Title

A METHOD FOR THE PRODUCTION OF CONCAVE GRADIENTS FOR USE IN COLUMN CHROMATOGRAPHY AND ITS APPLICATION TO THE SEPARATION OF PHOSPHOLIPID AND NEUTRAL LIPID MIXTURES WITH SILICIC ACID COLUMNS

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Publication Date

1962-05-01

University of California

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Contract No. W-7405-eng-48

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SUMMARY

An automatic method for the column chromatographic separation of lipid mixtures, particularly phospholipids, utilizing concave gradient elution is described in detail. The gradient in this system is produced by an automatic pumping system using accurate controlled volume pumps and variable pumping rates. A discussion of the theoretical aspects of gradient elution in relation to non-linear absorption isotherms is included, and several chromatographic run using the system are presented. The system is capable of excellent resolution of naturally occurring phospholipid mixtures and is quite reproducible. Considerable flexibility is available in shape of the gradient produced by the pumping system allowing modification for particular tissue extracts containing phospholipids in varying ratios. The system is completely automatic and, once a run is started, operates untended until completion.

Gradient elution, first introduced by Alm et al. (1,2) in 1948, has only recently been applied to column chromatography of lipids (3,4,5). Early studies with gradient elution systems considered only gradients in which the concentration of the polar or eluting solvent was increased very rapidly at the start of a run and then slowly approached 100% concentration asymptotically in latter part of the run; this is often termed a convex[1] gradient. This type of gradient is relatively simple to produce in the laboratory, but is necessarily a much poorer system than the corresponding linear or concave gradient in which the eluting solvent is introduced gradually at first and then more rapidly so that a continually increasing rate of change of concentration of the eluting solvent is presented to the column.

In the study of serum lipids being carried out in this laboratory (6,7), stepwise silicic acid column chromatography of phospholipids proved inadequate, and a system of concave gradient elution silicic acid column chromatography was developed. This paper will present the details of this system and certain aspects of column performance will be discussed and the results of several chromatographic runs on standard mixtures and natural samples will be shown.

THEORETICAL CONSIDERATION

Martin and Synge (8) developed a theory for partition chromatography which, while not rigorous for adsorption, can be adapted to adsorption processes. Lakshmanan and Lieberman (9) presented a discussion a gradient elution chromatography utilizing partition chromatography theory and concluded that concave gradient elution will provide the best possible elution characteristics if the substance has an adsorptivity described by a non-linear isotherm of the type proposed by Freundlich (10) for batch processes and mathematically represented by the equation:

$$C_s = K (C_m)^n \quad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

where C_s is the amount of solute adsorbed by a given amount of absorbent, C_m is concentration of the solute, and k and n are constants and n is less than 1.

A more rigorous treatment applying to adsorption chromatography per se has been proposed by de Vault (11) and provides additional theoretical support for the belief

that concave gradient elution will give optimum resolution in absorption column chromatography. If diffusion effects are neglected, a single solute with a Freundlich adsorption isotherm will elute off an adsorption column with a sharp front and a gradually tapering trailing edge. In practice diffusion effects will tend to spread the boundary further. deVault has shown that a substance with a linear adsorption isotherm will elute off a column with the shape identical to the initial absorption band on the column, which will be rectangular in a well packed column, if diffusion effects are neglected.

Concave gradient elution can be considered as a way to increase the linearity of a non-linear isotherm. For a single substance a gradient can be constructed to keep the adsorptivity of that substance almost always in the linear region of the absorption isotherm. With complex mixtures this is not possible, but by varying the shape of the gradient in critical regions much improved separations can be obtained over stepwise elution schemes, and "false peaks" caused by discontinuous solvent changes can be entirely avoided.

EXPERIMENTAL APPARATUS

Alm et al. (1) originally proposed a double reservoir system in which an eluting solvent[2] was added to a mixing chamber containing a carrier solvent. As the rates into and out the mixing chamber were equal, the volume in it remained constant. The gradient produced was convex and given by the equation

$$C_t = C_o \left(1-e^{\frac{-RT}{V}}\right) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

where C_t is the composition of solvent leaving the mixing chamber at time, t, in terms of the composition of solvent added, C_o. R is flow rate into (or out of) the mixing chamber and V is the volume of solvent in the mixing chamber.

Lakshmanan and Lieberman (9) added greatly to flexibility of the double reservoir arrangement by allowing the rate into, R_1 and out of, R_2, the mixing chamber to be independent of each other. In this manner they obtained concave, convex, or linear gradients depending on whether R_1 was greater, equal to or less than R_2, and devised rigorous mathematical solutions for each case. More recently several other authors have described apparatus for the production and maintenance of complex gradients (12, 13,14). Perhaps the ultimate in flexibility for the production of gradients was achieved in the "Varigrad" designed by Peterson and Sober (15).

During this study linear as well as convex gradients were found to be inferior to concave gradients, and, hence, will not be discussed further here.

Wren (3) proposed a system for the specific production of a concave gradient adapted from a proposal of Boman (16). While Wren's system gave a wide, uniformly varying gradient, it was not easily adaptable to close regulation of flow rates or an exact mathematical description of the resulting gradient. It was possible to modify this system, however, to allow better regulation as well as greatly increasing the flexibility.

The siphons and pressure differentials were replaced by controlled volume pumps. The shape of the gradient produced in this system is then given by the differential equation in terms of C_t the concentration of eluting solvent leaving the mixing chamber.

$$C_t = \frac{\int C_o R_1 \, dt - \int C_t R_2 \, dt}{V_o + \int R_1 \, dt - \int R_2 \, dt} \quad \ldots\ldots\ldots\ldots\ldots (3)$$

where R_1 and R_2 are as given previously, C_o is the concentration of eluting solvent added to the mixing chamber, and V_o is the volume of carrier solvent in the mixing chamber at time zero. This equation is similar to that given by Lakshmanan and Lieberman (9) except that R_1 is a variable of the form:

$$R_1 = at \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots (4)$$

where a is an arbitrary constant determined by a pump setting and has units of ml/min^2.

Substituting equation (4) into equation (3) and differentiating yields

$$\frac{dc}{(C_o - C)} = \frac{at\,dt}{\left(V_o + \frac{at^2}{2} - R_2 t\right)} \quad \ldots\ldots\ldots (5)$$

which can be solved immediately by integration. The forms of the solutions are:

If $2aV_0 = R_2^2$

$$C = C_0 \left[1 - \frac{R_2^2 e^{2\left(1 + \frac{R_2^2}{at - R_2}\right)}}{(at - R_2)^2}\right] \quad \ldots\ldots\ldots (6)$$

If $2aV_0 < R_2^2$

$$C = C_0 \left[1 - \frac{V_0 \left(\frac{R_2 + \sqrt{R_2^2 - 2aV_0}}{R_2 - \sqrt{R_2^2 - 2aV_0}}\right)^{\left(\frac{R_2}{\sqrt{R_2^2 - 2aV_0}}\right)}}{\left(V_0 + \frac{at^2}{2} - R_2 t\right)\left(\frac{at - R_2 - \sqrt{R_2^2 - 2aV_0}}{at - R_2 + \sqrt{R_2^2 - 2aV_0}}\right)^{\left(\frac{R_2}{\sqrt{R_2^2 - 2aV_0}}\right)}}\right] \quad \ldots\ldots\ldots (7)$$

If $2aV_0 > R_2^2$

$$C = C_0 \left[1 - \frac{V_0 e^{\frac{2R_2}{\sqrt{2aV_0 - R_2^2}} \tan^{-1}\left(\frac{-R_2}{\sqrt{2aV_0 - R_2^2}}\right)}}{\left(V_0 + \frac{a}{2}t^2 - R_2 t\right) e^{\frac{2R}{\sqrt{2aV_0 - R_2^2}} \tan^{-1}\left(\frac{at - R_2}{\sqrt{2aV_0 - R_2^2}}\right)}}\right] \quad \ldots\ldots\ldots (8)$$

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The three solutions are necessary because the radical $\sqrt{\pm(R_2^2 - 2aV_o)}$ appears and is indeterminate where $\pm (R_2^2 - 2aV_o)$ assumes a negative value or zero. Figure 1 gives examples of the curves obtained from each solution with various initial conditions. The curves are sigmoidal over the full range. The parameters a, R_2 and V_o can each be varied independently; thus, one solution is capable of yielding quite varied gradients. Figure 2 illustrates this using equation 7.

