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A SYSTEM FOR PRECISE GRADIENT-ELUTION CHROMATOGRAPHY: APPLICATION TO THE CHROMATOGRAPHY OF SMALL AMOUNTS OF PHOSPHATIDES ON COLUMNS OF SILICIC ACID

DONALD F. HOELZL WALLACH AND GORDON L. NORDBY*

Department of Biological Chemistry, Harvard Medical School and Protein Foundation, Boston, Mass. (U.S.A.)

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SUMMARY

An apparatus for column chromatography, using precise and predetermined linear or non-linear eluant gradients and constant rates of flow is described. A mathematical treatment of gradient production by differential-flow techniques, permitting selection of a wide variety of gradients for chromatographic elution is given. Illustrative examples showing gradient-elution chromatography of phosphatides on silicic acid are presented.

INTRODUCTION

The introduction of the technique of gradient elution chromatography by TISELIUS *et al.*[1] has greatly advanced the chromatographic separation of mixtures whose components have a wide range of elution requirements, as well as that of substances which, due to non-linear adsorption isotherms, tend to be eluted in broad, tailing peaks.

There are many methods for the production of eluant gradients for chromatography[2], but these are in general quite inflexible as far as eluant volumes and gradient shapes are concerned. On the other hand the versatile "varigrad" system of SOBER AND PETERSON[3] is not suitable for use with most organic solvents.

For our studies on the phosphatides of tumor cells[4,5], leukocytes[6] and the phosphatides involved in blood coagulation[7,8] it was essential to have a system for quantitative column chromatography on both a large scale and with as little as 2 mg total phosphatide. Furthermore, in order to achieve reproducible chromatograms devoid of artifactual peaks, it was necessary to have precise and predictable as well as flexible gradient elution at constant and controllable rates of flow. Since highly unsaturated phosphatides are readily oxidized, particularly on silicic acid[9], chromatography was carried out at regulated low temperatures and controlled gas tensions. Finally, for some studies[6] it was important to run simultaneous chromatograms of duplicate samples on duplicate columns.

* Current address: Biophysical Research Division, Institute of Science and Technology, The University of Michigan, Ann Arbor, Mich. (U.S.A.).

The apparatus to be described, by employing differential pumping rates[10,11] produced by high precision metering pumps, meets the above demands and also avoids the complications which arise in gravity systems[12] due to differences in solvent densities. Furthermore, the mathematical considerations applied to this apparatus provide a simple but precise approach to gradient elution chromatography in general.

METHODS

General description

The chromatographic system is shown schematically in Fig. 1. Solvent from a mixing chamber is forced through the column(s) at the desired rate of flow by a metering pump (I). When gradient elution is employed, the solvent gradient is formed in the mixing chamber by a second metering pump (II). Materials used for construction are glass, teflon and stainless steel.

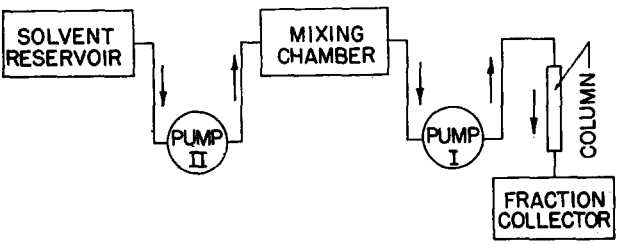

Fig. 1. Schematic of chromatographic system.

Apparatus

Columns (Fig. 2): Three types of columns have been employed:

(a) Columns of 0.125 in internal diameter and 10 in length, holding 1 g silicic acid and used for the chromatography of up to 6 mg phospholipid (Fig. 2A). These columns are constructed from stainless steel. The wall thickness is 0.112 in overall. The top and bottom ends are threaded for 0.5 in. The bottom end of the column is closed by a stainless steel nipple (4a) into which a 2-in piece of No. 18 gauge stainless steel tubing (2) has been welded. A disc of Whatman No. 42 filter paper with a diameter of 0.125 in over this outlet prevents escape of the column matrix. This tubing serves as column outlet. A gasket (3) made of 1/16 in teflon impregnated felt ("Armalon"—E. I. DuPont de Nemours and Co., Fabrics Division, Fairfield, Conn.) assures a solvent-tight seal between the column body and the outlet nipple.

The column eluate is collected by a time-actuated fraction collector. The column inlet (4b) is identical to the outlet, except that it is connected to the column feeding tube (5) which is identical for all columns used.

(b) Columns of internal diameter 0.25 in and 12–48 in length, holding 4–16 g silicic acid and used for up to 25 mg phospholipid, Fig. 2B. These columns are made of borosilicate glass piping (Fisher and Porter Co., Hatboro, Pa.). They have a wall thickness of 0.125 in. The top and bottom ends have expansions tapered at 15° for attachment of fittings.

