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INVESTIGATION OF PHOSPHOLIPID CHANGES IN ACTIVELY METABOLIZING CELLS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF ^{32}P-LABELLED PHOSPHOLIPIDS

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SUMMARY

A high-performance liquid chromatographic system was employed to study turnover rates of phospholipids in various types of cells, such as leukocytes, erythrocytes and yeast cells. In the extracts obtained from ^{32}P-labelled cells ten to twelve different phospholipids could be identified. Phosphatidyl glycerol and other minor phospholipids show a high specific activity, so that the lipids may be readily detected. The reproducibility and sensitivity of the method permits the determination of lipid changes during nutrient uptake, phagocytosis, or other cellular activities.

INTRODUCTION

The range of analytical methods in lipid biochemistry has been recently enlarged by the development of high-performance liquid chromatography (HPLC)[1–9]. This appears to be a versatile, rapid and sensitive method capable of good resolution. Most applications of modern HPLC for separating phospholipid mixtures use ultraviolet (UV) absorption[3,4] and flame-ionization detection[5,7]. However, even with these detection systems, it is difficult to determine minor phospholipid compounds in a total phospholipid extract. In the present paper a HPLC method is described for the analysis of the minor phospholipids after first labelling with radioactive phosphorus, for the purpose of investigating phospholipid changes in actively metabolizing cells. The results are compared with data obtained earlier by paper chromatography[10–12].

MATERIALS AND METHODS

Phospholipid extracts from various cell types were obtained by the method described by Folch *et al.*[13]. In the case of yeast a modified procedure was required, as previously described by Deierkauf and Booij[10]. The extracts were evaporated under nitrogen at a temperature below 20°. Methanol and water were removed by repeated addition of chloroform and subsequent evaporation.

Components were identified on the basis of known chromatographic da-

TABLE I

RELATIVE SPECIFIC ACTIVITY OF VARIOUS PHOSPHOLIPID FRACTIONS, IN REPRESENTATIVE EXPERIMENTS WITH SPECIFIC CELLS (LEUKOCYTES, ERYTHROCYTES AND YEAST CELLS)

Average of four determinations. For experimental conditions and abbreviations see text.

	PA + DPG	*PE*	*PG*	*LPE*	*PS*	*PI*	*PC*	*Sph*	*X**	*DPI*
Rabbit leukocytes	400 ± 30	90 ± 5	800 ± 60	**	50 ± 3	300 ± 25	120 ± 6	10 ± 1	1000 ± 100	–
Human erythrocytes	800 ± 60	180 ± 9	1800 ± 140	200 ± 20	20 ± 1	300 ± 25	15 ± 1	10 ± 1	–	**
Yeast	600 ± 50	100 ± 5	1000 ± 80	–	150 ± 8	200 ± 10	20 ± 1	–	–	150 ± 15

* Probably CDP-diglyceride.

** Not detectable with two-dimensional paper chromatography.

ta[10–12,14,15]. Cytidine diphosphate diglyceride was synthesized according to the method of Cronan and Vagelos[16]. Cytidine diphosphate-diglyceride-dipalmitoyl was purchased from Miles Lab., Slough, Great Britain.

The labelling of polymorphonuclear leukocyte phospholipids with [^{32}P]-orthophosphate was accomplished as follows. Rabbit polymorphonuclear leukocytes were obtained according to the method described by Deierkauf *et al.*[17]. Washed cells (10^8 cells per ml) in an isotonic Hanks' medium[18] containing 0.05% glucose were incubated with sodium [^{32}P]orthophosphate (Radiochemical Centre, Amersham, Great Britain) for 3 h at 37°; 100 μCi [^{32}P]orthophosphate were added per ml of cell suspension. After incubation the cells were washed three times with Hanks' solution and extracted. Similar procedures were used for yeast cells[10] and human erythrocytes[11].

Phospholipid extracts were subjected to paper chromatography by methods described earlier[10,11,19]. The specific activity of various components was determined by measuring the amount of radioactive phosphorus as compared to the total phosphorus for separate spots on two-dimensional paper chromatograms. The amount of phosphorus was determined according to Chen *et al.*[20]. In a two-dimensional procedure[12] phosphatides were separated in the first direction on formaldehyde-impregnated paper[21] and in the second direction on Whatman Sg 81 silicic acid-impregnated paper. The mobile phase in the first direction was the upper phase obtained after a butan-1-ol–acetic acid–water mixture (4:1:5) had been equilibrated with diethyl ether[21]. The mobile phase in the second direction was the diisobutyl ketone–acetic acid–water system (40:25:5) described by Marinetti[19]. The relative position of the spots was established by comparing the results of phospholipid staining by Rhodamine 6G with the black spots on X-ray film.

