Automated Gradient-Elution Column Chromatography of Lipids

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Abstract

An automated gradient-elution chromatography system was developed specifically for the separation of complex mixtures of naturally occuring lipids obtained from various tissues. It uses silica gel columns with concave gradients of increasing polarity. These gradients offer advantages over the convex type because of the absorptive properties of most lipid classes on silica gel.

The entire system is operated automatically once the charge is placed on the column. The flow rate is constant throughout the run and the shape of the gradient is predetermined by selecting known values for three parameters. The selection of the gradient is arbitrary but previous knowledge of the chromatographic behavior of the substances to be separated is useful in determining the shape of the gradient. The entire system is controlled by timers, pumps, and solenoid valves. Details of the construction of the apparatus are described and criteria for the selection of acceptable pumps for organic solvents are discussed.

Several examples of the separations obtained with the system are described. While silica gel was used as the adsorbent, there is no reason to believe that other adsorbers cannot be substituted for other application. In addition, column size and elution times can be changed to provide better resolution for the specific problems, greatly broadening the application of liquid column chromatography, particularly in the area of routine quantitative analysis of nonvolatile substances. Nongradient elution can also be performed by this apparatus.

Introduction

While instruments are commercially available that will perform column chromatographic separations with some degree of automation, it is not yet possible for an investigator to readily purchase an automated system to perform any chromatographic separation under consideration. In this presentation, I would like to describe a fully automated system for the separation of complex lipid mixture using packed silica gel columns. An early version of this system was described in 1963 (1).

Design of System

Production of Gradient

Concave-gradient elution was selected on the basis of the absorption characteristics of lipids on silica-gel columns. Lakshmanan and Lieberman (2), and earlier de Vault (3), showed that concave gradients should in theory provide optimal elution characteristics for substances having absorption isotherms of the Freundlich type (4). Fig. 1 illustrates the common types of isotherms encountered in nature. A substance obeying the Freundlich absorption relationship (curve III) is more strongly bound to the adsorber as the concentration of the compound is reduced. This effect produces the undesirable tailing commonly encountered in column chomatography.

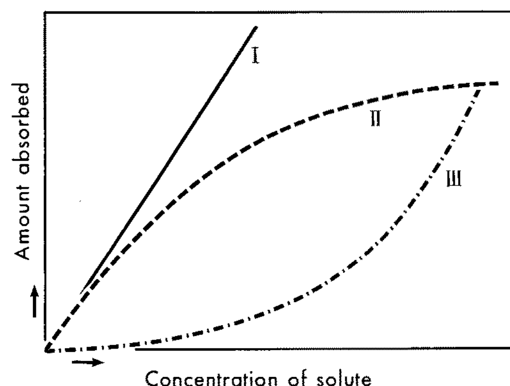

FIG. 1. Idealized absorption isotherm encountered in liquid-solid column chromatography. Curve I is a linear isotherm, II is an inverse isotherm, not commonly found in silica gel systems, III is the common Freundlich or Langmuir isotherm frequently observed with lipoid substances on silica gel columns.

Fig. 2 presents idealized elution patterns for substances obeying the absorption isotherms shown in Fig 1. It is obvious that pattern I is most desirable, yet pattern III is the result most often obtained in practice. Converting III to I can be accomplished with concave gradient elution, if one is dealing with a pure compound. Complex mixtures present a more difficult problem, but improved resolution can usually be achieved if conditions are carefully controlled and optimized for all parameters.

Fig. 3 is a schematic diagram of the system as operated in our laboratory. An important difference between the current model and that previously described is the replacement of the reciprocating piston with a constant infusion, cam-operated gradient pump, designed in our laboratory (Fig. 4), to force the polar solvent into the magnetically stirred mixing chamber. It has several unique features. It is a single-charge positive-displacement pump of a

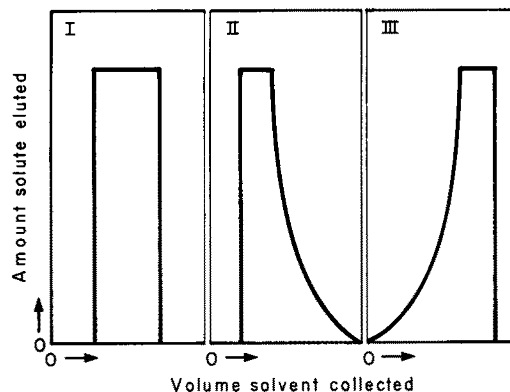

FIG. 2. Idealized elution patterns resulting from the three isotherms depicted in the first illustration. No diffusion effects were considered.