The outlet from this type of column is also via No. 18 gauge stainless steel tubing (7) which is welded into a stainless steel disc 1 in in diameter and 0.125 in thick (8) with a depression 0.250 in in diameter in the center. This depression has a conical

bottom leading to the outlet orifice and holds a 0.250-in diameter, 1/32-in perforated stainless steel disc (9). The disc acts as support for a 0.250-in filter paper disc (10) which is necessary to prevent escape of silicic acid from the column. This outlet assembly is connected to the column by a stainless steel fitting of the glass to glass type (11) (Fischer and Porter Co.). A 1-in teflon-impregnated felt gasket (12) with a 0.250-in central orifice assures a tight seal between column and outlet.

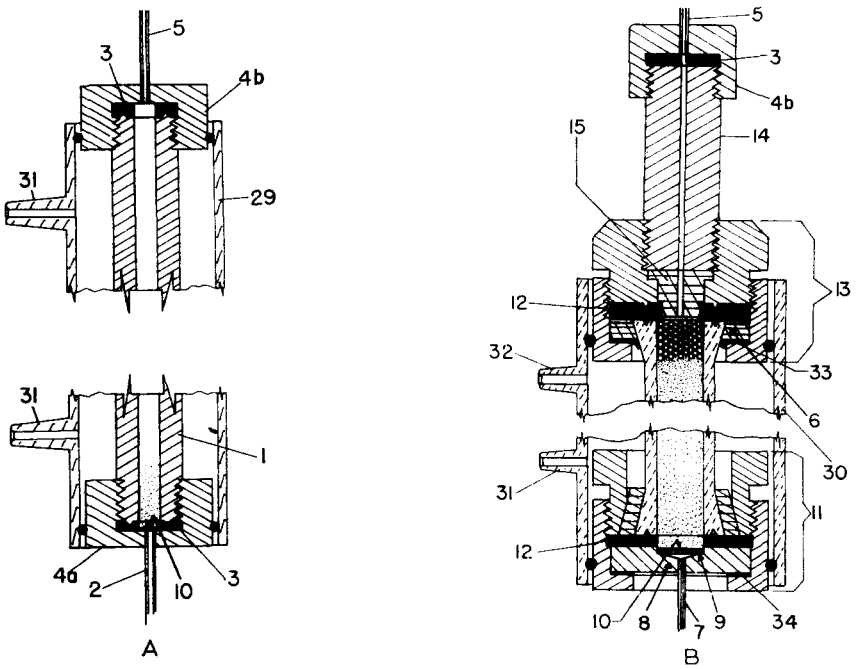

Fig. 2. (A) 1, stainless steel column; 2, outlet; 3, "Armalon" gasket; 4a, bottom fitting; 4b, top fitting; 5, inlet; 10, filter paper disk; 29, water jacket; 31, side arms of water jacket. (B) 1, glass column; 6, nylon split ring; 7, stainless steel disk; 8, outlet; 9, stainless steel screen; 10, filter paper disk; 11, bottom fitting; 12, "Armalon" gasket; 13, top fitting; 14, connector; 15, "Teflon" gasket; 30, water jacket; 31, 32, side arms of water jacket.

The column inlet is similar to the outlet and utilizes a "metal to glass" type stainless steel fitting (13) (Fisher and Porter Co.) except that a tapered split nylon ring (6) is used to hold the fitting to the tapered end of the column. A teflon-impregnated felt gasket is used here also.

The top fitting is connected to the pump by a stainless steel connector, 0.375 in diameter (14). This has a male pipe thread (5/16 in) at one end which screws into the fitting. A teflon gasket (15) is used here to make the junction solvent tight. The other end of the connector has a machine thread and connects to the pump output (4b, 5) just as with the smaller column. The orifice through the connector is 1/32 in in diameter.

(c) Columns of internal diameter 1 in and 12–48 in in length, holding to 64–256 g silicic acid. These columns are identical to the smaller columns except for size. They are made of 1 in I.D. borosilicate glass piping of appropriate length, with flanged ends (Fisher and Porter Co.) and utilize the matching stainless steel fittings. The

stainless steel screen at the bottom of these columns is 1-in in diameter as is the filter paper disc. The gaskets are 2 in in diameter and have a 1 in central orifice.

The bottom fittings can be modified to permit introduction of a thermistor into the column bed. By this technique it was shown that the silicic acid stayed at water jacket temperature under the conditions of operation described here. Differential thermometry[13] was also employed in order to measure temperature changes associated with the elution of phosphatide peaks. However, the heats of adsorption of phosphatides on silicic acid are very low and no significant temperature transients accompanied elution.

Water jackets (*Figs. 2A, 2B*): These are lucite pipes of length and diameter suitable to the column size employed (29, 30). They are held in place by O-rings on the top and bottom fittings. In the case of the larger columns a rubber diaphragm (33) and a rubber gasket (34) are required to have the system water tight. Water at desired temperature (4° for phosphatide chromatography on silicic acid) is circulated by a pump via the side arms (31, 32).

Pumps: Both the column feeding pump (I) and the gradient shaping pump (II) are single piston reciprocating pumps (Milton Roy Co., 1300 Mermaid Lane, Chestnut Hill, Pa.). Pump heads, valves and pistons are stainless steel; the packings are made of teflon. The pumps are driven in phase at 28 cycles/min by a common 0.25-HP motor. They have a maximum delivery of 340 ml/h at pressure of 250 lbs/in. The flow rates can be varied from 0 to 100% of maximum by adjustment of identical screws with vernier dials on the pump heads. This adjustment can be made even with the pump running.

