 5:1 to 15:1 with the final choice made on the basis of the amount of absorbent needed to hold and separate the solute and the desired flow rate. Some experimentation will be necessary before a final selection can be made.

Eluant

In contrast to chromatographic techniques where the column is extruded and the zones carved from the adsorbent, lipid applications involve displacement analysis. This is a special case of development where the eluant is used to displace less well adsorbed substances from the adsorbent causing them to emerge from the column in an orderly fashion, and in fairly distinct zones without appreciable tailing and mixing. Regardless of the adsorbent used, to accomplish the desired result one has to choose the developer or eluant very carefully. In the case of a mixture of lipids whose structures make their affinity for the adsorbent quite similar, the affinity of the adsorbent for the displacing solvent must be just greater than that of the material to be eluted but not great enough to cause displacement of the next solute in order of affinity. In many cases this is difficult to accomplish, particularly in the separation of molecules that may differ only in the number of carbon atoms in a chain.

From a practical standpoint a concept of polarity may be applied to define the relative degree of adsorbent affinity for solutes and solvents and offers a more convenient mode of expression of displacement development. For instance, the naturally occurring lipids of animal or plant tissues may be broadly divided into "neutral" lipids and "polar" lipids. The neutral group usually includes hydrocarbons, cholesterol esters, tryglycerides, cholesterol, mono- and diglycerides and free fatty acids; while the polar group is composed primarily of the phospholipids. It is apparent that free fatty acids are not neutral compounds, but they have been placed with that group because of their relative non-polarity with respect to the polar phospholipids. When mixtures of the lipids are considered in terms of polarity, displacement analysis is more readily understood, and the importance of the choice of eluant for column separation is quite evident.

If one considers a natural lipid mixture from the standpoint of polarity, the individual compounds may be rated with respect to degree of polarity beginning with the least polar hydrocarbons and concluding with the most polar phospholipid, lysolecithin. While the rating may differ with the adsorbent used, the following one based on silicic acid will serve to illustrate the point: hydrocarbons<cholesterol esters<triglycerides<fatty acids<cholesterol<diglycerides<monoglycerides < cephalins < lecithins < sphingomyelins <lysolecithins. From the foregoing it is obvious that the selection of a displacing solvent to separate cholesterol esters from the phospholipids would present no particular problem. However one would need to exercise great care in the selection of a system for the separation of monoglycerides and the other "neutral" lipids from the cephalins and other phospholipids. Even more critical would be separation of the individual members within these two broad classes.

When considering the choice of eluting solvents, one is not limited to use of a separate solvent for each material to be eluted. In practice, solvent pairs are often used and elution carried out with mixtures of the two. A solvent pair usually includes a polar and a non-polar material, the selection of which is based on their ability to elute the most polar and least polar members of the mixture to be resolved. An example of this is the use of petroleum ether (hexane)-benzene mixtures for separation of "neutral" lipids on silicic acid as carried out by Börgstrom (1). Once the selection of solvents is made, the investigator has the choice of two modes of application. These are step-wise elution and gradient elution. As with the choice of eluant, the mode of elution is dependent upon the separation desired.

If the problem is to separate as many individual compounds as possible or to separate a mixture of closely related solutes from the standpoint of their polarity, the choice should be that of gradient elution. In this process the column is developed by the addition of a continuously changing proportion of one solvent in the other member of the pair. Operations of this type require special equipment but are useful

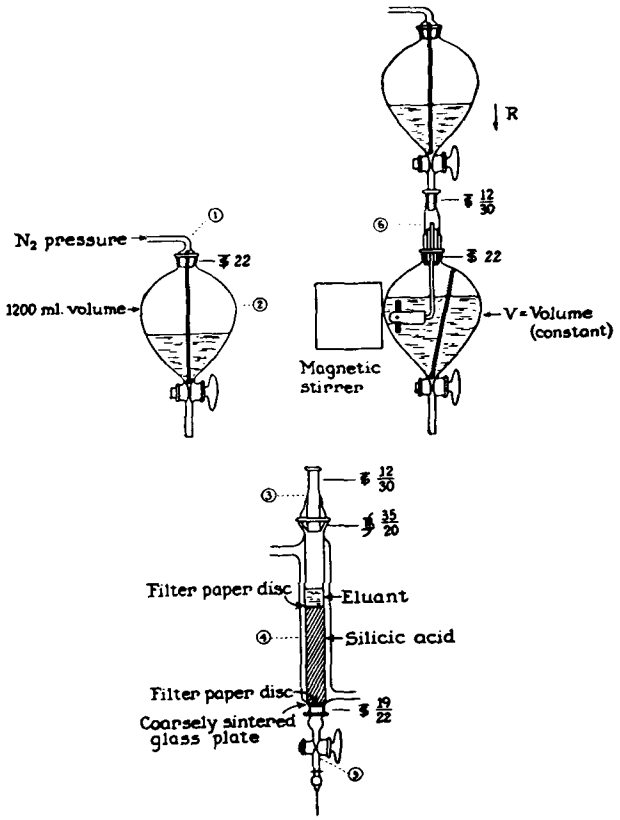

FIG. 1. Apparatus used for column chromatography with silicic acid consisting of a lower assembly and two types of upper assembly. Lower assembly: This shows the jacketed column and surrounding Pyrex parts. The top of this assembly fits into either type of upper assembly. Upper assemblies: The two arrangements shown here are for stepwise elution with a single flask (left) or for gradient elution with two flasks in tandem (right). Reprinted from reference (2).