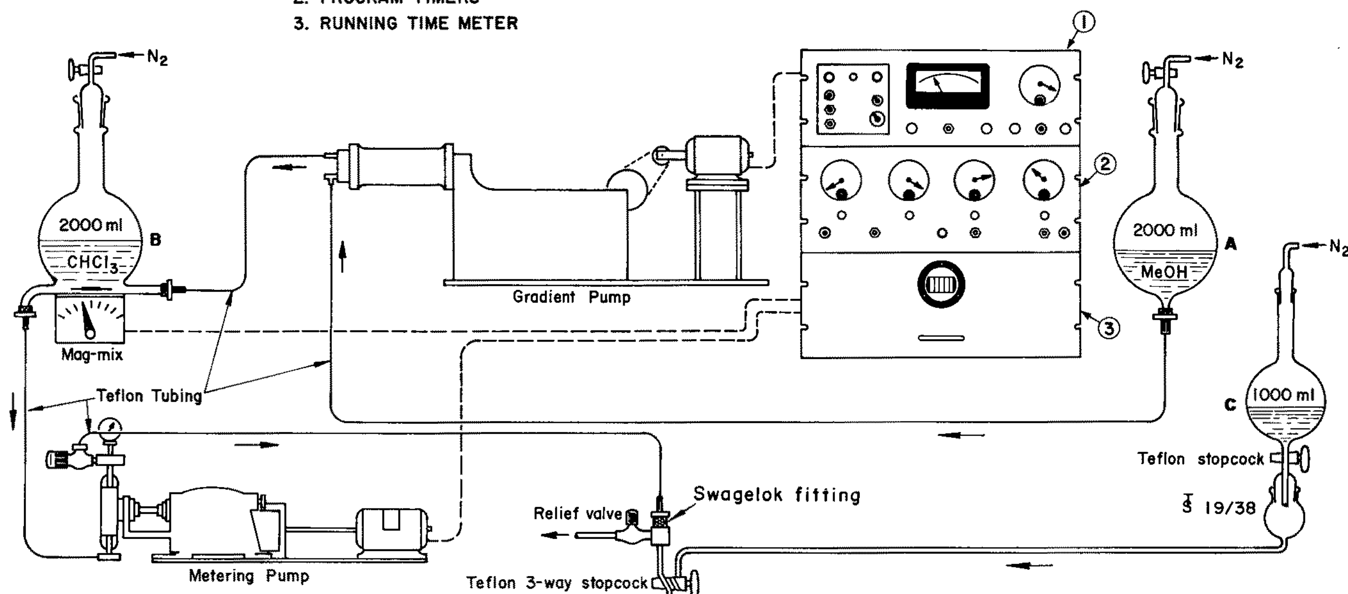

FIG. 3. Schematic diagram of the automated gradient elution column chromatographic system described in the text. *A*, a solvent reservoir to facilitate charging of the gradient pump, is not used during the run. *B* is the mixing chamber. *C* is an additional solvent reservoir for nongradient elution. Operational details are given in the text.

type sometimes used in hydraulic systems, except that it is driven by a special cam. The radius of the cam is given by the equation

$$r = a\theta^2 \qquad [1]$$

where θ is angle of rotation in radians and a is a dimensional constant. The pump is so designed that the rate increases with time, and this rate, R_1, along with the initial volume in the mixing chamber, determines the slope of the gradient. The volume pumped at time t is

$$V = \tfrac{1}{2}at^2 \qquad [2]$$

which is similar to equation of acceleration. The constant is determined by the physical parameters of the pump.

The solvent mixture leaving the mixing chamber is pumped through the column by a reciprocating piston pump, a chromatographic "mini-pump" (Milton-Roy Company, Philadelphia, Pa.). C_t, the composition of the solvent leaving the mixing chamber at time t, is given by

$$C_t = \frac{\int C_0R_1dt - \int C_tR_2dt}{V_0 + \int R_1dt - \int R_2dt} \qquad [3]$$

where R_1 is rate at which the polar solvent enters the mixing chamber, R_2 is the rate at which solvent is removed from the mixing chamber, C_0 is the concentration of polar solvent added to the mixing chamber, and V_0 is the initial volume of solvent in the mixing chamber. R_2, C_0, and V_0 are constant in this system. R_1 is the differential of Equation 2:

$$R_1 = at \qquad [4]$$

With the appropriate substitutions and differentiations, Equation 3 can be solved explicitly. The forms of the solutions are:

If $2aV_0 = R_2^2$

$$C_t = C_0\left[1 - \frac{R_2^2\, e^{2\left(1+\frac{R_2}{at-R_2}\right)}}{(at-R_2)^2}\right] \qquad [5]$$

If $2aV_0 < R_2^2$

$$C_t = C_0\left[1 - \frac{V_0\left(\frac{R_2+\sqrt{R_2^2-2aV_0}}{R_2-\sqrt{R_2^2-2aV_0}}\right)^{\left(\frac{R}{\sqrt{R_2^2-2aV_0}}\right)}}{\left(V_0+\frac{at^2}{2}-R_2t\right)\left(\frac{at-R_2-\sqrt{R_2^2-2aV_0}}{at-R_2+\sqrt{R_2^2-2aV_0}}\right)^{\left(\frac{R_2}{\sqrt{R_2^2-2aV_0}}\right)}}\right] \qquad [6]$$

If $2aV_0 > R^2{}_2$

$$C_t = C_0\left[1 - \frac{V_0 e^{\frac{2R_2}{\sqrt{2aV_0-R_2^2}}\tan^{-1}\left(\frac{-R_2}{\sqrt{2aV_0-R_2^2}}\right)}}{\left(V_0+\frac{a}{2}t^2-R_2t\right)e^{\frac{2R}{\sqrt{2aV_0-R_2^2}}\tan^{-1}\left(\frac{at-R_2}{\sqrt{2aV_0-R_2^2}}\right)}}\right] \qquad [7]$$

The three solutions are necessary because the radical $[\pm(R_2^2 - 2aV_0)]^{1/2}$ appears and is indeterminate where $\pm(R_2^2 - 2aV_0)$ assumes a negative value or zero.

In the first case the gradient produced is independent of the volume of solvent in the mixing chamber. In each of the other solutions the initial volume in the mixing chamber enters the equation. The equations are rather complicated to handle manually but if one is fortunate enough to have access to a computer, they present no problem. One can predetermine various gradients graphically and select the corresponding parameters for the flow rate, initial volume in the mixing chamber, and the pump constants with the help of the computer.

Fig. 5 is a photograph of the apparatus in a unitized housing, while Fig. 6 is a close-up view of the control panel showing the mixing chambers, solvent reservoirs and program timers as well as the control for the gradient pump motor. The gradient pump motor is a 1/50 hp DC motor (Bodine Electrical Company, Chicago, Ill.). The control, a feedback-tachometer, silicon-rectified DC power-supply (de-