The parallel arrangement of the two pumps is of particular advantage for the mode II gradient operation described below, since the pump ratio (ρ) is given directly by the ratio of the settings of the two pump adjustment screws. Pump adjustment is ordinarily done by hand. However, since the screws can also be readily driven by servomotors it is possible to set up electronically a program for any one or any combination of the eluant gradients described by the mode II operation.

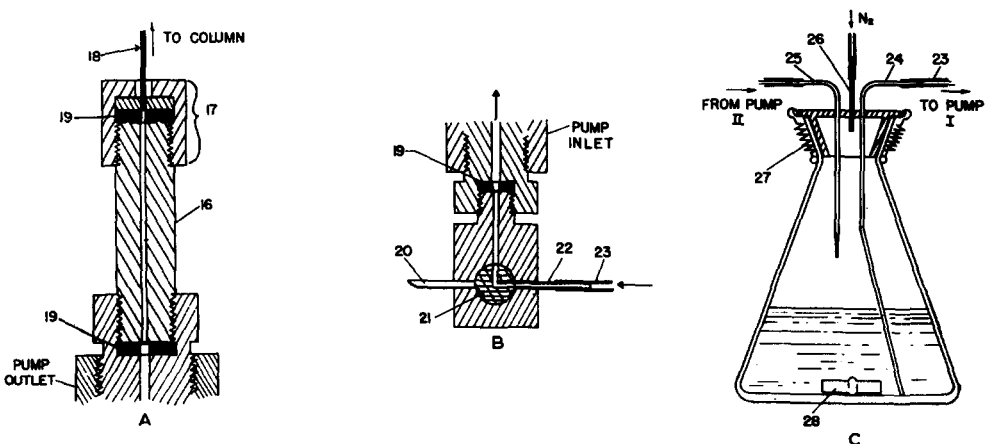

Fig. 3. (A) Outlet of pump I. 16, Stainless steel connector; 17, stainless steel union; 18, stainless steel tubing conduit to column(s); 19, "Teflon" gasket(s). (B) Inlet to pump I. 20, Priming inlet; 21, "Teflon" stopcock; 22, solvent inlet; 23, "Teflon" tubing conduit from mixing vessel. (C) Mixing chamber. 24, Output to column feeding pump; 25, Inlet to mixing chamber; 26, Inlet for nitrogen gas; 27, Steel springs; 28, Magnetic stirring bar.

The outlet of pump I is shown in Fig. 3A. A stainless steel piece (16) with pipe thread at one end and machine thread at the other connects the pump outlet to a stainless steel union (17) which has a piece of No. 18 gauge stainless steel tubing 4 in long (18) welded into it. Teflon gaskets (19) between pump and connector and connector and union are essential. At the other end of the output tubing is the stainless steel nipple with a 0.375-in female machine thread, and a teflon-impregnated felt gasket (4b). This fits the tops of all the columns. A stainless steel "Y" tube can be introduced at this point if it is desired to run two columns in parallel.

The input to the pump I is shown in Fig. 3B. There are two inlets to the pump, one a 2-in (18 gauge) stainless steel needle (20) and the other a 2-in piece of stainless steel tubing 1/16 in outer diameter (22). Both are welded into a stainless steel connector fitting into the pump inlet. A teflon stopcock (21) in the connector selects one inlet or the other. The solvent from the mixing chamber reaches the pump via heavy walled teflon tubing 0.058 in I.D. (Pennsylvania Fluorocarbon Co. Inc., 1115 N. 38th St., Philadelphia, Pa.) which fits over the 1/16-in stainless steel tubing. The other inlet is used to prime the pump and is one of the ways in which samples can be introduced.

No high pressure fittings are needed for pump II. The output of the pump is by a stainless steel connector to teflon tubing 0.058 in I.D. leading to the mixing chamber. The input to the pump differs from that to the column feeder only by the fact that the solvent input tube is 0.125 in diameter and receives solvent via 0.125 in I.D. teflon tubing. A priming inlet is used here also.

Mixing chamber (*Fig. 3C*): This is a 1-l borosilicate glass Erlenmeyer flask provided with a No. 24/40 ground glass neck. The stopper of the flask is made of stainless steel and is held down by two steel springs (27). The inlet to the mixing chamber (25) is a 1/16 in O.D. stainless steel tube welded into the top. The output of the gradient pump connects to this. The inner opening of this tube is sharply restricted, which is necessary so that the pump will always work against sufficient pressure to assure proper valve functioning. The output to the column feeding pump (24) is a 1/16 in O.D. stainless steel tube angling to the bottom of the flask and connected on the outside of the teflon tubing (23) leading to the column feeding pump. The stopper has a third opening (26) (No. 16 gauge tubing) which for phosphatide chromatography connects to a tank of pure nitrogen. The atmosphere in the flask is pure nitrogen under about 2 lbs/in^2 pressure. Positive nitrogen pressure in the mixing chamber serves three purposes: The first is to minimize oxidation of highly unsaturated lipids. The second is to provide additional counterpressure to the action of pump II. The third is to overcome the frictional resistance offered by the small bore input to pump I (so chosen to minimize dead space). In the case of pump II, a large diameter input is permissible and the pump can be driven by hydrostatic pressure alone. Stirring is via a teflon coated magnetic stirring bar (28). The mixing chamber is in a water bath kept at the same temperature as the columns.

