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Continuous Solvent Gradient Extraction of Lipids from Erythrocyte Membranes (Ghosts)

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(1) The lipids of erythrocyte membrane (ghosts) were analyzed employing a continuous solvent gradient extraction in a column where the support, Sephadex G-10 or 100–200 mesh glass beads, was coated with the ghosts. (1.1) The method includes a multistage closed vessel solvent gradient arrangement permitting a continuous one-phase gradient from hexane through benzene and chloroform to ethanol; (1.2) Monitoring of the column effluents by means of a circulating chain detector ("X-chromatogram"); (1.3) Analysis of the lipids of the eluted fractions by thin layer chromatography ("Y-chromatogram"); (1.4) Presentation of the combined analyses as a three-dimensional lipid map.

(2) The lipids of the erythrocyte ghosts were found to consist of three major X-fractions. (2.1) These were eluted in typical ranges of the dielectric constant (ε) of the eluent, namely fraction X1 at $\varepsilon = 1.8$, X2, $\varepsilon = 1.8-2.5$ and X3, $\varepsilon = 2.5-3.7$; (2.2) Furthermore some minor lipid fractions were detectable at $\varepsilon = 3.7-7.0$; (2.3) The major lipid constituents of fractions X1-3 were in order of relative magnitude: Cholesterol, phosphatidyl ethanolamine (PE), phosphatidyl choline (PC), lyso-PE (LPE), lyso-PC (LPC) and sphingomyelin (Sph) in X1; PE, PC and Sph in X2; Sph, LPE, PC and PE in X3. Phosphatidyl serine (PS) and Sph were found in the most polar X effluent.

(3) Total recoveries and cholesterol:phosphorus ratios were found to agree with those obtained with single-step $CHCl_3$-MeOH extraction of ghosts.

(4) It is suggested that the method may prove useful in the study of lipids of biomembranes and other subcellular structures.

Usually extraction methods for lipids from biological material aim to a single step quantitative removal of the lipids from the original sample. The amount and composition of the extracted lipids depend very much on the solvent. Hence during recent years only a few extraction systems have been used as standard procedures. The advantage of this obviously is to attain quantitatively and qualitatively comparable yields and results. On the other hand single-step quantitative extraction — and this is true also in respect to natural products other than lipids — does not give information about how the extracted compounds were bound to the original natural structure.

The primary objective of the present paper is to investigate the usefulness of a continuous solvent gradient extraction in the study of biomembrane lipids. To illustrate the principle of a "chromatographic" gradient extraction of lipids we have chosen to study the lipids of human erythrocyte ghosts, as these represent a reasonably well known and suitable object. For more detailed information about ghost lipids as related to membrane structures, the reader is referred to, *e.g.*, van Deenen,[1] Green and Tzagoloff,[2] and VandenHeuvel.[3]

The extractability of a compound, say lipid, depends on the nature of the bonding between the compound and the membrane structure. Coulombic bonding between phospholipids and proteins has been postulated (Das and Crane,[4] Das, Haak and Crane,[5] Green and Tzagoloff,[2] Salem [6]). Also the possible role of divalent metal ions in the formation of electrostatic protein-lipid interactions seems possible.[4,7] Nonpolar interaction of the London-van der Waals type between the hydrophobic moieties of lipids [3] as well as between lipids and proteins [6] are known to exist.

Parpart and Ballentine [8] divided the ghost lipids in three main groups; the loosely, the weakly, and the strongly bound fraction. The "loosely" bound fraction, comprising 44 % of the total lipid, was extractable from vacuum dried ghosts by means of dry ethyl ether. This fraction consisted mostly of cholesterol and a part of phospholipids. Correspondingly the "weakly" bound lipids, 14 % of total lipids, were extractable with wet ethyl ether or acetone-ether and consisted of cephalins. The "strongly" bound lipids, 42 % of total, were extracted with ethanol-ether 3:1 and the fraction contained the major part of all phospholipids.

It seemed to us well justified to extend the specific stepwise cathegories of Parpart and Ballentine [8] to a continuous axis of increasing extractant efficiency (polarity or dielectric constant of solvent). It was suggested that a suitable arrangement of such a continuous solvent gradient extraction would result in the elution of certain defined lipid fractions from the erythrocyte membranes. Here the solvent polarity at the time of elution of a given fraction would be related to the nature of the original bonding between the lipid and the "matrix". These fractions, named in the following the X-fractions, can then be analyzed, *e.g.* with thin layer chromatography on which the mobilities can be illustrated as a Y-chromatogram. The amounts of each X- and Y-fraction can then be presented on the Z-axis to complete a three-dimensional map of the analysis.