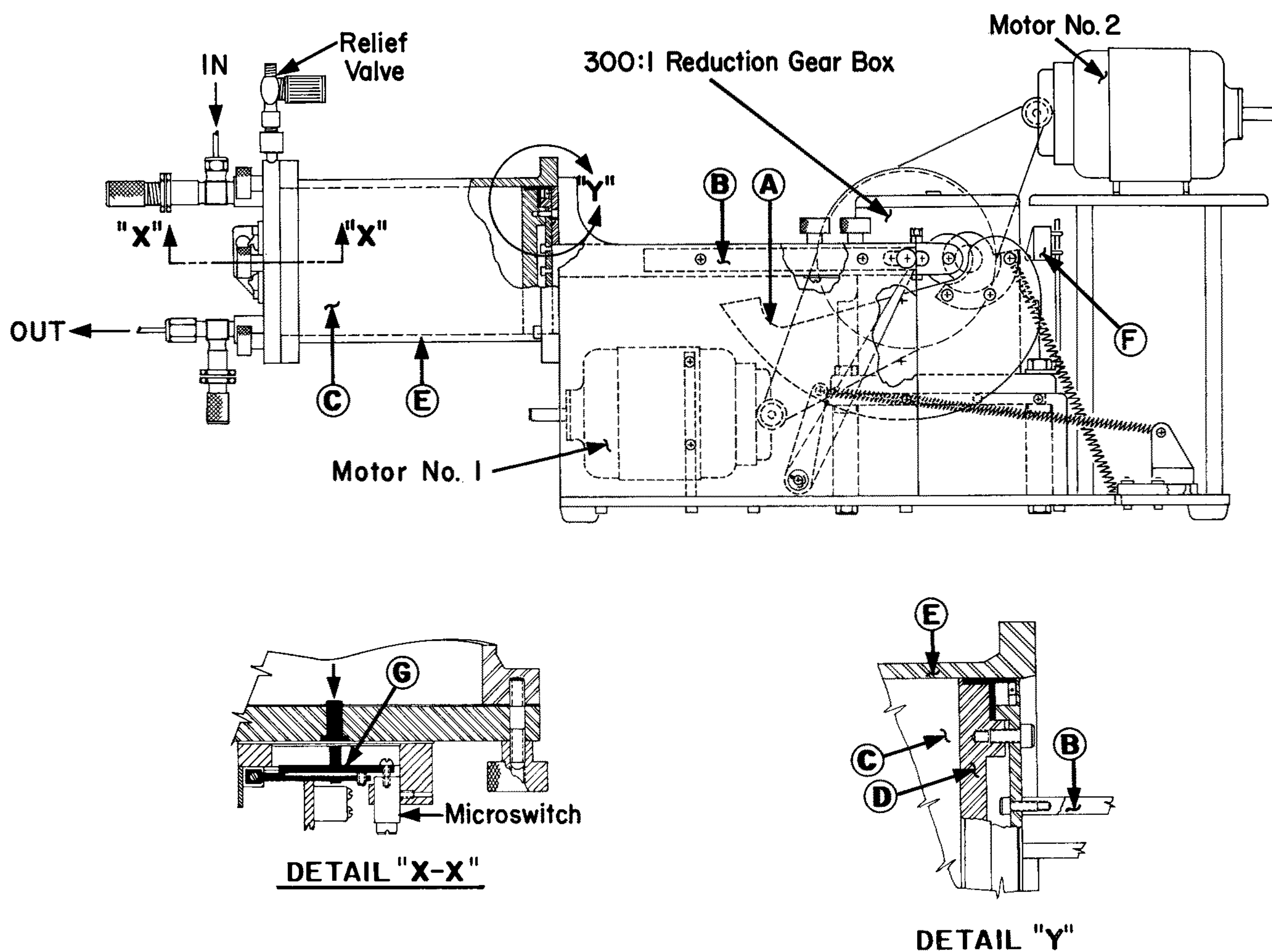

FIG. 4. Simplified design drawing of the gradient pump. *A* is the cam that drives the piston rod *B*. Motor No. 1 is used during the run to drive the cam, and motor No. 2 is a fast reverse motor for charging the pump chamber, *C*, before the run; an interlocking switch on the control panel (see Fig. 3) prevents simultaneous operation of both motors. The piston, *D*, has a Teflon gasket that forms the seal between the piston and the cylinder wall, *E*. The cylinder is honed to within 0.0005 in. of true round. Limit switches at *F* and a combined pressure and limit switch at *G* prevent the cam from overriding the follower. Detail "X-X" is an enlarged cross-sectional view of the combined forward-limit and pressure switch on the chamber face plate. Detail "Y" is an enlarged sectional view of the piston and cylinder showing the Teflon seal assembly between the piston and the cylinder wall. Teflon is shown in solid black and stainless steel in diagonal shading in all views. All parts of the pump in contact with the solvent are either stainless steel or Teflon.

signed by G. K. Heller of Las Vegas, Nev.), insures constant rpm during the operation of the system.

Fig. 7 presents typical gradients produced by this system using various parameters. Fig. 8 presents three more unusual gradients. Curve I necessitates the addition of nonpolar solvent to the mixing chamber at the time of the discontinuity (the addition can be accomplished automatically with our system).

Column Design

Initially the system used a chromatographic column housing similar to that originally described by Hirsch and Ahrens (5). This type of column housing is convenient in many respects, but in this system it had several disadvantages. With certain solvents, its flow rate was too fast for the pumping rate of the metering pump, and, because of the relatively large diameter, it was difficult to add the charge to the column in a uniform layer. We have recently discarded this type of column in favor of narrow, long columns (90 cm) of precision-bore glass tubing of 2–10 mm internal diameter. A convenient column size is 6 mm I.D. by 90 cm in length, which will hold approximately 18 to 20 g of 100- to 325-mesh silica gel. The column is thermostated by a slipover condenser jacket and connected to inlet and outlet lines by stainless steel Swagelok fittings as shown in Fig. 9. Teflon ferrules in the Swagelok fittings insure adequate sealing and minimize breakage of the columns. A perforated Teflon disk supports the packing column. The internal resistance to flow is very high, 150–300 psi in a well-packed column, as shown by a pressure indicator on the output side of the pump. This pressure is not a disadvantage because the "mini-pump" actually works better against a high back-pressure.

The preparation of the silica gel has been described (1). Briefly, a large quantity of silicic acid (Mallinckrodt) was resieved to 100- to 325-mesh, then washed with successive portions of distilled water, methanol and acetone. The dry powder was stored in sealed jars. Before use, a weighed portion was activated to 130C for 48 hr in a vacuum drying oven, cooled in a desiccator, added to the column housing in dry form, and packed into the column with a vibrator.