FORMAL DESCRIPTION OF ELUANT GRADIENTS

The chromatographic system described above is exceedingly versatile in producing eluant concentration gradients of a variety of shapes. The gradients are, in general, independent of the physical properties of the solvents and column matrix. In the

subsequent discussion, the effect of two modes of pump operation on the shape of the eluant gradient will be described. In mode I, both pumps operate to deliver identical volumes of solutions. In mode II, the pump delivering eluant to the chromatography column operates at a greater rate than the pump delivering solute from the reservoir to the mixing chamber. The alternative condition for mode II, characterized by an increasing mixing chamber volume, is practically unimportant, although analytically interesting.

Mode I operation

In mode I the eluant solution pump (pump I) operates at a rate, r (ml/min), which is identical to the rate, R (ml/min), at which the reservoir solution pump (pump II) is operating. Hence the mixing chamber volume, V (ml), remains constant. The composition of the eluant, c (concentration units) can approach but cannot be identical to the composition of the reservoir solution, C (concentration units), since the mixing chamber volume is constant at a finite volume. The eluant concentration as a function of time, t (min), is given by Eqn. 1:

$$c = c_0 + [rC\int_{t_0}^{t} (\mathrm{d}t) - r\int_{t_0}^{t} c(\mathrm{d}t)]/V \tag{1}$$

The initial composition of the mixing chamber solution is given by the concentration c_0; the term in brackets represents the net change in amount (not concentration) of the component measured by the concentrations C and c in the mixing chamber solution. An explicit function in c can be obtained by first differentiating Eqn. 1 with respect to time:

$$V(\mathrm{d}c/\mathrm{d}t) = r(C - c) \tag{2}$$

and then, following suitable transposition of terms,

$$(\mathrm{d}c)/(C - c) = r(\mathrm{d}t)/V \tag{3}$$

integrating over the appropriate concentration and time intervals. Thus either

$$\ln [(C - c)/C - c_0)] = [-r(t - t_0)/V] \tag{4}$$

or

$$c = C - (C - c_0) \exp [-r(t - t_0)/V] \tag{5}$$

express the desired concentration–time relationship. At time t_0 the independent variables C, c_0, r, and V are defined. Although these variables can be unequivocally specified for a stated experiment, they cannot easily be specified in a manner which predicts an eluant gradient of the desired shape. Not only is the geometric consequence of this set of variables obscure, but also an uncountable number of different sets of variables can generate curves of the same shape.

It is suggested, therefore, that Eqn. 5 be modified to reflect changes in concentration as a function of changes in time.

$$\Delta c = [C - c_0] [1 - \exp (r\Delta t/V)] \tag{6}$$

In this equation $r\Delta t_{max}/V$ is defined as μ (dimensionless) and Δt_{max} is the maximum

in the first stages of germination. Since this finding is contrary to available information and since it is of primary importance to our knowledge of seed germination, a study was made of the changes in total nucleic acids (RNA and DNA) in cotyledons of peanuts during germination. This is a report of such a study on four varieties of mature and immature peanut seeds. The nucleic acid changes in the corn scutellum were reinvestigated with a different method of nucleic acid analysis and starting with resting seed; the pattern of change is compared to that for peanuts.

MATERIALS AND METHODS

Four varieties of peanut seeds* (Ga. 61-42, S. E. Runner, Dixie Spanish, and Virginia 56-R) were lightly dusted with 2,3-dichloro-1,4-napthoquinone, a fungicide, and germinated in a dark, humid atmosphere at 30° in vermiculite. Hybrid corn seed (WF9XM14) were dusted with chloranil and were germinated on absorbent paper. The nucleic acids were determined by a method[6] previously found satisfactory for peanut cotyledons. The tissue (about 400 mg) was excised and homogenized in cold methanol. The insoluble pellet was subsequently washed with methanol, 0.2 N $HClO_4$, and ethanol with operations being performed at 0–4°. This insoluble pellet was then defatted with ethanol–ether (2:1) at 50° for 30 min. Following this step the nucleic acids were extracted with 5% $HClO_4$ at 70° for 40 min. Absorbancy difference at 260 and 290 mμ of the cleared supernatant was referred to a standard curve obtained with a similarly-treated yeast RNA to estimate the content of nucleic acid. DNA was determined by measuring the deoxyribose[7]; the RNA content was obtained by difference.

All data presented in this paper represent averages of two or more experiments.

RESULTS

Changes with germination of mature seeds

It has been reported that as corn, rice, and bean seeds germinate there is a reduction in RNA in the cotyledonary tissue. Contrary to the findings in these seeds, Fig. 1 shows that as peanut seeds germinate, the RNA content increases two-fold within about a week after planting. The pattern of RNA metabolism shows that during the early stage of germination, the content reaches a maximum at about 4–6 days after planting with a decrease in RNA content thereafter. At 15 days after planting, the RNA content is at a level about the same or lower than that for ungerminated seeds.