DESIGN OF APPARATUS: Figure 3 is a diagramatic sketch of the apparatus capable of producing the gradients described above mathematically. The volume of solvent chambers shown in the figure are those that were found convenient for the 20 gm columns most commonly used in this laboratory. The critical parts of the assembly are the gradient and monitor pumps and the control for the gradient pump. Both pumps are "chromatographic miniPumps" No. CH-MM-29B designed by the Milton Roy Company, Philadelphia, Pa. These pumps have no organic soluble substances in the liquid head and are capable of pumping quite accurately liquids of low viscosity and high vapor pressure. The pumping action is accomplished by a reciprocating piston whose stroke can be varied from zero to 100% of capacity by a micrometer adjustment.

The motor on the gradient pump is a 1/15 h.p.-D.C. motor made by Bodine Electrical Company, Chicago, Illinois, and is connected to an automatic control which increases the rpm of the motor linearly with time, starting at very low rpm. This control was designed by Frank Upham of this laboratory's Electronics Division. Figure 4 is the schematic circuit diagram of this control.

A linear increase in rpm of the motor produces a linear increase in pumping rate. The total volume, V, delivered by the pump at time, t, is

$$V = \frac{1}{2} at^2 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (9)$$

a can be calculated from this relationship by collecting the volume delivered by the pump during a specific interval. The time for the pump motor to reach its maximum rpm is taken as t_{max}. t_{max} is varied by a gear change on the motor control. a can

be varied independently of t_{max} by changing the length of the piston stroke. Generally t_{max} is constant for columns of the same size.

The motor is started, however, at an rpm somewhat greater than zero, approximately 1/20 of its maximum rpm. This produces a small deviation in the value of a predicted by equation (9), and consequently in the gradient for times close to zero. Figure 5 presents a calculated and observed gradient over the first 400 minutes of a run in which t_{max} had a value of 1000 minutes. For runs with t_{max} equal to or greater than 1000 minutes the effect of this small deviation from the theoretical gradient should be negligible. However, on short runs corrections must be applied.

The second or monitor pump, also a "chromatographic miniPump" identical to the gradient pump except that a constant speed A.C. motor is used in place of variable speed motor on the gradient pump, is used to transfer the eluting solvent from the mixing chamber to the column at a constant rate, R_2. Originally, gravity and pressure feeds were used in place of this pump but were inadequate to maintain a constant flow rate for any extended time period. A metering value[3] attached to this pump at the outflow increases its accuracy. Experimentally, this pump delivered 1 ± .08 ml/min when pumping a gradient which varied from 100% chloroform to 100% methanol during 1000 minutes.

COLUMN HOUSING: A glass column housing which was water jacketed and similar to the design of Hirsch and Ahrens (17) was used. The laboratory water supplied was found to vary only 3°C, making thermostatic control unnecessary. A medium porosity fritted glass disc[4] held in place by a carefully machined Teflon gasket which in turn rested on a slight constriction at the bottom of the column supported the packing in the column. A coarse porosity disc[4] was used to protect the top of the column from disturbance after packing. Teflon stopcocks[5] were used throughout the apparatus to prevent leakage and eliminate the need for stopcock grease.

PREPARATION OF COLUMN PACKING: As the monitor pump is capable of maintaining a constant flow rate against high back pressures, no filter aid was added to the silicic acid packing. Columns were packed with pure silicic acid obtained from Mallinckrodt Chemical Co., 100 mesh, which was re-sieved to give a fraction passing 100 mesh but retained by a 325 mesh screen. The sieved silicic was then washed with successive portions of distilled water, methanol[6], and acetone and dried for 24 hours at 120°C, then stored in a sealed jar.

To pack a column, 20 gm of silicic acid was weighed and heated between 110° to 130° C for 48 hours in a vacuum oven immediately before use, then cooled and slurried with a solvent and poured as the slurry into the column housing. A minimum amount of additional solvent was used to wash the packing down the column and the coarse porosity glass disc placed on top of the packing. The disc was pressed down until the column appeared closely packed under visual inspection. The extra solvent above the level of the disc was removed, and the charge added to the column.

CHROMATOGRAPHIC PROCEDURE: The lipid extract was added to the column in the same solvent used to prepare the column packing. If neutral lipids were being analyzed, normal hexane was used; if only the phospholipids were of interest, chloroform was used. The charge was added to the column in 2 ml of solvent and washed onto the column with an additional 2 ml. When neutral lipids were being fractionated, gradient elution with a gradient of ethyl ether in normal hexane is begun immediately after the charge is placed on the column. When the gradient reaches pure ethyl ether, a methanol in chloroform gradient can be started to fractionate the phospholipids.

If, as was the case in most studies carried out here, neutral lipids were not separated in the same run, the column was prepared with chloroform. Before the gradient separation of the phospholipids was begun, 1000 ml of chloroform were run through the column to remove all neutral lipids in a single fraction (8). The flow

rate through a 20 gm column was 1 ml per minute. The charge added to the column yielded good resolution for most mixtures when it was between 3 and 4 mg of phospholipid per g of silicic acid. A slightly higher value was found for the neutral lipids. The resolution of the neutral lipid and phospholipids fractions was found to be independent and, hence, a 50-50 mixture of phospholipids and neutral lipid could be added to the column at 8 to 12 mg per g of silicic acid. No single component should exceed 3 mg per g of silicic acid in this system.

In most runs where multiple fractionation was carried out 10 ml aliquots were collected. As the flow rate through the column was constant at 1 ml per minute, time indexing was used to collect fractions[7].

LIPID EXTRACTION: Lipids from serum and liver were extracted as previously reported (8, 18). Red cell lipids were extracted in essentially the same manner except the cells were first resuspended and washed twice with 0.9N saline solution. 20 ml of packed cells were then transferred to 180 ml. of 50-50 methanol-chloroform solution and homogenized for two minutes in a Waring Blendor. The subsequent extraction steps were carried out as described (18).

Standard samples of l-α dipalmitoyl phosphatidyl ethanolamine and l-α dipalmitoyl phosphatidyl choline were obtained from California Corporation for Biochemical Research, Los Angeles, California. Sphingomyelin standards were prepared by the method of Rapport and Lerner (19) and rechromatographed on silicic acid. Lysolecithin was prepared by the method of Long and Penny (20). Monophosphoinositol was prepared from mouse liver (18). No pure phosphatidyl serine or cardiolipin standards were obtained.

Neutral lipid standards were obtained from the following sources. Palmitic acid and tripalmitin, the Hormel Foundation, Austin, Minnesota. Free cholesterol, Armour Laboratories, Chicago, Illinois. Cholesteryl laurate was synthesized from the free cholesterol and lauric acid by Dr. D. Kritchevsky. Saturated mono-and diglycerides were prepared from hydrogenated cottonseed oil by partial hydrolysis in 1N NaOH for 1 hour at 37° C and isolated by silicic acid chromatography.

ANALYTICAL METHODS: Neutral lipids were characterized by infrared spectrophotometric techniques (21,22) and thin layer chromatographic methods (23). Phospholipids were also analyzed by these methods and fractions were monitored by chemical phosphorus determinations performed as described previously (24). The solvent system used for separation of the phospholipid mixture on the thin layer chromatograms was diisobutyl-ketone, acetic acid, water (70-20-10 V/V). Spots were developed by spraying the dried plates with concentrated H_2SO_4 and charring (18).

In addition, specific chemical determination were performed on aliquots taken from various chromatographic fractions by the methods listed below: Glycerol was determined by the method of Van Handel and Zilversmit (25) as modified by Van Handel (26). Ethanolamine and serine by the method of Dittmer et al. (26). Choline by the procedure of Wheeldon and Collins (28), and sugars were analyzed by the anthrone reaction of Radin et al. (29). Sphingosine was determined by its infrared spectra after isolation from the neutralized aqueous phase of the transmethylation procedure (7) with chloroform.

These tests were run only on a qualitative basis to indicate the absence or presence of the various compounds. No quantitative results were obtained and no criterion of purity was established. Naturally in any specific study more rigorous control procedures would be necessary, but exact procedures would vary with the purpose of the separation.

RESULTS

Figure 6 shows the elution pattern of standard mixture of neutral lipids from a 20 gm silicic acid column. Table I gives the recoveries of the various component on a weight basis. The cholesteryl esters and triglycerides were fully resolved, while the free fatty acids, cholesterol and diglycerides peaks overlapped slightly which is reflected in the lower recovery of free fatty acids and diglycerides while the cholesterol is greater than 100%. This column was not fully activated which probably decreased its resolving power. Satisfactory methods of neutral lipid silicic

chromatography have been developed by other workers (17), so no further attempt was made to improve this system for neutral lipids.