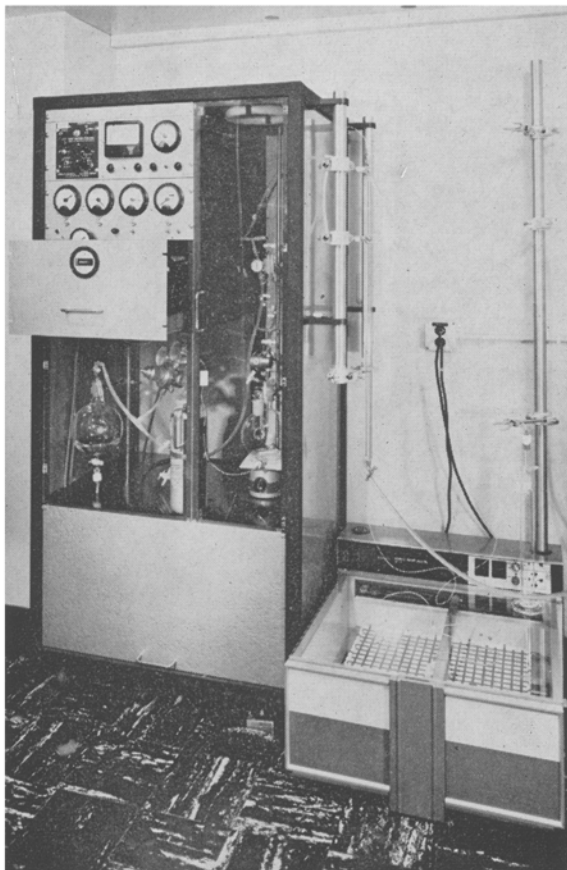

FIG. 5. Photograph of the automated system in a unitized housing in the laboratory.

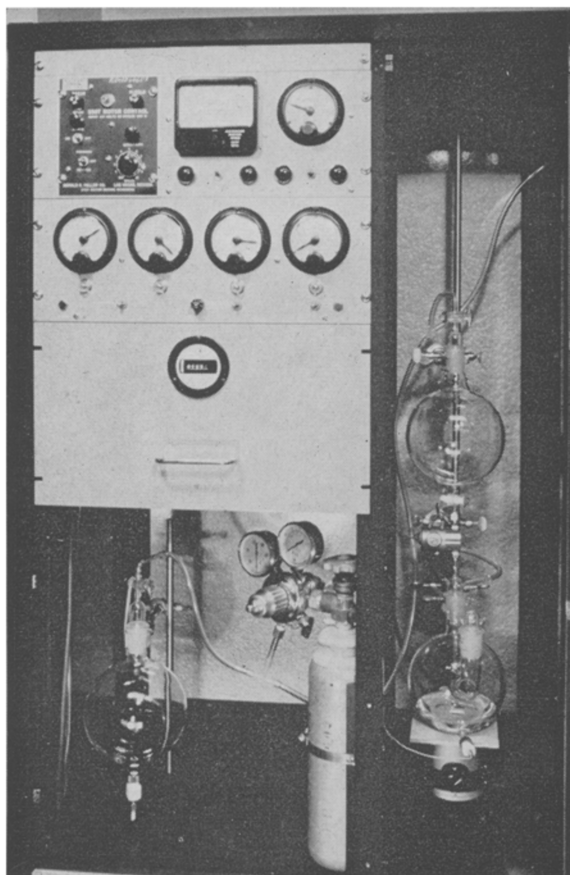

FIG. 6. Close-up view of the control panel and solvent reservoirs. The program timers and gradient pump motor control are clearly visible.

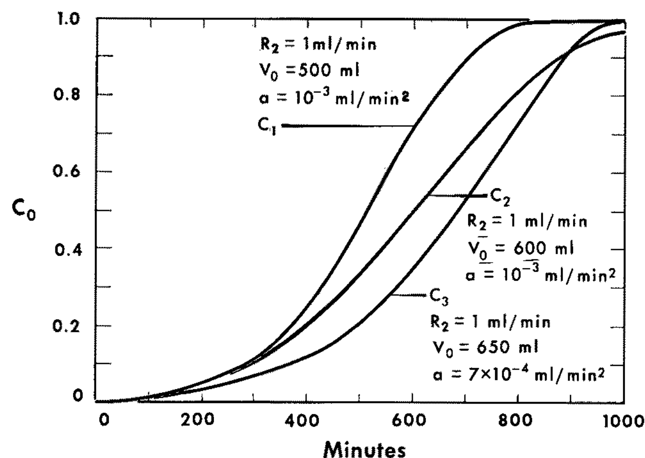

FIG. 7. Gradients obtained using values for the three parameters as indicated on the figure. Curves C_1, C_2, and C_3 are solutions of Equations 5, 6, and 7, respectively.

Methods

Chromatography

Lipid samples for chromatography were extracted with chloroform-methanol (2/1) solutions and washed with 20% water as previously reported (6). Portions of the total lipid extracts were analyzed by various chemical procedures described below before column chromatography to determine the quantitative recoveries from the column and to obtain a mass balance on the components of the sample. The remainder of the sample was dissolved in chloroform (0.5 ml/100 mg of sample) and washed onto the column with two additional 10-ml portions of chloroform. For a 20-g column, the loading factor was 6 to 7 mg lipid per gram of packing. Elution was begun with chloroform at a rate of 1 ml/min and continued until a minimum of 1000 ml of chloroform has passed through the column. Chloroform was found to have little or no effect on the migration of polar and acidic lipids on the column (6). Even in runs in which larger volumes of chloroform were used, no changes were detected in the elution properties of the substances eluted later. Recoveries of phosphorus as well as sugars, nitrogen and sulfate in the chloroform fraction were insignificant.

When sufficient chloroform had passed through the column to remove the neutral lipids, gradient elu-

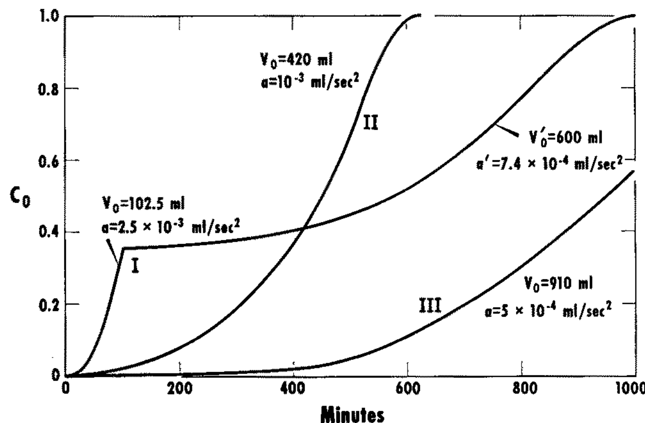

FIG. 8. Some additional types of gradients that can be produced by this system. All curves are solutions of Equation 6. Curve I necessitates the addition of nonpolar solvent to the mixing chamber at the time of the discontinuity. Values for the parameters are indicated on the figure.