The pattern of RNA metabolism was similar for 4 varieties of mature peanut seeds. However, the point of germination when the highest level of RNA was reached varied from 4 to 6 days depending on the variety and the size of the seed. Virginia 56-R seed, the largest seed, reached the highest level of RNA at 6 days (Fig. 1A), whereas the smallest seed, Dixie Spanish, reached the highest level at 4 days (Fig. 1B).

The DNA content (μg/cotyledon) of Virginia 56-R seed remained constant during germination (Fig. 1A) whereas the content increased with germination for the other seeds (Fig. 1B).

The initial level (0 day) of RNA and DNA varied with the variety and seed size. No difference in the pattern of RNA metabolism was found in the same variety of seeds produced in two locations (Fig. 1A).

* The author is indebted to W. K. BAILEY and K. T. HOLLEY for the peanut seeds provided in this study.

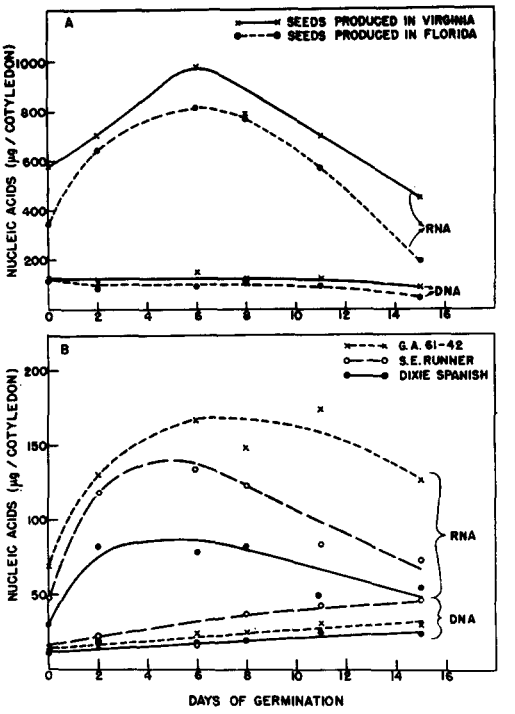

Fig. 1. Nucleic acid changes in the cotyledon of mature peanut seeds during germination. The seed size (dry weight/ cotyledon) of the largest seed (Virginia 56-R) was 370 and 445 mg, respectively, for those produced in Virginia and Florida (A). The seed weight of Dixie Spanish, GA. 61-42 and S. E. Runner were 170, 240, and 235 mg/cotyledon, respectively (B). Nucleic acids are given as µg per single cotyledon.

Changes with germination of corn seed

The above results show that during the germination of large peanut seeds, ranging from 0.5 to 1 g in weight, the RNA content doubles during the first part of germination and then declines. Since the corn scutellum is roughly about 1/15 the size of a single peanut cotyledon and since corn seedlings grown under the described conditions (METHODS) deplete their storage materials by 5–6 days of germination[8], it seemed possible that there might be an increase in RNA of the scutellum between 0 to 1 day. Hence the previous experiments with corn[1–3] would not have detected this change. Therefore, the nucleic acid changes in corn scutellum during germination (0–4 days) was reinvestigated. Fig. 2 shows that there is a 20 % increase in RNA of the scutellum one day after planting. Subsequently, the RNA content declines as previously reported[1–3]. The DNA content of the scutellum remained constant.

Effect of seed maturity on nucleic acid changes

It is known that bean (*Phaseolus vulgaris*) seeds[9] have their maximum number of cells within 20 days after flowering, which is about one-half the time necessary to complete seed development. In the last half of the development period, most of the storage material is formed (86 % of seed dry weight). To determine if the pattern of

reservoir and mixing vessel pump rates by the relation $r = \rho R$ where ρ is a dimensionless constant greater than unity in value. When ρ has a value greater than two, a (Φ, Ω) eluant gradient with a convex upward shape is produced; these curves are somewhat similar to those produced in mode I operation. As $\rho \longrightarrow \infty$ the reservoir pump rate R becomes limiting and a stepwise gradient is approached. When ρ is equal to two, a linear eluant gradient is produced. For ρ between (but not equal to) two and one, concave upward gradients are produced.

The solute concentration in the eluant as a function of time is given by

$$c = [a_0 + RC \int_{t_0}^{t} (\mathrm{d}t) - r\int_{t_0}^{t} c(\mathrm{d}t)]/[V_0 + (R - r)\int_{t_0}^{t} (\mathrm{d}t)] \qquad (9)$$

where a_0 is the initial amount (not concentration) of solute in the mixing chamber of initial volume V_0.