Figure 7 is the elution pattern of a known mixture of phospholipids obtained from the sources listed above. All components except lecithin and sphingomyelin are well resolved. And even these hard to separate compounds are fairly well resolved. Infrared spectrophotometric measurements indicated that the collection tubes 80 through 95, the sphingomyelin peak, contained 5% of the lecithin added to the column while tubes number 69 through 86, the lecithin peak, contained approximately 3% of the sphingomyelin. Most of the contamination is in tubes 77 through 85. Table II gives the recoveries of the various phospholipid in terms of moles percent phosphorus added to the column.

In figures 8, 9 and 10 the phospholipid elution patterns obtained from normal human serum, normal human red blood cells, and mouse liver are shown. The shape of the gradient, column size and other conditions were the same in each case. It can be seen from these runs that over a wide variation in the amount of individual components present, the major phospholipids are eluted at the same point in the system, plus or minus 2 collection tubes (20 ml of solution). This allows batch collection of the major phospholipids eluted by such a system after initially determining the elution position of the various components which will depend on the shape of gradient used.

Regardless of the shape of the gradient however the order of elution of different phospholipids from silicic acid column remains constant. Figure 11 summarizes this sequence as determined by the analytical methods listed above for the gradient elution from 20 gm columns in this laboratory. Aside from phosphatidyl ethanolamine and phosphatidyl serine, and the plasmalogen analogues of various phospholipids most naturally occurring phospholipids can be separated by this scheme. Lecithin and sphingomyelin overlap slightly. Variations in the ratios of the phospholipids present in a mixture will effect the resolution to some extent although perhaps less than one would expect and certainly less than in non gradient systems.

In the samples investigated here no material eluted off the column after lysolecithin, although the triphosphoinositol reported recently by Tomlison and Ballou (30) and Dittmer and Dawson (31) would be expected to elute after lysolecithin/if it can be eluted from silicic columns at all.

In serum and liver, little or no cerebrosides or ganglioside are present, and, hence, would not contaminate the various phospholipid fractions obtained by chromatography. In erythrocytes and other tissues various amounts of these compounds are present and have been observed to elute with the phospholipids. In chromatography of the red blood cell phospholipids shown in Figure 9 cerebroside like material was found in tubes 15 through 20 and 45 through 60, and amounted to approximately 10% of the total lipids extracted from the erythrocytes by methanol-chloroform. While no detailed studies of the elution characteristics of the cerebrosides were attempted, it is feasible that these sphingolipides can be also resolved from phospholipids by this procedure.

The phospholipids which eluted with low concentrations of methanol from the column exhibit much more symmetric elution peaks than those needing higher concentrations. Thus, the primary reason for the overlapping of the lecithin and sphingomyelin peaks is not the similarities of the migration rates, but the tailing of lecithin fraction into the sphingomyelin peak caused by this asymmetry. The synthetic lecithin containing only palmitic acid produced an asymmetric elution peak as well as the natural lecithins, hence this tailing can not be attributed solely to differences in the fatty acid moieties of the molecule. More likely it is a result of the non linear adsorption isotherm of lecithin on silicic acid.

Conversely the distortions of phosphatidyl ethanolamine elution peak at its base observed in natural samples is most probably due to other, unidentified lipids, as the pure synthetic phosphatidyl ethanolamine gave a very symmetrical elution pattern. Generally, the symmetry of the elution peak is inversely related to concentration of methanol needed to remove the substance from the column. The shape of the elution gradient can also influence the shape of the elution peak.

DISCUSSION

The results presented in this study indicate that concave gradient elution providesa technique capable of separating lipid mixtures with good resolution and without the necessity of multiple solvent changes during the run. Not only are so-called "false peaks" avoided but the method lends itself to complete automation. Once a run is started no attention need be payed to it until it is finished, thus, allowing a run to continue overnight and for extended periods not feasible in manual operations.

While Hirsch and Ahrens (17) as well as Hanahan et al.(32) had previously indicated that gradient elution column chromatography did not offer any advantages over stepwise schemes and, in fact, actually reduced the resolving power of the column, only convex gradients were investigated by these workers. Conversely, the present study indicates that concave gradient elution of phospholipid mixtures yields better separations than any constant solvent or stepwise system operated under comparable conditions.

The activation of the column packing to the same state is absolutely necessary to obtain reproducible results from run to run, and a packing is good for only one run in this system. No solvent rejunvenation was able to return a packed column to its initial degree of activation. Furthermore,[a] column packing that was not fully activated showed poorer resolving power and shorter elution times than fully activated columns. The most prominent change observed with deactivated columns was a shortened retention time for the various lipids, either neutral lipids or phospholipids.

Several authors (33,34,35) have added water to their solvents in stepwise elution systems. In the recent scheme of Newman, Chiu and Zilversmit (35) the authors indicated that additions of 3% H_2O to their methanol greatly improved the resolution of the system. Addition of 3% H_2O to the methanol (the commerical reagent grade methanol used here had .05% H_2O) produced no noticeable improvement in resolution with the gradient elution system reported here.

The initial choice of a gradient is arbitrary. When some prior knowledge of the elution characteristics of the system is available, a gradient picked to approximate a stepwise scheme can be choosen. If two components are not well resolved, a reduction in the slope of the gradient will often provide a better separation. Sometimes it is possible to separate compounds having identical elution times under one set of conditions by appropriate changes in the shape of the gradient.

The total cost of duplicating such equipment should be under 2,000 dollars which, while high relative to most chromatographic systems, is not particularly expensive compared to many commonly used analytical instruments found in modern laboratories. The superior performance of the system is believed to warrant the added cost.

In operation the system was found to be very reliable. A reliable automatic fraction collector and indexing device is a necessity for monitoring runs during developmental stages of an investigation. Batch collection can be used after the reproducibility of the system for a given mixture has been determined. It is advantageous to use a master timer to supply power to all components of the system. The timer then automatically shuts off all apparatus at the end of the run.

The use of this system for producing concave gradients is not restricted to silicic acid column chromatography of lipids. Any column chromatographic system would be applicable as would many ion exchange separations. Piez and Morris (36) have already indicated that gradient elution of amino acid hydrolysates on ion exchange column using buffered solutions of different ionic strength greatly enhances the operation.

Another modification of this system involves the addition of a second gradient pump leading to the mixing chamber, which would add a third solvent to the mixing chamber, and lead to tertiary, rather than binary gradients. The mathematics of this type of system have already been investigated by the author and present no unusual difficulties. Whether such complex gradients can provide additional advantages to the chromatographic separation of lipid mixtures remains to be investigated.

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* This work supported in part by the United States Atomic Energy Commission and the U.S. Public Health Service, Grant Number H-2029 (C-6).

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1. Alm et al. (1) proposed the termed "gradient elution" to cover the method of chromatography discussed in their paper. The authors discussed a gradient having a large initial slope and which apprcached an abscissa assymptotically. Lakshmanan and Lieberman (9) later showed that a gradient of this type had definite disadvantages compared to a gradient whose slope was initially low and gradually increased as the chromatography was continued. Later investigators have tended to differentiate these two types of gradients by designating the first as a "convex gradient" and the second as a "concave gradient". A third type of gradient, called "linear", has a constant slope during the chromatography. Mathematically the second derivative of convex gradient is negative; of a concave gradient, positive; and of a linear gradient, zero.

2. The solvents used in gradient elution systems have a confused nomenclature. Some authors refer to different solvents by letters or numbers, others "polar" or "non-polar" or "relatively more polar". In this paper a solvent which will not cause a noticeable migration of a compound on a column during the usual period of a chromatographic run is termed a "carrier solvent" referring to the fact that this solvent is a carrier or diluent for the solvent producing the migration of the compounds on the column. This latter solvent is referred to as the "eluting solvent".

3. Obtained from Nuclear Products Co., Cleveland 10, Ohio.

4. Obtained from Fischer and Porter Co., Warminster, Pennsylvania.

5. Kimax brand Teflon Stopcocks obtained from Kimble Glass Co. through various laboratory supply houses.

6. All solvents used in all phases of this work were reagent/commerical grade solvents. The chloroform contained 0.75% ethanol.