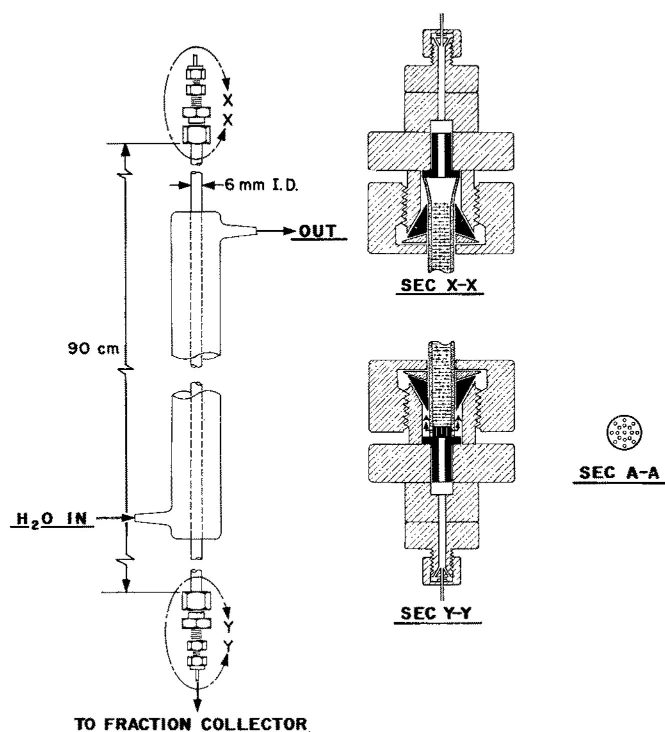

FIG. 9. Cutaway drawing of the column housings currently used with the gradient elution system. Swagelok fittings with Teflon ferrules are used to seal the inlet and outlet tubing to the column. A machined Teflon perforated plug (A-A) is inserted in the bottom of the precision bore tubing before the Swagelok fitting is attached to support the packing. The upper end of the housing is flaired slightly to prevent slippage of the fitting (X-X) due to pressure; the cooling jacket is slipped over the bottom end of the housing before the lower fitting (Y-Y) is attached. Teflon tubing (1/16 O.D.) is used throughout the system to minimize dead volumes. All Swagelok fittings are 316 stainless steel. Teflon is shown in solid black, stainless steel in diagonal shading, glass in dotted shading, and silica gel in broken cross-hatching.

tion was begun. (The choice of a gradient is arbitrary. When some prior information is available on the elution characteristics of the mixture, a gradient can be chosen to approximate a stepwise scheme. After a trial run, appropriate changes can be made in the shape of the gradient to provide better separation.) The flow rate through the column was maintained at 1 ml/min during the gradient elution.

In initial runs, fractions containing 10 ml of solvent were collected during the course of the gradient elution by a time-indexed automatic fraction collector. The eluate was collected in screw-cap vials with Teflon-lined caps and all tubes were stored at low temperature under N_2 until further analysis.

Analytical Procedures

Appropriate aliquots from each collection tube were subjected to the analyses described below. A portion of the chloroform fraction was rechromatographed on a small silica-gel column (7) and the resulting fractions were analyzed by infrared spectroscopy for triglycerides and cholesterol esters (8,9).

Phosphorus determinations were performed on 0.5-ml aliquots by the method described previously (9). Galactose analyses were performed by the anthrone method of Radin et al. (10), on 1.0-ml aliquots from the various collection tubes. Sulfate was estimated by a modification of the barium-chloranilate procedure of Bertolacini and Barney (11). Briefly, the sample was first hydrolyzed in 1 N HCl at 90C for 2 hr. Then 2.5 ml of pH 4 acetate buffer, 7 ml of isopropyl alcohol, and 5 mg of Ba chloranilate were added. The tube was shaken intermittently for 3 min and centrifuged. The optical density of the supernatant was read at 320 mμ and compared with a standard curve prepared in a similar manner.

The tests defined the elution pattern of the chromatographic run. From this information, the various components were located and the collection tubes were combined into fractions, or peaks, as indicated in Table II. In this manner enough material from each fraction was isolated for additional analyses to establish its identity. The infrared spectra of the combined fractions were determined, and the material isolated from each fraction was analyzed further. Sphingosine was analyzed by the method described previously (12). Plasmalogens were not analyzed quantitatively but were qualitatively estimated from the recovery of fatty aldehydes from dimethyl acetals after transmethylation of the various fractions.

The homogeneity of the combined fractions was established by thin-layer chromatography. Plates were prepared as described elsewhere (6). Solvent systems of varying proportions of methanol in chloroform-water were selected so that each fraction was chromatographed with a solvent of approximately the same composition as that that had eluted the fraction from the silica gel column. Spots were developed either by charring with concentrated sulfuric acid or by spraying the plate with 2′, 7′,-dichlorofluorescein and viewing under ultraviolet light. In addition, spots of unsaturated lipids were detected by exposure to iodine vapor and free amino groups by spraying with ninhydrin in acetone-lutidine solution.

Results

Fig. 10 presents an elution pattern obtained from an analysis of a total lipid extract from rat brain,

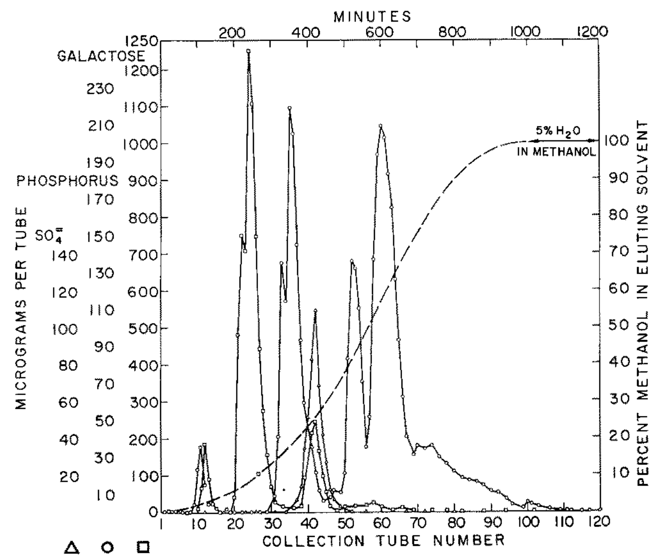

FIG. 10. Elution pattern of polar lipids extracted from whole rat brain and chromatographed on a 20-g silica gel column. The shape of the gradient is indicated by the dashed line and its composition is given along the right ordinate. Neutral lipids were removed from the column by elution with chloroform prior to the gradient elution with methanol in chloroform. Data are shown for analyses of galactose, phosphorus, and sulfate from individual collection tubes.

along with the results of analyses for phosphorus, galactose and sulfate from the individual collection tubes. This gradient had a duration of 1000 min, after which 5% H_2O in methanol was passed through the column. As can be seen, the resolution of the components is not exceptional. Of interest in this pattern are the small shoulders on the main galactose peak at tube 23 and on the phosphorus peak at tube 33. Infrared analysis indicated that the material in these tubes differed from that in the adjacent tubes. Hence, it appeared that the main peaks included two components that could perhaps be separated by changing the shape of the gradient.