The experimental conditions for the above principle are available from conventional liquid-chromatographic methods. The extraction can be arranged to occur in a chromatography column with inert support that carries the ghosts like a stationary phase. This technique has very recently been successfully used in a chromatographic study of the glucose binding principle of erythrocytes.[9,10]

Principal arrangements for sophisticated gradient elution are available (see, *e.g.*, Bock and Ling,[11] Peterson and Sober [12]) as well as methods to monitor and locate fractions in liquid chromatographic effluents.[13,19]

EXPERIMENTAL

Fresh human ACD-blood was obtained from healthy blood donors through the local Red Cross Blood Bank.

Erythrocyte ghosts were prepared according to Dodge, Mitchell and Hanahan [20] by hemolysis of washed erythrocytes in 20 milli-osmolar phosphate buffer at pH 7.4 followed by successive washings and centrifugation in the same buffer. The resulting material was a pale opalescent suspension.

The content of ghosts in each sample was estimated by taking the dry weight residue per millilitre of duplicate aliquots of the ghost suspension, corrected by the phosphate residue. Typically, stock suspensions contained 2.0—3.2 mg of ghosts per ml. For standardization and comparisons one batch was subjected to total protein and total lipid determinations by conventional methods (micro-Kjeldahl-Nessler and chloroform-methanol extraction followed by gravimetric determination, respectively). The values obtained were as follows: protein (nitrogen × 6.25) 56 % of dry weight; lipid 48 %; lipid to protein ratio 1:1.2. The values were considered satisfactorily to agree with known data (see, *e.g.*, Dodge *et al.*[20] and van Deenen [1]).

Two millilitres of the ghost suspension were added onto 1.5 g of dry Sephadex G-10, the slurry was gently mixed and the beaker was then placed in a vacuum desiccator. The slurry was dried *in vacuo* for about 20 min until 1.0-1.2 g of weight (water) loss was recorded. At this stage the slurry has turned to a slightly moist "silky" powder. More recently glass beads, 100—200 mesh, were used as support instead of Sephadex in order to exclude possible partitioning effects in the column during elution (see below).

A precision column 9 × 150 mm (No. 4200 A-13, LKB Produkter A.B., Stockholm, Sweden), with all-teflon parts and fittings, was packed to a height of 15 mm with Sephadex G-10 in hexane. The ghost-Sephadex sample was then lightly tapped on this layer; hexane, in no excess to avoid dead volume formation, was added to wet the sample, and bubbles were vibrated off the support. No special technical difficulties were met in the preparation of these columns.

The column was then connected to a gradient device that delivered a continuous eluotropic gradient from hexane to ethanol or methanol. The device consisted of five closed vessels with magnetic stirrers, connected in series with teflon capillary tubing (Table 1). Constant flow was assured by a peristaltic pump (LKB No. 4912 A-2), placed between the gradient device and the column. The pump operated with a "Viton" (LKB No. 3095-01) tubing, resistant to organic solvents. Flow rates were adjusted to 0.5 ml/min. The dielectric constant (ε) was chosen to represent the eluotropic efficiency of the multicomponent eluent solvents, consisting of variable relative amounts of hexane, benzene, chloroform, methanol (or ethanol). The usefulness of this parameter (ε) is obviously limited to solvent mixtures of same or similar components, as used in the present study. The dielectric constant (ε) of the eluent was measured at +20°C by means

Table 1. Solvents employed for the development of continuous eluotropic gradients. Gradient type numbers refer to those of Fig. 1. (M = methanol, E = ethanol, C = chloroform, B = benzene, H = hexane; mixtures are 1:1 v/v; volumes in millilitres).

Direction of flow ↓	Bottle	Gradient type							
		1		2		3		4	
		Solvent	Volume	Solvent	Volume	Solvent	Volume	Solvent	Volume
	VI			M	∞			E	∞
	V	M	∞	C—M	60	E	∞	C—E	60
	IV	E	30	C	30	C—E	30	C	30
	III	C—E	30	B—C	30	C	30	B—C	30
	II	C	30	B	30	B—C	30	B	30
	I	H	30	H	30	H	30	H	30

of a WTW Dekameter (Weilheim, Germany, Type DK 03) using measuring cells MFL1, MFL2, and MFL3 for dielectric constant ranges of 1.8—7.8, 7—21 and 21—90, respectively.