7. The automatic fraction collector with time indexing units was obtained from Research Specialities Co., Richmond, California.

FIGURE CAPTIONS

Figure 1. Graphical presentation of the gradients obtained under the conditions indicated on the figure for the three forms of the solution to equation 3 in the text. Curves C_1, C_2 and C_3 are solutions of equations 6, 7, and 8 respectively. The values of the parameters a, V_o, and R_2 are listed for each solution.

Figure 2. Some of the possible variations in the gradients produced by this system are shown. Solutions are obtained from Equation 7. R_2 = 1 ml/min. in all cases. Curve I presents a case in which additional solvent was added to the mixing chamber 100 minutes after the start of the run. The values for a and V_o are listed for each solution.

Figure 3. A diagramatic sketch of the gradient elution column chromatographic system described in the text. A is the eluting solvent reservoir from which the solvent is transferred to the mixing chamber (B) by the gradient pump. A specific volume of carrier solvent is placed in the mixing chamber before the start of the run. The exact amount is determined by shape of the gradient and length of the run and calculated by equations 6, 7, or 8 given in the text. The outflow of the mixing chamber is pumped to the column (D) by the monitor pump. C is an additional solvent reservoir for non gradient elution.

Figure 4. Schematic circuit diagram for the gradient pump motor control.

Figure 5. Curves showing experimentally observed deviation of gradient from ideality in the low concentration range caused by the gradient pump's initial rpm being greater than zero. Dotted curve is experimentally observed gradient. Solid curve is gradient calculated by equation 7 in the text.

Figure 6. The experimentally determined elution pattern obtained by gradient elution chromatography of a known mixture of neutral lipids from a 20 g silicic acid column. The gradient was diethyl ether in normal hexane. The shape of the gradient is shown on the figure. The left ordinate is the optical density of a 0.15 ml solution of the material, recovered from 1.0 ml of

FIGURE CAPTIONS (Cont'd)

eluant solvent, in CCl_4 in a 3.0 mm path length cell. The various peak heights are dependent on the specific absorption coefficient of each substance and not directly comparable to each other in the Figure. The right ordinate is the percent of diethyl ether in the gradient. See text for details of method and recovery data.

Figure 7. The experimentally determined elution pattern obtained by gradient elution chromatography of a known mixture of phospholipids obtained from sources listed in the text. 20 g silicic acid column. The shape of gradient is indicated by the dotted line. The gradient was methanol in chloroform, and determined theoretically by equation 7 of the text. The values for the various parameters were: $a = .0007$ ml/min^2; $R_2 = 1$ ml/min; $V_o = 650$ ml; and $t_{max} = 1000$ min. After 1000 min. elution was continued with pure methanol for an additional 400 min. Collection tubes 40 to 50, 69 to 85, 75 to 97, and 98 to 120 contained phosphatidyl ethanolamine, lecithin, sphingomyelin, and lysolecithin respectively. See text for detail of method and recovery data.

Figure 8. Experimentally determined elution pattern obtained by gradient elution chromatography of phospholipids extracted from normal human serum. Points on the curve represent phosphorus determinations on samples from every odd number collection tube. The methanol in chloroform gradient was produced by conditions identical to those described in the caption to Figure 7. Weight added to column was 2.89 mg of P, and 2.94 mg. were recovered as determined by chemical P analysis. Main components can be located by reference to Figure 7. See text for details of method.

Figure 9. Experimentally determined elution pattern obtained by gradient elution chromatography of phospholipids extracted from normal human erythrocytes. Chromatographic conditions are described in the captions to Figures 7 and 8. Weight added to column, 2.73 mg P; recovery, 2.77 mg P. Collection tubes 18 to 30 and 56 to 66 contained polyglyceryl phosphatides and monophosphoinositol respectively. Main components can be located by reference to

FIGURE CAPTIONS (Cont'd)

Figure 7. See text for details of method.

Figure 10. Experimentally determined elution pattern obtained by gradient elution chromatography of phospholipids extracted from normal mouse liver. Chromatographic conditions are described in the text and in the captions to Figures 7 and 8. Weight added to column, 2.95 mg P; recovery, 2.90 mg P. Components can be located by reference to Figure 7, 8, and 9.

Figure 11. A schematic presentation of the relative elution characteristics of different phospholipids in the gradient elution system described in the text as determined by chromatography of reference compounds and specific chemical tests on the fractions obtained from chromatography of natural phospholipid mixtures extracted from serum, red cells and liver. Fully activated column eluted with methanol in chloroform gradient. The order of the sequence will be constant in any gradient elution although the particular concentration of methanol at which any individual phospholipid will elute from a column depends on the size of the column, the flow rate of the solvent and the shape of the gradient.

Table I

Recoveries of Neutral Lipid From Chromatography of Standard Mixture Shown In Figure 6

(Gravimetric Determination)

Lipid	Collection Tube Numbers	Wt. Added to Column mg.	Wt. Recovered mg.	Percent Recovery
Cholesteryl laurate	2-10	58.3	58.1	99.6
Tripalmitin	11-20	59.1	60.7	102.8
Palmitic acid	21-30	21.2	19.8	93.4
Free Cholesterol	31-46	37.9	40.3	105.9
Diglyceride, Monoglyceride	47-110	12.3	11.1	90.2
Total		188.8	190.0	100.7

Table II

Recoveries of Phospholipids from Chromatography of Standard Mixture Shown In Figure 7

(Mole % Phosphorus Added to the Column)

Phospholipid	Collection Tube Numbers	µgm P added to column	µgm P recovered	Percent Recovery
L-α (dipalmitoyl) phosphatidyl ethanolamine	35-55	880	885	100.7
L-α (dipalmitoyl) phosphatidyl choline	69-85*	1976	1940	98.1
Sphingomyelin	75-97*	735	738	100.4
Lysolecithin	98-120	370	394	106.5
Total		3961	3957	99.9

* Overlapping tubes analyzed by infrared spectrophotometry

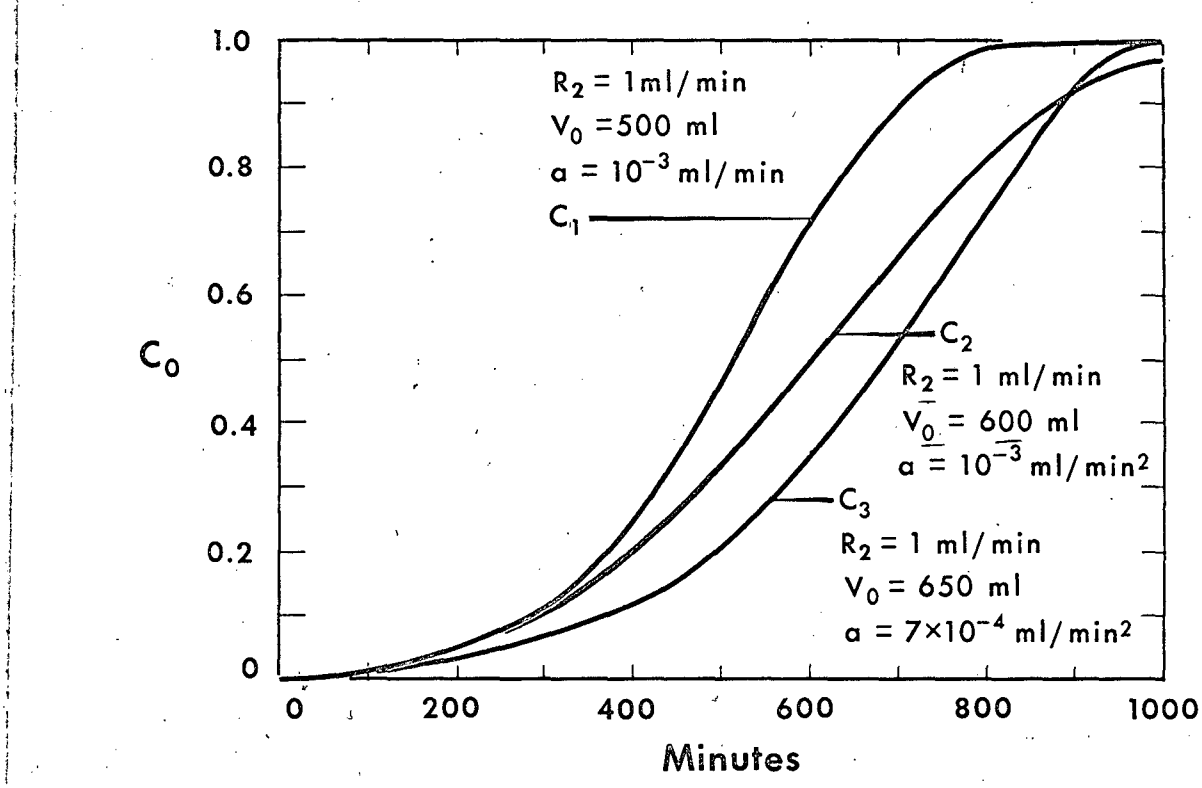
1.0
0.8
0.6
0.4
0.2
0
C_0
0
200
400
600
800
1000
Minutes
R_2 = 1ml/min
V_0 =500 ml
a = 10^{-3} ml/min
C_1
C_2
R_2 = 1 ml/min
V_0 = 600 ml
a = 10^{-3} ml/min^2
C_3
R_2 = 1 ml/min
V_0 = 650 ml
a = 7×10^{-4} ml/min^2