For the separation in Fig. 11 the conditions were identical except that the time of gradient elution was extended to 1200 min. This more gradual gradient produced better resolution of the shoulders on the galactose and phosphorus peaks at tubes 33 and 45, respectively. Also, the sulfate peak at tube 58 was less overlapped with the neighboring phosphate peak, and the phospate peaks at tubes 85 and 95 were better resolved.

Table I gives the results of analyses on the total lipid extract for the various components and the recovery of material from the silica gel column, while Table II presents a detailed analysis of the chromatographic fractions from the run shown in Fig. 11. Fractions 1, 5, 6, 8, 9, and 10 contained essentially all of the phosphorus applied to the column, while fractions 2, 3, 4, and 7 contained essentially all of the galactose. Fractions 1 and 2 contained only a

TABLE I
Analysis of Methanol-Chloroform Extract of Whole Rat Brain and Recovery from Silica-Gel Column

Compound or analysis	Weight, mg	Recovery, %
Total weight	139.0	105
Cholesterol	24.5	110
Phosphorus	3.18	98
Galactose	4.56	103
Sulfate	0.37	90
Sphingosine	8.85	97
Fatty acid and fatty aldehydes	66.7	100

small fraction of the material applied to the column and were not identified. Fraction 3 consisted of cerebroside; its galactose-to-sphingosine ratio was 0.91 and its infrared spectrum agreed with that of a known phrenosine sample. Fraction 4 contained the largest amount of galactose and its galactose-to-sphingosine ratio was 0.87; however, the total percentage of galactose was less than that in fraction 3. The infrared spectra of this fraction were quite different from fraction 3, whose spectrum indicated no ester absorption but considerable amide and hexose absorption. The amide absorption in this compound appeared higher than could be accounted for by the amino group of sphingosine; hence, this material may have contained hexosamine. The data of Weiss (13) also indicated excess nitrogen in this fraction.

Fraction 5 was not definitely identified. Its phosphate-to-fatty-acid ratio was 1.94 and its infrared spectrum, while similar in some respects to that of phosphatidyl ethanolamine, yet differed sufficiently

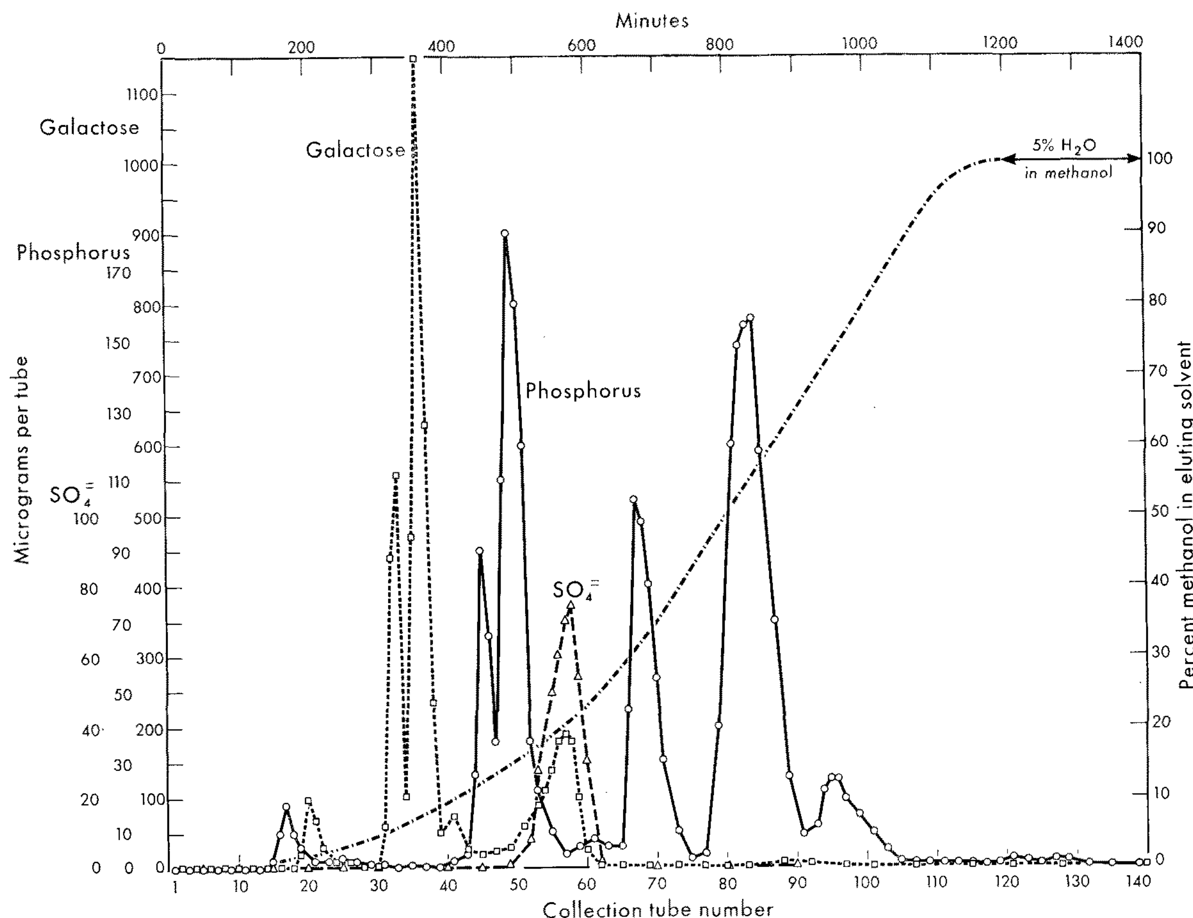

FIG. 11. Elution pattern of polar lipids extracted from whole rat brain. Conditions were as for Fig. 10 except that the duration of gradient elution was increased to 1200 min. Note the improved resolution of the components that appeared as small shoulders at tubes 23 and 33 in Fig. 10. See text for details.