The effluents of the column were monitored with a circulating chain detector.[13-15] Simultaneous fraction collection was performed manually in test tubes on the basis of monitor curve or time (X-chromatogram).

Equal aliquots (by volume) of recovered X-fractions were subjected for thin layer chromatography on Silica Gel G (E. Merck, Darmstadt, Germany). One-dimensional chromatograms (Y-chromatograms) were developed with chloroform-methanol-7 N ammonium hydroxide (CMAm) (65:25:4) (Nichols [21]) to analyze the components of each X-fraction as given in the following diagrams. To obtain evidence about the composition of found Y-fractions it was necessary to base the identification of each Y-fraction on its behaviour in a two-dimensional TLC in a solvent system of (I) CMAm (65:25:4) and (II) chloroform-methanol-acetic acid (CMAc) (85:25:1) as described by Nichols.[21]

Commercial reference compounds were available as follows: Phosphatidyl ethanolamine (Batch No. 20741), phosphatidyl inositol (Batch No. 1520), cerebrosides (Batch No. 24779), and sphingomyelin (Batch No 20.688) from Koch-Light Laboratories, Colnbrook, Bucks., G. B.; phosphatidyl choline (β-α-dipalmitoyl-α-lecithin, synth., Lot No. 73B-0540) from Sigma Chemical Co., St. Louis, Missouri, U.S.A.; phosphatidyl serine (Lot No. M1606) from Mann Research Laboratories Inc., New York, N.Y., U.S.A.; "Mixture TLC2" (cholesterol, cerebrosides, sulphatides, sphingomyelin, Lot 367—19) and "Mixture TLC 3" (cholesterol, phosphatidyl ethanolamine, lecithin, lysolecithin, Lot 656—22) from Applied Science Laboratories, State College, Pa., U.S.A.

Solvents used in the present study were redistilled or *pro analysi* grade from E. Merck, Darmstadt, Germany.

Visualization of fractions on developed thin layer chromatograms was carried out by spraying with sulphuric acid-bichromate, followed by charring on a hot plate in conventional manner. These Y-chromatograms were then scanned in a Photovolt densitometer Model 52C (Photovolt Corp., N.Y.)*. The densitometer tracings were subjected for base-line corrections based on scanning of the neighbouring blank areas of the samples on the TLC plates. At the present stage the exact quantification of the X- and Y-chromatograms was not considered as actual. For specific staining of phosphatidyl ethanolamine (PE), phosphatidyl serine (PS) and their derivatives as well as those of phosphatidyl

Table 2. The dielectric constant (ε) ranges of the solvent eluting chief (X) fractions of ghost lipids.

(X) fraction	(ε) range	Corresponding Y-chromatogram number in Figs. 2 and 3.
1	1.8	1 2
2	1.8—2.0 2.0—2.2 2.2—2.5	3 4 5
3	2.5—3.0	6
4	3.0—3.7 3.7—4.3 4.3—5.1 5.1—7.0	7 8 9 —

* The authors are thankful to Department of Pharmacology of Messrs Leiras Pharmaceutical Manuf., Turku, Finland, for permitting the use of the densitometer.

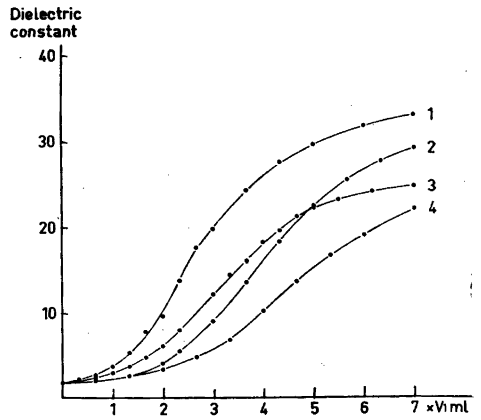

Fig. 1. Continuous eluotropic gradients used in the present study. The gradient device consisted of five closed bottles in series, the volume of the first bottle at the exit end designated as V1. A list of volumes and solvents is given in Table 1. The gradient employed for the X-chromatogram of Fig. 3 is illustrated by curve 3.