The integrals in the numerator represent the change in the amount of solute in the mixing chamber during a stated time; whereas the integral in the denominator represents the corresponding change in mixing chamber volume. An explicit equation in c is derived by first differentiating Eqn. 9 with respect to time

$$V(\mathrm{d}c/\mathrm{d}t) + Rc + Rt(\mathrm{d}c/\mathrm{d}t) - rc - rt(\mathrm{d}c/\mathrm{d}t) = RC - rc \qquad (10)$$

Then, following rearrangement,

$$(\mathrm{d}c)/(C - c) = R(\mathrm{d}t)/(V + Rt - rt) \qquad (11)$$

integrating over suitable limits of concentration and time. Both Eqn. 12 and Eqn. 13

$$\ln [(C - c)/(C - c_0)] = -[R/(R - r)] \ln [(V + (R - r)t)/(V + (R - r)t_0)] \qquad (12)$$

$$c = C - (C - c_0) \exp [[R/(R - r)] \ln [(V + (R - r)t_0)/(V + (R - r)t))]] \qquad (13)$$

express the desired function of eluant concentration. Eqn. 13 can be expressed in a more general form by considering changes in concentration and in time.

$$\Delta c = (C - c_0) [1 - \exp [(R/(R - r)) \ln (V/(V + (R - r)\Delta t)]]] \qquad (14)$$

In this equation R $(R-r)$ can be defined as ν; if Φ be the concentration change ratio $\Delta c/\Delta c_{\max}$, and if Ω be the time change ratio $\Delta t/\Delta t_{\max}$, then the above equation reduces to:

$$\Phi = (C - c_0) [1 - \exp [\nu \ln (1 - \Omega)]] \qquad (15)$$

Implicit in the above equation is the relation $(R - r)\Delta t_{\max} = -V$; that is, the mixing chamber volume becomes zero at the end of the experiment. The dimensionless parameters ν and ρ are related by either $\rho = (\nu - 1)/\nu$ or $\nu = 1/(1 - \rho)$.

Table III and Fig. 5 are tabular and graphic representations, respectively, of Eqn. 15. For purposes of illustration $-\nu$ has been set to a number of values, as specified in the table appended to Table III. For $-\nu > 1$ the curves are convex upward and somewhat resemble the mode I curves. For $-\nu = 1$ a linear gradient is obtained. And for $-\nu < 1$ curves which are concave upward are produced. The function becomes discontinuous for $-\nu \longrightarrow \infty$ or $-\nu \longrightarrow 0$. In the former limit $\rho \longrightarrow 1$ and the two rates become equal; since at this limit there is no rate difference,

the initial mixing chamber volume is constrained to be zero and a stepwise type gradient results. In the latter limit $\rho \longrightarrow \infty$, which means that the very large mixing chamber volume ($V \longrightarrow \infty$) is run onto the column with no concentration change since R is effectively zero.

TABLE III

TYPICAL (Φ, Ω) ELUANT GRADIENTS FOR CHROMATOGRAPHY SYSTEMS OPERATING IN MODE II

Ω	$-ln(1-\Omega)$	Φ_1	Φ_2	Φ_3
0.0	0.0000	0.0000	0.0000	0.0000
0.1	0.1054	0.0513	0.0260	0.0131
0.2	0.2231	0.1056	0.0543	0.0275
0.3	0.3567	0.1633	0.0853	0.0436
0.4	0.5109	0.2254	0.1199	0.0619
0.5	0.6932	0.2929	0.1591	0.0830
0.6	0.9163	0.3675	0.2048	0.1082
0.7	1.2040	0.4523	0.2599	0.1397
0.8	1.6094	0.5528	0.3313	0.1823
0.9	2.3026	0.6838	0.4377	0.2501

Ω	$-ln(1-\Omega)$	Φ_4	Φ_5	Φ_6
0.0	0.0000	0.0000	0.0000	0.0000
0.1	0.1054	0.1900	0.3439	0.5695
0.2	0.2231	0.3600	0.5904	0.8322
0.3	0.3567	0.5100	0.7599	0.9424
0.4	0.5109	0.6400	0.8704	0.9832
0.5	0.6932	0.7500	0.9375	0.9961
0.6	0.9163	0.8400	0.9744	0.9993
0.7	1.2040	0.9100	0.9919	0.9999
0.8	1.6094	0.9600	0.9984	1.0000
0.9	2.3026	0.9900	0.9999	1.0000

Constants for Table III

i	1	2	3	4	5	6
ν	$-1/2$	$-1/4$	$-1/8$	-2	-4	-8
c_0	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
C	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000

From preliminary experiments, a suitable pump rate r and a total eluate volume v can be specified (for example, 2 ml/min and 500 ml, respectively). With the help of Fig. 5 the appropriate eluant gradient curve can be selected ($-1/4$, for example). Implicit in this information is the maximum lapse-time Δt_{max} (250 min) for the experiment, the reservoir pump rate R (0.4 ml/min), and the initial mixing chamber volume (400 ml). The initial mixing chamber volume and the volume delivered from the reservoir total the eluant volume. For the case of a gradient running from 0.1 M to 0.6 M, Δc_{max} is 0.5 M; in mode II the reservoir solute concentration is always equal to the maximum solute concentration required at $\Omega = 1.0$. Several intermediate

values for Δt, Δc and c are presented in Table IV for the example under discussion.