Fig. 1

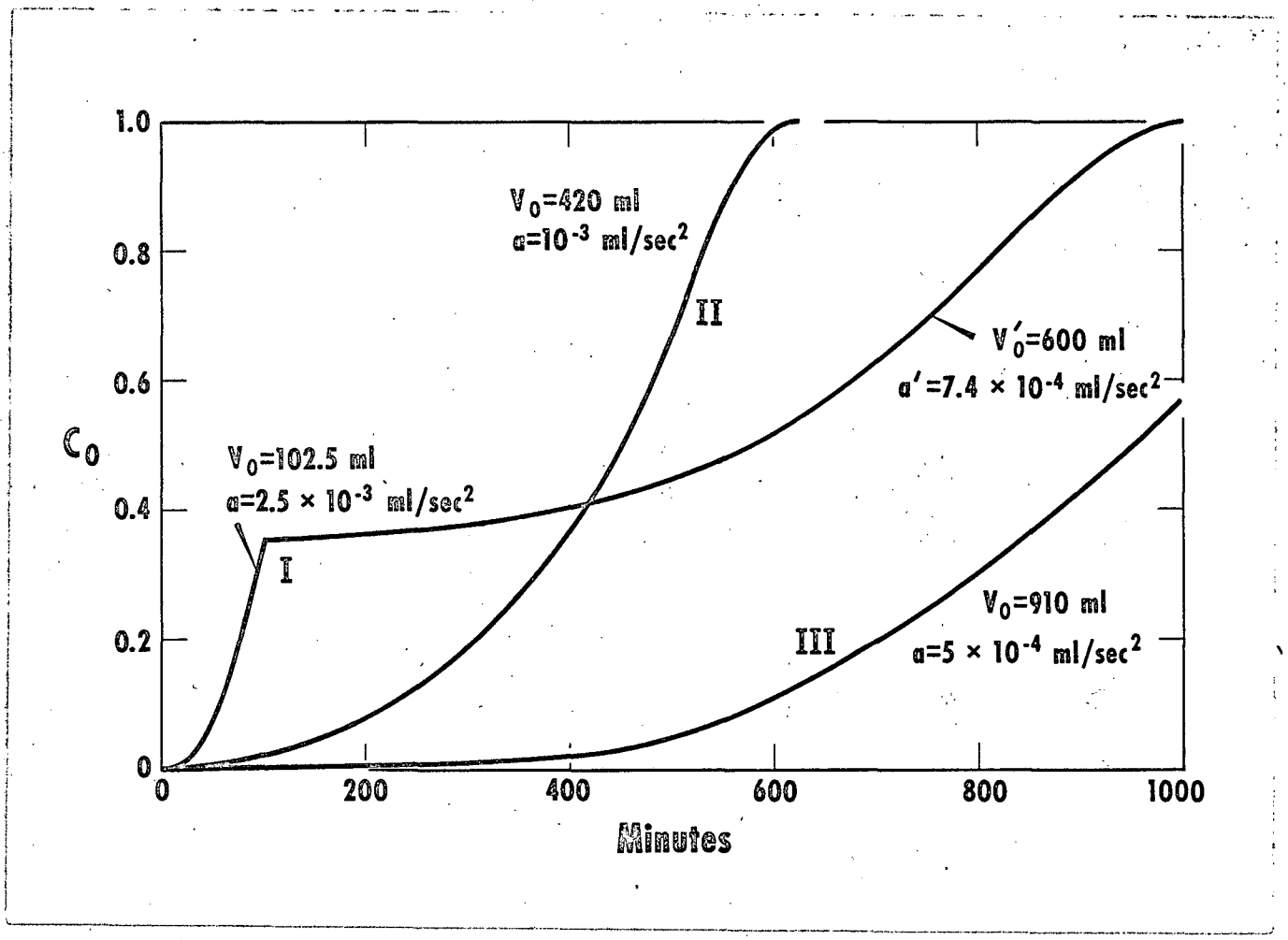
1.0
0.8
0.6
0.4
0.2
0
C_0
V_0=420 ml
a=10^{-3} ml/sec^2
II
V_0'=600 ml
a'=7.4 × 10^{-4} ml/sec^2
V_0=102.5 ml
a=2.5 × 10^{-3} ml/sec^2
I
V_0=910 ml
a=5 × 10^{-4} ml/sec^2
III
0
200
400
600
800
1000
Minutes

Fig. 2

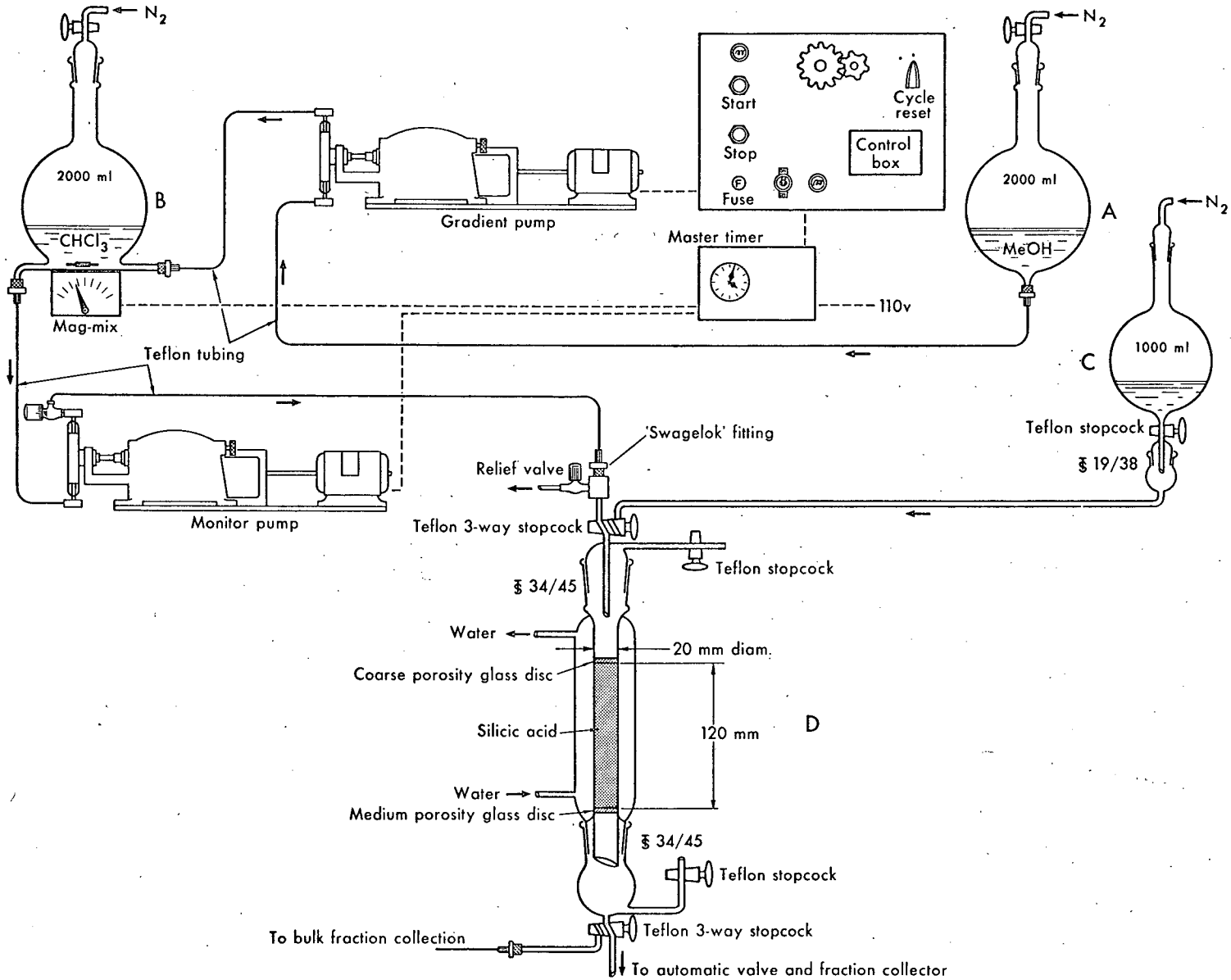
N2
2000 ml
B
CHCl3
Mag-mix
Teflon tubing
Gradient pump
Start
Stop
Fuse
Cycle reset
Control box
Master timer
110v
N2
2000 ml
A
MeOH
N2
1000 ml
C
Teflon stopcock
§ 19/38
'Swagelok' fitting
Relief valve
Monitor pump
Teflon 3-way stopcock
Teflon stopcock
§ 34/45
Water
20 mm diam.
Coarse porosity glass disc
Silicic acid
120 mm
D
Water
Medium porosity glass disc
§ 34/45
Teflon stopcock
To bulk fraction collection
Teflon 3-way stopcock
To automatic valve and fraction collector
MUB-891