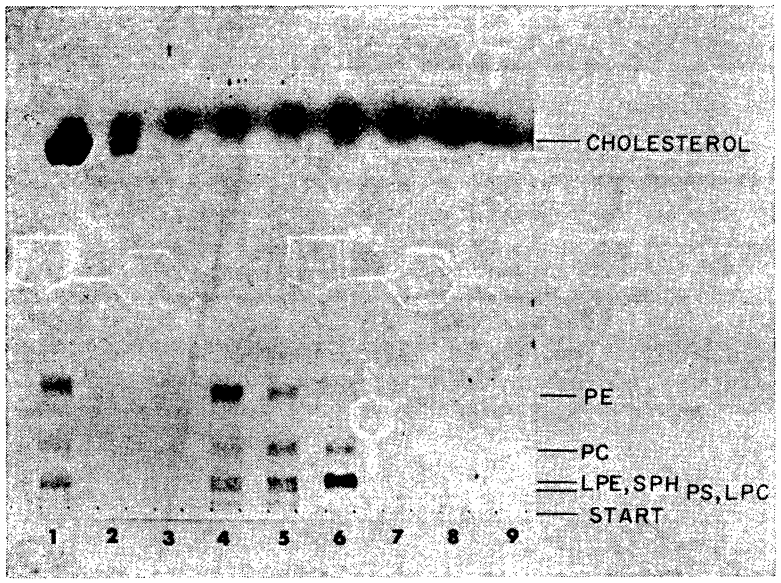

Fig. 2. Thin layer chromatography (Y chromatograms) of erythrocyte ghost lipids collected from continuous gradient extraction column. Samples represent equal (by volume) aliquots of corresponding X-fractions. For conditions of analysis, see text.

choline (PC) the visualization was performed with ninhydrin and the Dragendorff reagent, respectively, as described, *e.g.* by Stahl.[22]

The obtained information was illustrated by preparation of a three-dimensional map of the recorded fractions.

RESULTS AND DISCUSSION

Fig. 1 illustrates the eluotropic gradients used in this study. The corresponding types and volumes of solvents used to produce these gradients are listed in Table 1.

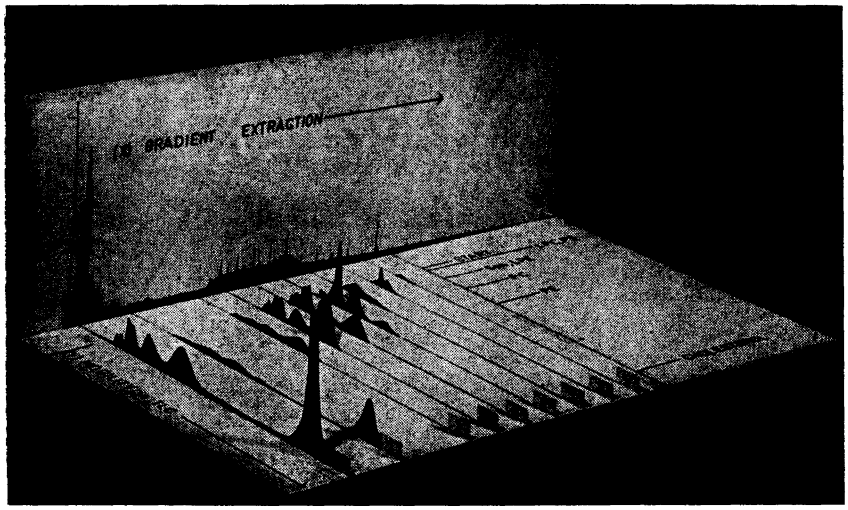

Fig. 3. Three-dimensional presentation of erythrocyte membrane lipids. Gradient extrac tion effluents (X-chromatogram) monitored with circulating chain detector. Total time of X-chromatogram 3 h. Thin layer (Y) chromatograms 1—9 were run of (X)-eluates of successive 15, 45, 15, 15, 15...min. For other details of analysis, see text.

Fig. 2 shows a TLC analysis of collected lipids of the gradient extraction. The ranges in dielectric constant (ε) of the (X) eluent corresponding to these Y-chromatograms are listed in Table 2.

Fig. 3 illustrates an analysis of erythrocyte ghost lipids obtained with the above technique. The X-chromatogram represents the order of extractability and thus gives information about the nature of the bond that existed between the extracted compounds and the "matrix" structure (van Deenen,[1] p. 64—74). When total (by chloroform-methanol extraction) lipids of ghosts were applied onto a Sephadex G-10 or glass bead column, prepared as described above, and subjected to a solvent gradient elution with effluent monitoring, all the lipids were eluted with hexane as one tailing fraction. Considering the small length of the column, the low solute concentrations and the relatively fast solvent flow, the effect of the Sephadex support as a partitioning factor was thus neglected. To assure this the Sephadex has been replaced by 100—200 mesh siliconized glass beads. The recoveries of lipids from the ghost columns were compared to yields of conventional chloroform-methanol extractions of ghosts, and the elution was found quantitative. Chloroform-methanol (2:1) did not elute further any detectable lipids from the support.