TABLE II

Analysis of Chromatography Fractions Recovered from Gradient Elution of Total Lipid Extract of Normal Rat Brain (See Fig. 11)

Fraction number	Combined collection tubes[a]	P µg	Galactose, mg	SO_4 µg	Sphingosine, mg	Free amino group	Weight mg	Fatty acids[b] mg	Fatty acid to P ratio	Galactose-to-sphingosine ratio	Wt % total lipid
1	15–21	51				—	0.35	N.A.[c]			0.6
2	22–29	11	0.10		0.13	—	0.51	N.A.		0.85	0.9
3	30–33	3	0.38		0.53	—	2.04	0.69		0.91	3.7
4	34–42	1	1.12		1.49	—	6.43	2.37		0.87	11.6
5	43–46	97				++	2.35	1.50	1.94		4.2
6	47–53	323	0.04	19		++	9.23	6.72	2.02		16.6
7	54–62	27	0.24	114	0.30	+	3.79	1.43		1.2	6.8
8	63–75	219				++	5.11	2.25	1.17		9.2
9	76–90	443				—	13.59	8.30	1.93		24.4
10	91–105	70			0.98	—	2.94	1.43			5.3

[a] Four ml portions from each 10-ml collection tube were pooled to form combined fractions; hence, values given are 1/2.5 of total recovery.
[b] Includes dimethyl acetals from plasmalogens.
[c] N.A.: not analyzed.

to rule out identity. Since this fraction was not completely resolved from fraction 5, it was probably a mixture of the main component with phosphatidyl ethanolamine from fraction 6. It also contained appreciable quantities of plasmalogens, although less than the adjacent fraction 6. It gave a positive test for free amino groups. This fraction was probably phosphatidyl serine and serine plasmalogen.

Fraction 6 appeared to consist largely of phosphatidyl ethanolamine and its plasmalogen analogue, Its infrared spectrum was typical, and it gave a positive test for free amino groups. This fraction was slightly contaminated by the adjacent fractions. Fraction 7, while considerably overlapped by fraction 6, was primarily cerebron sulfate, on the basis of its galactose, sphingosine and sulfate content as well as its infrared spectrum. Fraction 8 was probably lysophosphatidyl ethanolamine; its ninhydrin reaction was positive and its phosphate-to-fatty-acid ratio was 1.17. It infrared spectrum was not compared to a standard but was consistent with this assumption. No galactose or sulfate were found in this fraction. A small amount of monophosphoinositol (less than 5% of the total material in the fraction) might have been present. Fraction 9 was phosphatidyl choline. Its infrared spectrum was identical to that of pure synthetic phosphatidyl choline except for differences expected from the variation in fatty acid composition. The ratio of phosphate to fatty acid was 1.93, and no ninhydrin-positive substances, galactose, or sulfate were present. Very little choline plasmalogen was detected in this fraction.

Fraction 10 was primarily sphingomyelin (on the basis of its infrared spectrum, phosphorus, and sphingosine content), with some lecithin apparently tailed into the fraction. This fraction accounted for 5% of the total phosphorus applied to the column; no galactose or sulfate were detected.

No definite elution peaks were detected following sphingomyelin in any of the chromatographic runs, although a small tailing of phosphorus occurred. No lysolecithin or di- or triphosphoinositol was detected. These latter compounds are known not to elute from silica gel with methanol (C. E. Ballou, personal communication) and are lost in the aqueous wash of the organic extract.

Fig. 12 is the elution pattern obtained with the same system but with an initial loading of only 3 mg of total lipid per gram of silica gel. The major peaks occur at approximately the same time. Resolution is somewhat better, but the improvement was not great enough to warrant the reduced loading. The volumes of solvent were inconvenient and the amount of material recovered in any one peak approached an unworkably small quantity. If smaller quantities are being used, TLC is the method of choice.

Better resolution appears possible with longer gradients. Fig. 13 shows that another rat brain lipid chromatographic pattern in which the gradient was run for 1400 min and the load factor was approximately 7 mg of total lipid per gram of silica gel. There is, however, the danger of decomposition of the lipid on columns as the duration of chromatographic run is extended. We have therefore cooled our column to 10C with a circulating water bath, since longer runs appear to produce better resolution. In earlier work, the column temperature was controlled at 20C; temperatures in this range appeared to have relatively little effect on migration rate. Still lower temperatures could cancel the beneficial effects by increasing the time on the column. This figure represents the limit of resolution we have currently been able to achieve with our system.

Discussion

Small variations in the shape of the gradient appear not to produce noticeable effects on resolution. Nevertheless, the pumps used to produce the gradient must be accurate and reproducible; otherwise the position of elution peaks can be so shifted as to render batch collection procedures useless. In this respect the metering pump that forces the effluent through the column is the most important; it also

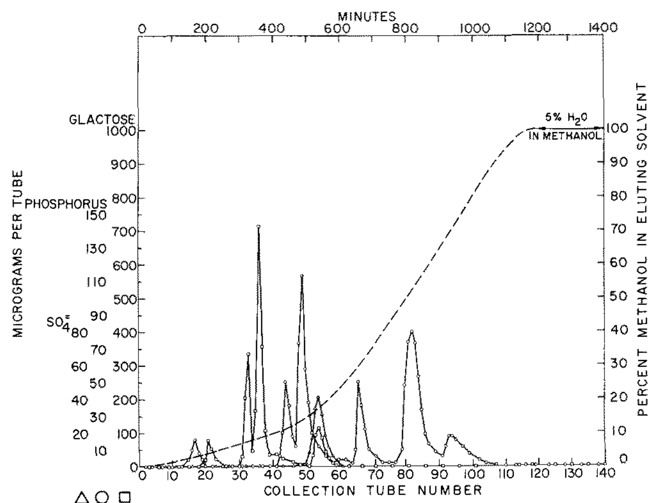

FIG. 12. Elution pattern of polar lipids extracted from whole rat brain. Conditions were similar to those in Fig. 10 and 11 except that the load of lipid was half that in Fig. 11.

has a much greater effect on the shape of the gradient than does the gradient pump. An error of 5% in the pumping rate of the metering pump will produce drastic changes in the shape of the gradient in the latter periods of the chromatographic run. Since our Milton Roy Chromatographic "mini-pump" works best against a high back pressure, we have installed a metering valve on the outlet to increase the back pressure. Unfortunately, all of the variety of pumps we have investigated are sensitive to the viscosity of the fluid being pumped; the major advantage of the Milton Roy pump is its lack of sensitivity to changes in back pressure. Without an adequate pump, this system is for all practical purposes inoperable.