Fig. 3.

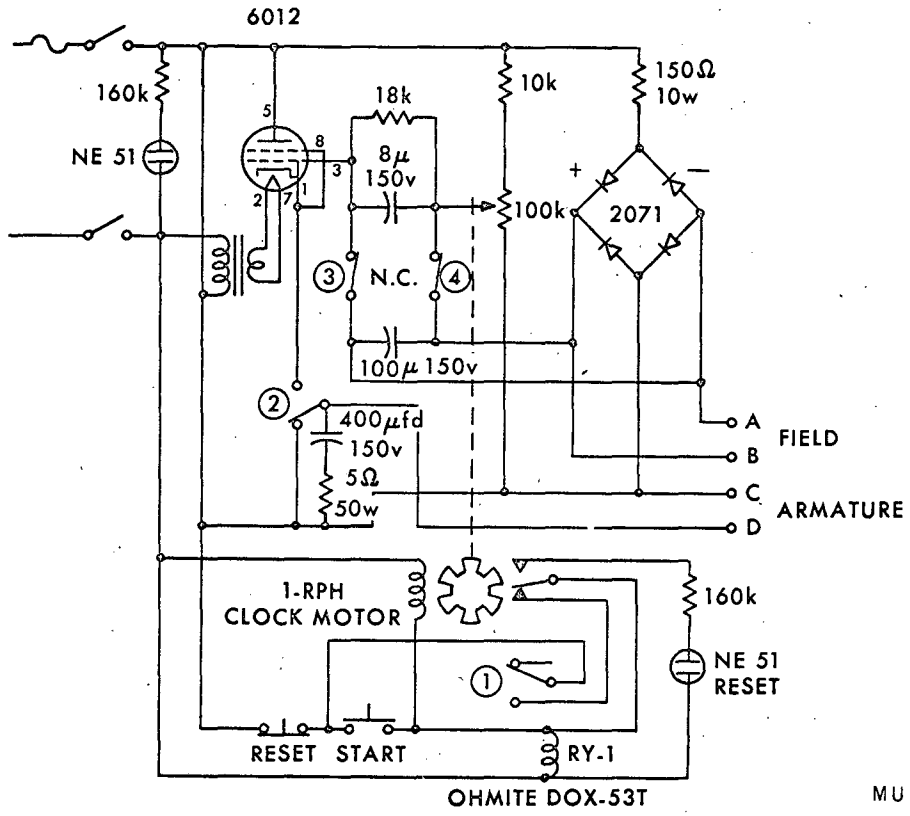
6012
160k
NE 51
18k
8μ
150v
10k
150Ω
10w
100k
2071
+
—
N.C.
100μ 150v
400μfd
150v
5Ω
50w
A
B
C
D
FIELD
ARMATURE
1-RPH
CLOCK MOTOR
160k
NE 51
RESET
RESET START
RY-1
OHMITE DOX-53T
MU—24308