It is not necessary to rearrange reservoir and mixing chamber contents at intermediate points in a run if an eluant gradient of more complex shape than those depicted is desired. At any time the pumps may be reset at different rates and ratios to change the gradient shape. If the indicated changes be done intermittently, then discontinuous points in the composite curve will exist, but all other points will be those predicted by suitable use of Eqns. 14 or 15.

TABLE IV

AN EXAMPLE OF THE APPLICATION OF TABLE III TO A SPECIFIC PROBLEM IN MODE II CHROMATOGRAPHY ($\nu = -1/4$)

Ω	Δt	Φ	Δc	c
0.0	000	0.0000	0.0000	0.1000
0.2	050	0.0543	0.0272	0.1272
0.4	100	0.1199	0.0600	0.1600
0.6	150	0.2048	0.1024	0.2024
0.8	200	0.3313	0.1657	0.2657
1.0	250	1.0000	0.5000	0.6000

TECHNIQUE OF OPERATION

Since this apparatus has been used primarily for chromatography of phosphatides on silicic acid, this particular operation is here described in detail. However, the apparatus has also been satisfactorily employed with other chromatographic systems such as cellulose, modified celluloses and alumina.

Preparation of column for silicic acid chromatography of phosphatides

Silicic acid (Mallinckrodt) 100 mesh has been used in these studies. Columns were packed dry as follows: After assembling the bottom fitting, a disk of Whatman No. 41 H filter paper of appropriate size was placed over the outlet opening, the gasket inserted and the fitting attached to the bottom of the column and tightened by hand.

The silicic acid was then slowly introduced with constant downward tapping. This process requires considerable care, particularly in the case of the small metal columns, to prevent cavity formation. Filling is continued until no further settling occurs. A filter paper disk is then placed over the top of the column and the top fitting is applied.

The column is then cleared of adsorbed oxygen as follows: The column is placed into an oven at 105° and kept at this temperature for 1 h or more with a constant stream of pure nitrogen passing through it via the top fitting. Usually the silicic acid is added and the process repeated. The total amount of silicic acid in each column is recorded.

After deoxygenation the column is allowed to cool, with the nitrogen stream continuing during this period. It is then attached to the column feeding pump and washed with the nitrogenated solvents appropriate for the intended chromatographic procedure. The flow rate should be not more than 1 ml/min/cm^2 cross sectional area.

During this process the silicic acid usually compresses somewhat more. The resulting dead space is filled with either glass or teflon powder after the completion of the washing procedure. The column is then reassembled and a constant flow of chloroform maintained, if desired, by re-cycling. During this period the column can be checked for leaks and the flow rate calibrated.

When it is desired to run duplicate columns in parallel, the column pair must be of identical diameter and length and must contain equal amounts of silicic acid. They are deoxygenated and washed in parallel by means of a "Y" adaptor on the output of pump I. They should then have identical flow rates.

Sample application

The technique of sample application depends on the sample volume. If this is large, the sample is placed into the mixing chamber in nitrogenated starting solvent and pumped on, followed by at least three 10-ml portions of initial solvent. For small samples, especially with the micro-columns, the sample is placed into a syringe which is attached to the needle inlet of pump I. The sample is then injected with the pump running and washed on with initial solvent. Both procedures are quantitative and, provided the system has been properly assembled, no losses can occur.

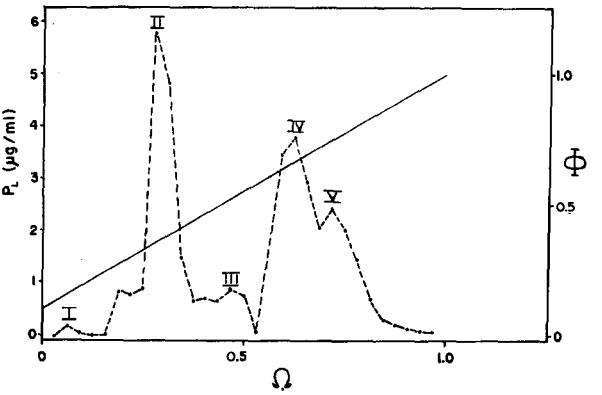

Fig. 6. Gradient elution chromatography of guinea-pig leukocyte phospholipids on a micro-column of silicic acid. Column: 0.32 cm^2 × 30 cm. 1 g silicic acid washed in sequence with hexane, chloroform – methanol (1:1, v/v) and chloroform. 1.8 mg phospholipid applied in chloroform – methanol (9:1, v/v). Elution by linear gradient from chloroform – methanol (9:1, v/v) to methanol. (total volume = 90 ml). Flow rate, 18 ml/h; fractions, 2 ml; recovery, 94%. Identifications: I, phosphatidic acid; II, phosphatidylethanolamine; III, phosphatidylserine; phosphatidyl inositol; IV, phosphatidyl choline; V, sphingomyelin.

Sample application in the case of parallel duplicate columns is carried out *seriatim*, care being taken to prevent drying of the resting column. After sample application the columns are again connected in parallel and eluted in parallel.