The Y-chromatograms in the present model experiments show the composition of the eluted X-fractions to follow the expectable pattern and to correspond partially to the results of Parpart and Ballentine.[8] Fig. 3 reveals obviously, that the X-chromatogram falls in three major fractions. When other solvent gradients were employed (No 1, 2, and 4 in Fig. 1) these chief fractions emerged at the same dielectric constant ranges. The first fraction

is eluted by solvent having (ε) of 1.8 (hexane). This peak corresponds to the "loosely bound" fraction of Parpart and Ballentine [8] and consists of cholesterol, phosphatidyl ethanolamine (PE) and phosphatidyl choline (PC), and sphingomyelin (Sph), as tentatively identified by comparisons with reference compounds on TLC. The X1 fraction can be interpreted as being bound to the native structure by hydrophobic attraction. Additional injections of cholesterol into the column did not elute any further phosphatides.

The second and third fractions appear as a double-peak pattern but so far we have not been able to completely resolve these two fractions. As can be seen from the corresponding Y-chromatograms (Figs. 2 and 3) there is a difference in composition of the phospholipids of these unresolved X-fractions. Y-chromatograms 3—6 (Fig. 3) demonstrate five chief components according to the TLC analysis: phosphatidyl ethanolamine (PE), phosphatidyl choline (PC), sphingomyelin (Sph), and phosphatidyl serine (PS) fractions. Spots corresponding to LPE and LPC were detectable by means of specific staining of the plates. When larger amounts of samples are run, faint zones ahead of phosphatidyl ethanolamine corresponding to cerebrosides or sulphatides can be observed in fractions corresponding to TLC-grams Y1-Y5. Furthermore the X-chromatogram of our experiment shows in the polar end at (ε) = 3.7—7.0 some lipids (PS and LPC) emerging from the column but above this polarity range no lipids were detectable. The lyso-phosphatides may represent decomposition products of native erythrocyte membrane phosphatides; the preparation of the ghost has taken one day's work at room temperature (repeated centrifugations in hypotonic phosphate buffers) and it is known [25] that aging causes considerable changes in the phosphatide pattern of ghosts.

The gradient extraction method presented here gives roughly the same X-fractions as were found by Parpart and Ballentine [8] by extraction of lyophilized ghosts with various solvents. In the present procedure the X-fractions are, however, eluted earlier in terms of dielectric constant of the eluent. The dielectric constant (ε) of the solvent (hexane) eluting the fraction X1 in the present study is 1.8 and this fraction obviously resembles the "loosely bound" fraction of the above authors; their extractant was dry ethyl ether, (ε) = 4.3. Similarly the (unresolved) X2 and X3 fractions of our experiments were eluted in dielectric constant ranges of (ε) = 1.8—2.5 and 2.5—3.7, respectively. The corresponding values for the "weakly" and "strongly" bound fractions of Parpart and Ballentine [8] are (ε) = 5.4—12.4 (wet ethyl ether and dry acetone-ether) and 21.7—22.3 (wet acetone-ether and wet alcohol-ether), respectively. For comparison the experiments described by Parpart and Ballentine [8] were repeated in our laboratory. It was found that, at least using a single over-night extraction with the named solvents, the extractability of the ghost lipids did not very clearly fall into corresponding cathegories.

We think that the continuous solvent gradient extraction as principally described here may prove useful in the study of biological lipid associations. The column geometry makes it simple to study effects of, *e.g.*, temperature and various solutes or ions on the biological "stationary phase". The extraction efficiency, in terms of extractant dielectric constant, is better in the column

than in batch extraction, and the continuity of the solvent gradient makes it possible to estimate the exact dielectric constant of the solvent at the elution of the different X-fractions. These values, again, are known to reflect the nature of bonding of the lipids in the biological structure to be studied.

It is obvious that most conventional primary extraction methods of group substances — not only lipids — are carried out in a way, which almost totally destroys the information of the basic underlaying molecular anatomy. The development of methods for the isolation and analysis of compound classes, specific in respect to the molecular anatomy of the biological microstructures, seems to us of vital interest.

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