Fig. 4

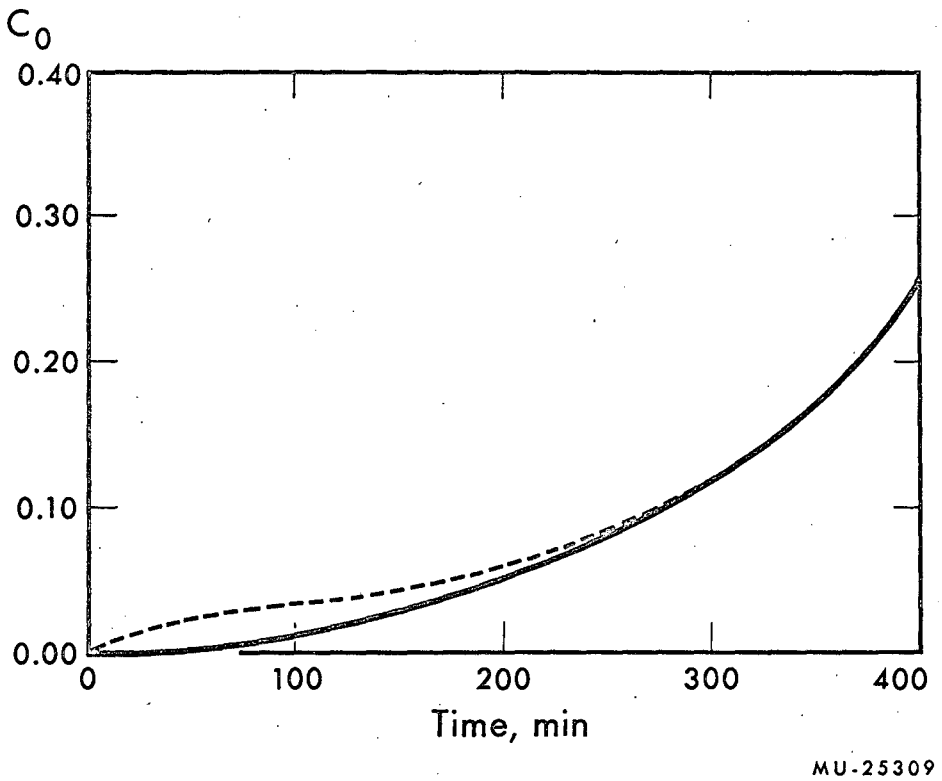

Fig. 5

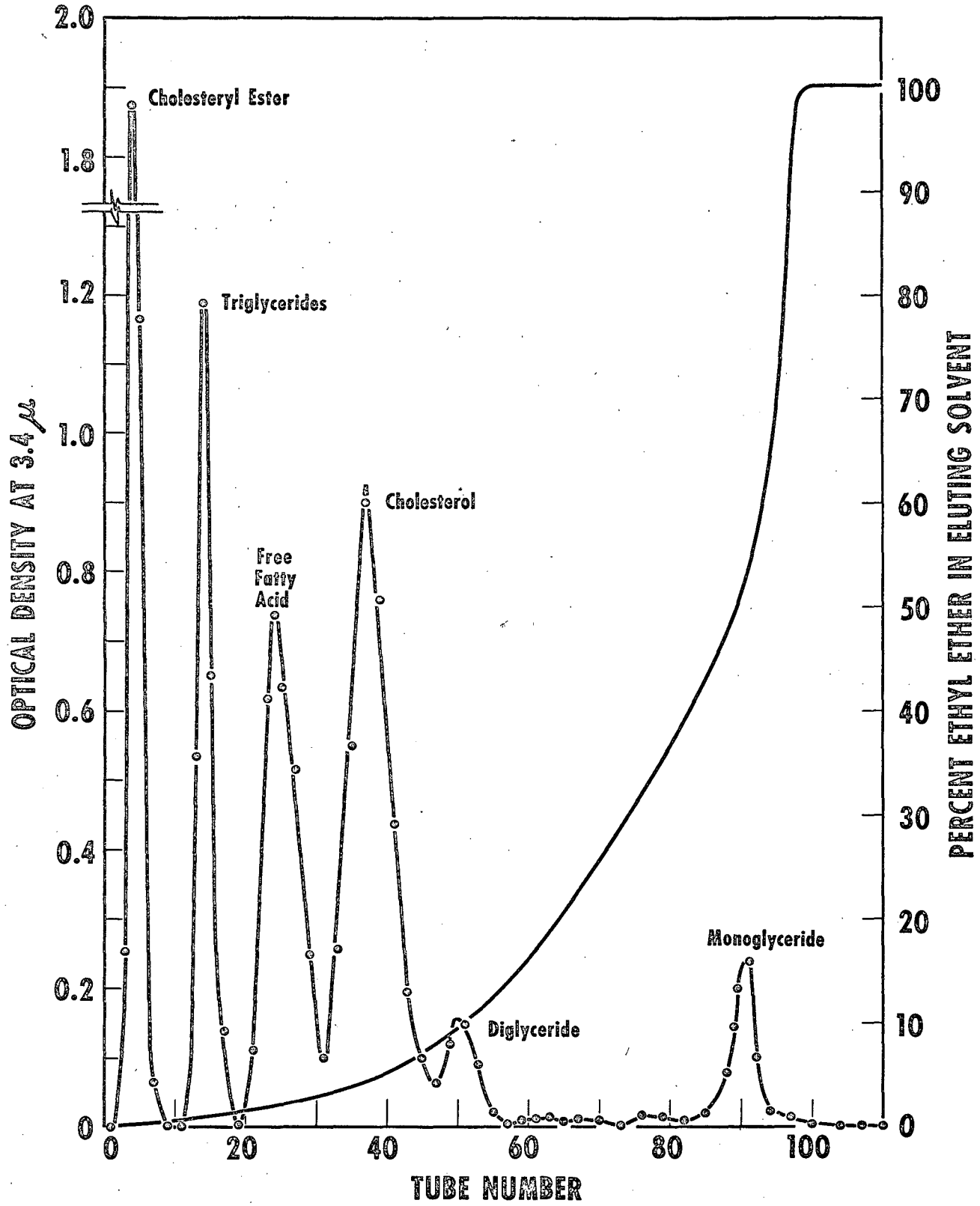
Cholesteryl Ester
Triglycerides
Free Fatty Acid
Cholesterol
Diglyceride
Monoglyceride
OPTICAL DENSITY AT 3.4 μ
PERCENT ETHYL ETHER IN ELUTING SOLVENT
TUBE NUMBER
2.0
1.8
1.2
1.0
0.8
0.6
0.4
0.2
0
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
100

Fig. 6

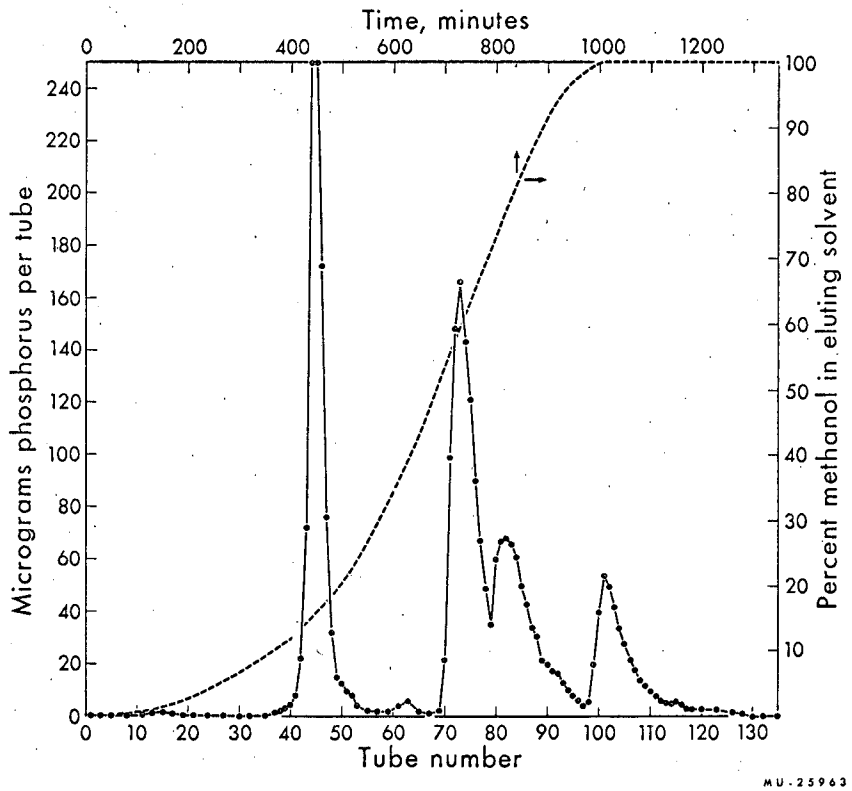
Time, minutes
0
200
400
600
800
1000
1200
Micrograms phosphorus per tube
0
20
40
60
80
100
120
140
160
180
200
220
240
Percent methanol in eluting solvent
10
20
30
40
50
60
70
80
90
100
0
10
20
30
40
50
60
70
80
90
100
110
120
130
Tube number
MU-25963

Fig. 7

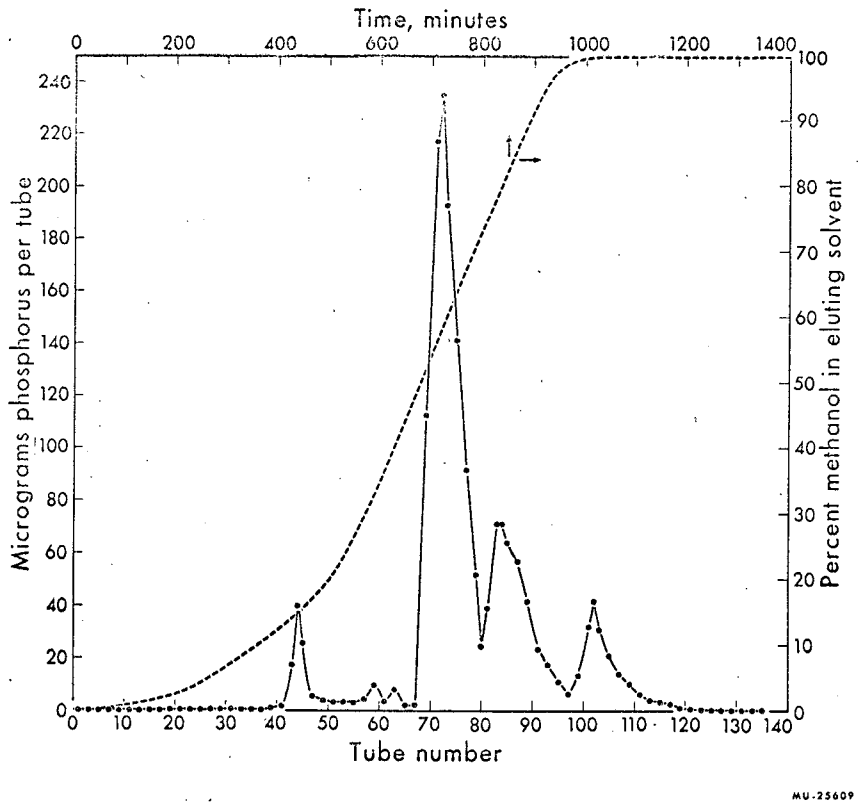
Time, minutes
0
200
400
600
800
1000
1200
1400
Micrograms phosphorus per tube
Percent methanol in eluting solvent
Tube number
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
MU-25609