Elution

After sample application, elution proceeds according to a program selected on the basis of the considerations discussed (*vide supra*). The appropriate volume (V) of the initial solvent mixture is placed into the mixing chamber and is nitrogenated, and the rate of pump I set to a desired rate (which should not exceed 1 ml/cm^2 cross

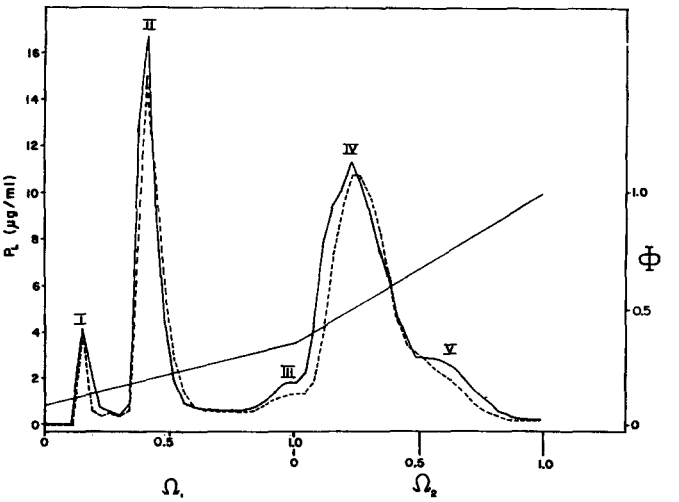

Fig. 7. Two-step gadient elution chromatography of Ehrlich ascites carcinoma phospholipids on two identical columns run in parallel. Columns: 1.16 cm^2 × 30 cm; 4 g silicic acid per column, washed in sequence with hexane, chloroform – methanol (1:1, v/v) and chloroform. 12 mg phospholipid applied to each column in chloroform. First elution: linear gradient from chloroform – methanol (9:1, v/v) to chloroform – methanol (6.5:3.5, v/v) (total volume 280 ml). Second elution: linear gradient from chloroform (6.5:3.5, v/v) to methanol (total volume 280 ml). Flow rate, 50 ml/h/column. Fractions, 5 ml. Recoveries 97% and 94%. Identifications of fractions: I, phosphatidic acid; II, phosphatidyl ethanolamine; phosphatidyl serine; III, phosphatidyl inositol; IV, phosphatidyl choline; V, sphingomyelin.

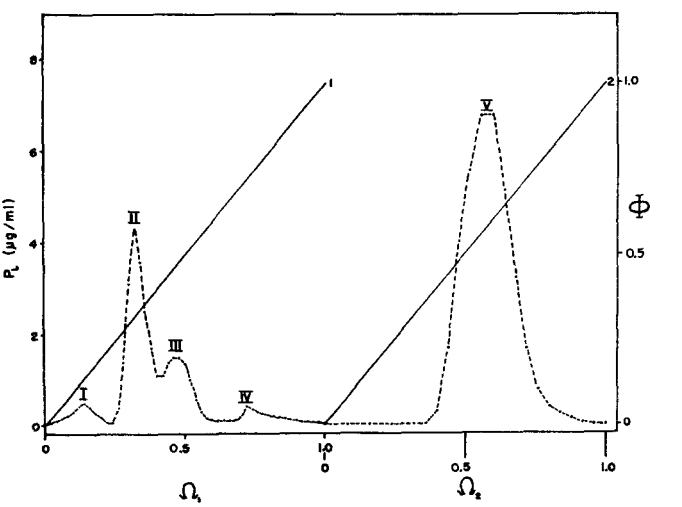

Fig. 8. Two-step gradient elution chromatography of Ehrlich ascites carcinoma phospholipids using two solvent pairs. Column: 1.16 cm^2 × 30 cm; 4 g silicic acid, washed in sequence with hexane, acetone, ethanol–cyclohexane (1:1, v/v) and cyclohexane. 12.5 mg phospholipid applied in cyclohexane. First elution: linear gradient from cyclohexane to ethanol (total volume, 250 ml). Second elution: linear gradient from ethanol to methanol (total volume, 120 ml). Flow rate, 50 ml/h. Fractions, 5 ml; recovery, 97.5%. Identification of fractions: I, phosphatidic acid; II, phosphatidylethanolamine; III, phosphatidylethanolamine, phosphatidylserine; IV, phosphatidylinositol; V, phosphatidyl choline; sphingomyelin.

sectional area per min). The pump ratio (ρ) is then set to give the desired gradient shape and elution initiated.

Results obtained with this chromatographic system have been presented previously[4-8]. However, illustrative examples are presented for phospholipid chromatography on a micro-column (Fig. 6), for chromatography using duplicate columns (Fig. 7) and for two-step gradient elution chromatography using two solvent pairs (Fig. 8).

CONCLUSION

The chromatographic system herein described permits the application of precise and predictable gradient elution to a wide variety of chromatographic problems. Although the illustrative examples have been chosen from the field of phospholipid biochemistry, the technique can be equally well applied to adsorbants other than silicic acid, and to almost any combination of miscible solvents. Furthermore, although the mathematical treatment was developed for the chromatographic system here described, it applies fully to any method for gradient development, using precise, differential flow rates, be this the production of eluant gradients for chromatography or of density gradients for centrifugation or column electrophoresis.

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