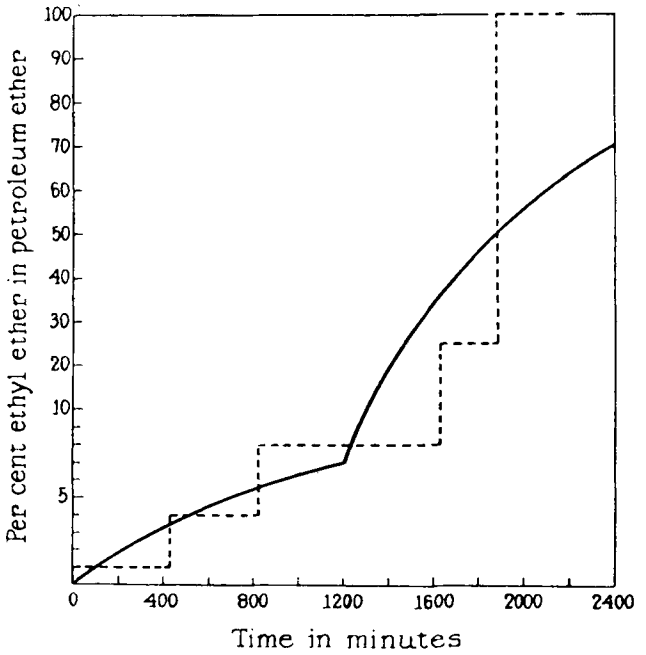

FIG. 2. Solvent composition change with time for a typical gradient (solid line) and stepwise (broken line) elution. In the gradient scheme, 10 per cent ethyl ether in petroleum ether is added at a constant rate to a constant volume of petroleum ether for a period of 20 hours, followed by the addition of ethyl ether (2).

for specific separations. An excellent apparatus for this purpose has been described by Hirsch and Ahrens (2) and is pictured in Fig. 1.

When compounds of somewhat different polarity are to be separated, step-wise elution offers a simple and adequate approach. In this technique the column is simply eluted with pre-determined quantities of different specific mixtures of the solvent pair. An illustration of the solvent changes with time for these two approaches is seen in Fig. 2.

Obviously a third choice is available which utilizes a combination of the continuous gradient and step-wise elution techniques. An operation of this type could be applicable to special situations, but generally one chooses one or the other as a starting point.

In both step-wise and gradient elution, the usual practice is to start with the least polar mixture or member of the pair and increase the polarity by the addition of the more polar material.

In addition to the above considerations, other factors play an important role in the selection of the eluting solvents. The absorbent may have a high affinity for the eluant but the value of the eluant may also be enhanced if it is a good general solvent for the materials to be eluted. Other general requirements are that the eluant be easily removed from the sparated material and that it should not react so as to irreversibly change the nature of the solute.

In this discussion we have considered only the major elements of column chromatography. There are many factors involved in the selection or development of a specific technique and it should be understood that this cannot be considered to be a complete treatise from the standpoint of theory or application. However it will serve as a means of introduction of the subject and to outline some of the problems associated with its use as a laboratory tool. For those who wish a more thorough and authoritative treatment of fundamentals, the work of Cassidy (3) is highly recommended as a starting point.

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Isolation of Neutral Lipids by Column Chromatography

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THE USE of column chromatography for the separation of lipids has been a valuable addition to the techniques for study of these important biological compounds. Historically alumina was the first adsorbent to be applied to lipid problems (1, 2, 3, 4, 5, 6, 7). With the introduction of silicic acid chromatography by Trappe in 1940 (8, 9), a new concept of the use of columns for lipid separations was initiated. More recently (10), Florisil has been introduced as an adsorbent potentially valuable for neutral lipid class separations. Although alumina may have some value in certain specific investigations, its use has been largely supplanted by silicic acid for lipid studies. Therefore this discussion will be oriented mainly toward the use of the latter for neutral lipid separations, but Florisil will also be discussed since it appears to have some advantages over silicic acid.

The value of silicic acid as an adsorbent for lipid separations is clearly demonstrated by a number of methods presently available. After the introduction of this adsorbent, Börgstrom (11) presented a method for separation of several lipid classes by elution with various mixtures of petroleum ether and benzene. This development was followed by other adaptations (12, 13).

Despite the efforts of many investigators, silicic acid separations were often found unreliable. Published methods worked well in the hands of their authors but their use by others often required modification. This prompted Hirsch and Ahrens to initiate a systematic study of all factors involved and to develop a standardized method for application of the technique to lipid class separations (14). This method is one of the most effective yet described. Using mixtures of