Fig. 8

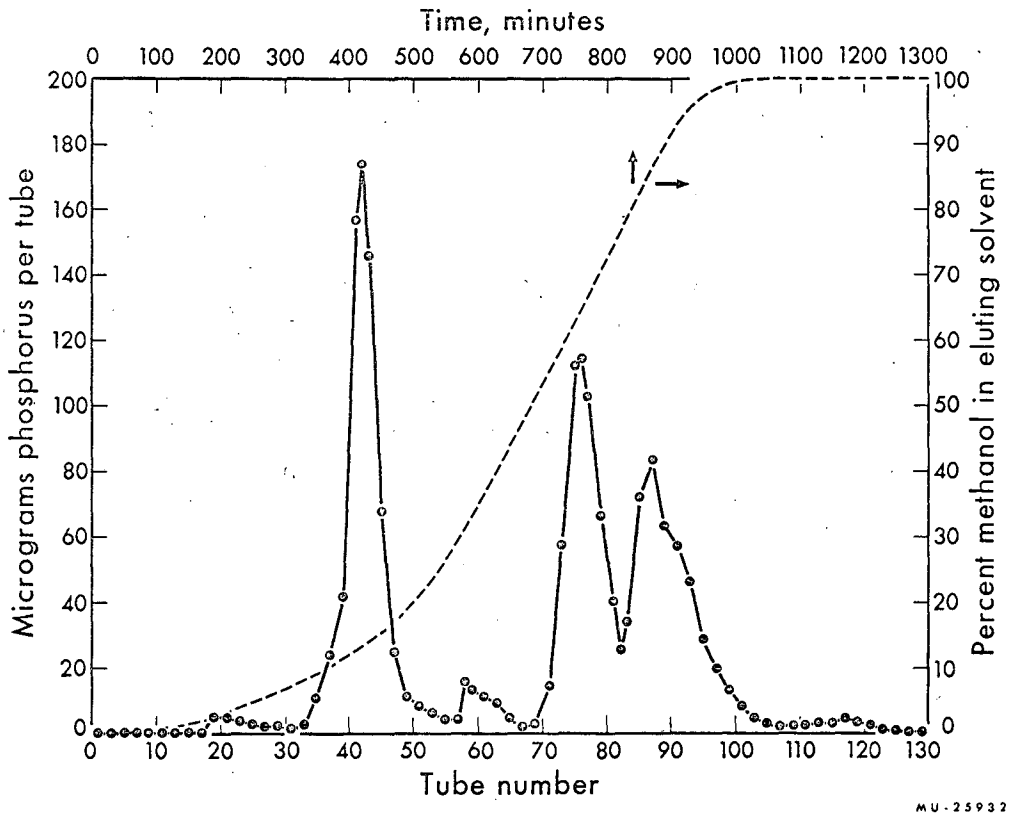
Time, minutes
0 100 200 300 400 500 600 700 800 900 1000 1100 1200 1300
Micrograms phosphorus per tube
200 180 160 140 120 100 80 60 40 20 0
Percent methanol in eluting solvent
100 90 80 70 60 50 40 30 20 10 0
0 10 20 30 40 50 60 70 80 90 100 110 120 130
Tube number
MU-25932

Fig. 9

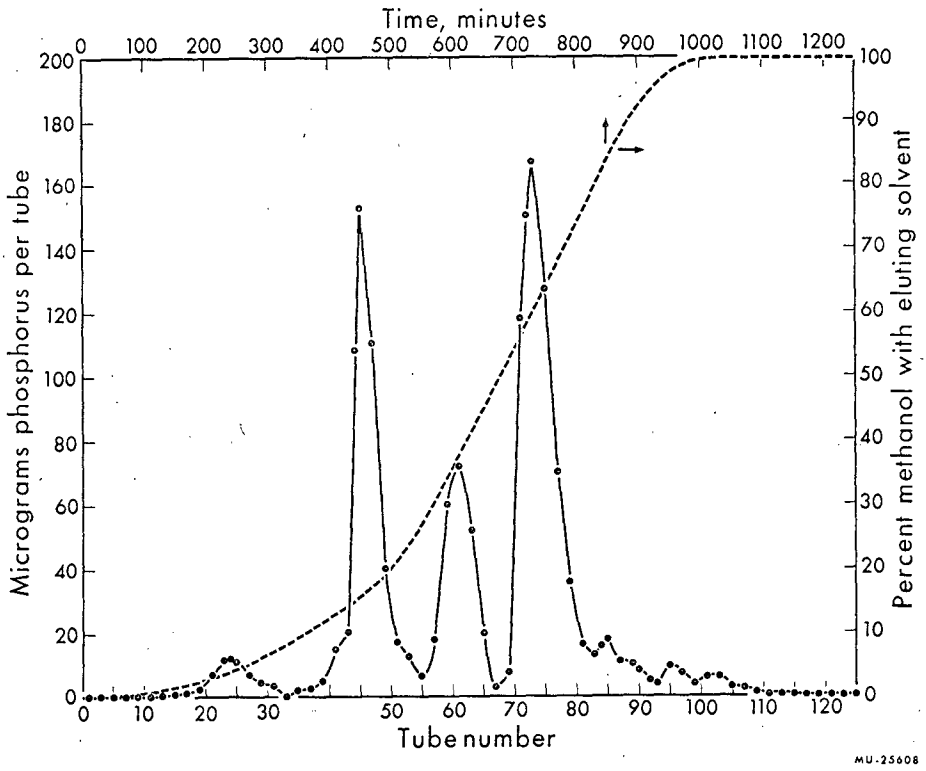
Time, minutes
0 100 200 300 400 500 600 700 800 900 1000 1100 1200
Micrograms phosphorus per tube
0 20 40 60 80 100 120 140 160 180 200
Percent methanol with eluting solvent
0 10 20 30 40 50 60 70 80 90 100
0 10 20 30 50 60 70 80 90 100 110 120
Tube number
MU-25608

Fig. 10

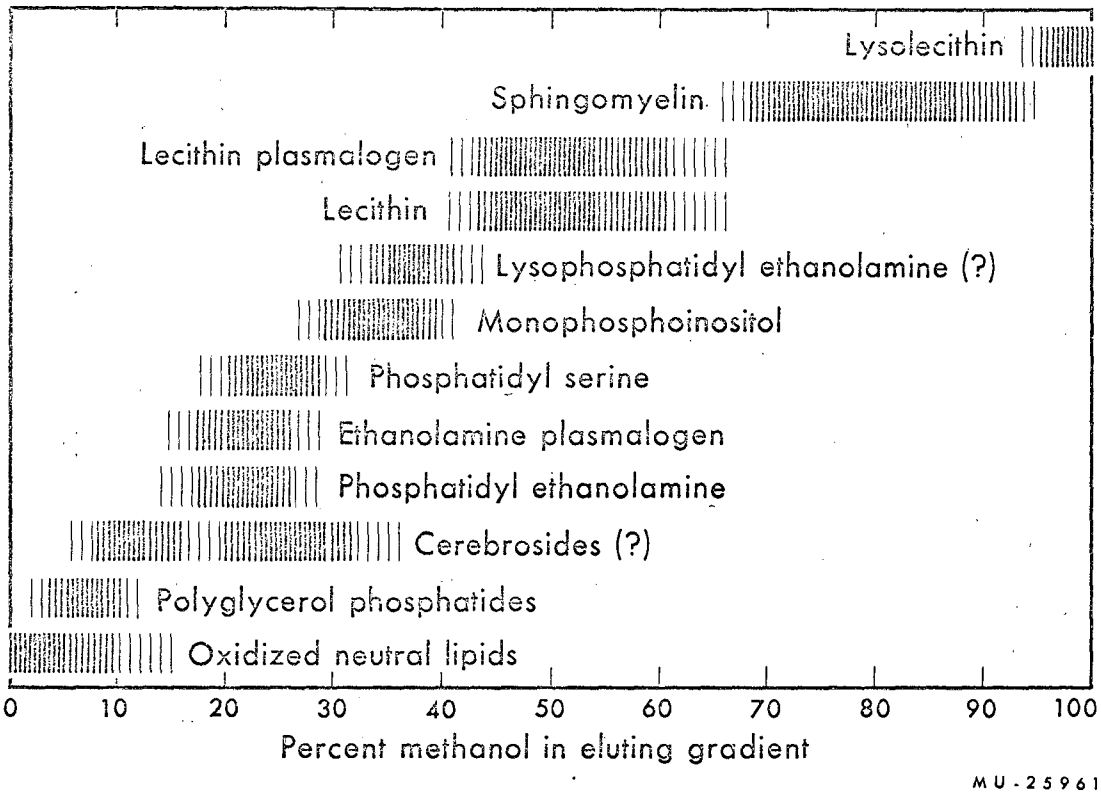
Lysolecithin
Sphingomyelin
Lecithin plasmalogen
Lecithin
Lysophosphatidyl ethanolamine (?)
Monophosphoinositol
Phosphatidyl serine
Ethanolamine plasmalogen
Phosphatidyl ethanolamine
Cerebrosides (?)
Polyglycerol phosphatides
Oxidized neutral lipids
0
10
20
30
40
50
60
70
80
90
100
Percent methanol in eluting gradient
MU-25961

Fig. 11