The gradient pump, on the other hand, is much less critcal than would be expected; the pump designed for this system is excellent but expensive. Another method of achieving the gradient, described in my earlier paper (1) will not be described here; several other methods have been reported (2,14–18) and several commercial units are available.

One point that deserves emphasis is that in systems using organic solvents, only stainless steel, glass, and Teflon can be used to connect the vessels. Particularly, all stopcocks and glass to metal or glass to tubing should have Teflon fittings; in our experience, only Teflon provides the qualities necessary for this type of apparatus. Polyethylene tubing tends to swell and leak when used with chlorinated hydrocarbon solvents.

The silica gel should be packed tightly into the column; any channeling will render the column useless. The load should be applied to the column in a minimum amount of solvent. This tends to keep the initial band of material narrow, to approach the ideal condition of an infinitely thin layer at zero time, with resulting narrower eluting peaks.

Oxidation of the unsaturated fatty acids of a lipid is known to change its migration rate on the column (19,20). The system is therefore kept under nitrogen during the run and the solvents are deoxygenated by bubbling nitrogen through them before use.

The resolution of a chromatographic column often appears to depend on a particular batch of silica gel (21). We have tried to overcome this difficulty by purchasing a large batch and then using the standardized conditions and activation treatment described previously. Even so, from time to time anomalies appeared that could not otherwise be accounted for. Silica gel from other sources frequently behaved quite differently from our material.

Heat activation appears to be essential for reproducible results. Variation in humidity changes the water content of silica gel (22,23), and adsorbed water tends to increase the migration rate of substances on the column (24,25). In our experience, the best results are produced by the highest stage of activity (hence, the lowest water content) that can be obtained.

In actual operation, assuming that all other factors are optimal, the resolution of the column appears to depend only upon the shape of the gradient and the flow rate. The flow rate must be relatively low; 1 ml/min appears to be an upper limit with this system, using a 20-g column; when larger columns are used, higher flow rates are possible. The exact mechanism of the effect of flow rate on performance of the column is not clear. It can be assumed that conditions of true equilibrium occur along the column. If the flow rate is too high, molecules in solution may have insufficient contact with the adsorber to achieve a true equilibrium condition. If the flow is too low, back diffusion may occur; however, the limiting rate is very likely far below those used in practice. A more important consideration is that as the flow rate is decreased, the lipid molecules remain in contact with the adsorber for longer periods and are more likely to be degraded on the column (26). From a purely practical standpoint, it is usually inconvenient to have chromatographic runs which last a week or longer. When 2400 ml of eluent is passed through the column at 1 ml/min, three days are required for completion; this is already approaching a practical limit.

In our experience, an important factor in the performance of the column is that the elution volume of solvent passing through the column be sufficiently large that its composition on entering the column is essentially the same as that on leaving the column at any given time. In other words, with a column that has a retention volume of 20 ml, the gradient must be chosen so that the difference in composition of the eluent in a 20 ml portion is small. When the ratio of polar solvent is increased too rapidly, additional compounds will begin to elute and some resolution is lost. A change of 5% may be acceptable in some instances, but 1 or 2% is preferable for optimum performance. However, the nonlinearity of the adsorption isotherms of the lipids on the silica gel column precludes the use of a stepwise elution scheme, and, unfortunately, the lowest concentration of methanol that will begin to elute a compound will not remove it from the column completely within a reasonable time. Further, the same concentration may, in fact, eventually elute a second compound while to a limited degree the first one is still retained on the column.

There still remains the problem of compounds with identical elution times. This cannot be solved by simply changing the shape of the gradient, but different solvent systems can be used and new packings developed; the work of Rouser and his associates (27–29) has been perhaps the most interesting in this regard in recent years. Some of the complex lipids will probably never yield to simple methods of separation. I see little or no hope of separating the plasmalogen analogues of the various glycerol phospha-

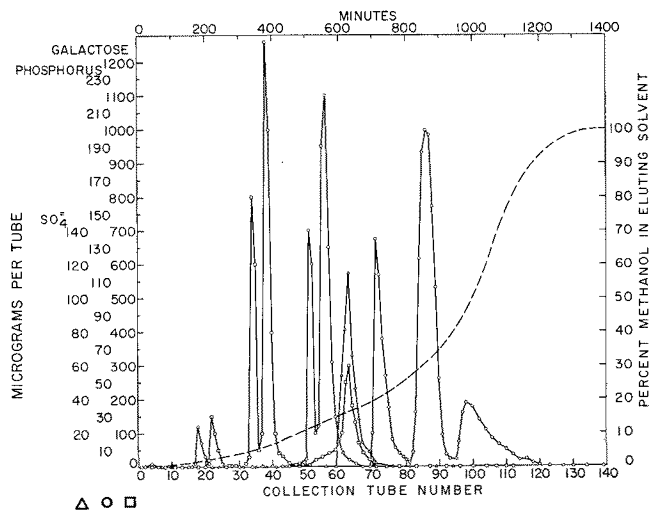

FIG. 13. Elution pattern of polar lipids extracted from whole rat brain. Conditions were similar to those in Fig. 10 and 11 except that the duration of the gradient was 1400 min.

tides by column chromatography of the unaltered molecules. The ether-linked phospholipids reported by Hanahan et al. (30) is another case in point; structural differences between molecules are so unimportant relative to polarity and configuration of the molecule as a whole that the chromatographic techniques discussed here are of little avail.

Theoretically, it would seem that if chromatographic column conditions are extremely carefully controlled, and all necessary precautions are taken to protect the sample, any compounds that can be separated by one-dimensional thin-layer chromatography with a binary solvent system can be separated on a column.

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