EXPERIMENTAL

Continuous detection of phospholipids in column eluates by UV absorption spectroscopy at 203–214 nm has been used in the case of eluents which do not absorb in this region[3,4]. With this method the range of eluents was necessarily limited. Other authors have used flame ionization detection[5,7]. Both detection systems are not sensitive enough to detect "minor phospholipids" in a total phospholipid extract. Therefore we employed another detection system, based on the incorporation of [^{32}P]orthophosphate in phospholipids. Most "minor phospholipids" rapidly incorporate [^{32}P]orthophosphate[22,23]. When cell suspensions are incubated with radioactive phosphate the specific activity of several minor lipids is much higher than that of other phospholipids (see Table I). This facilitates the detection of minor phospholipids in the HPLC column eluates.

The chromatographic system is schematically represented in Fig. 1. The apparatus contained a LiChrosorb Si 60-10 (25 cm × 4.6 mm I.D.) column (Chrompac, Middelburg, The Netherlands), a diaphragm pump (Orlita DMP 1515), a manometer (Haenni 121637), a HPLC sample loop valve (Kipp & Sons, Delft, The Netherlands) with a 50- or 100-μl sample loop and a drain. A liquid gradient was obtained with an LKB gradient mixer (Ultrograd 11300). The optimal separation of complex phospholipid mixtures requires a systematic change of the distribution coefficients of the components in the mixture, as can be achieved by a carefully controlled elution gradient[6,7]. The volume of the mixing chamber, pump and tubing before the column

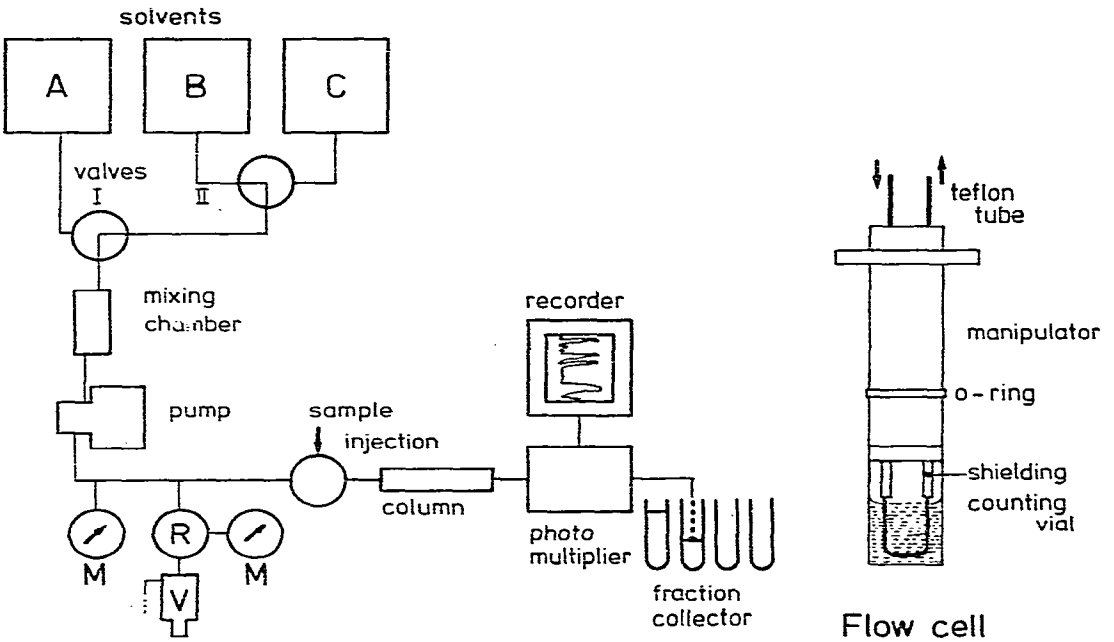

Fig. 1. Schematic representation of the chromatographic system. R = Reducing valve; M = manometer; V = micrometer valve with overflow.

Fig. 2. Flow cell for detection of radioactivity (^{32}P). The loop volume in the counting vial is 30 μl. Counting efficiency for ^{32}P is 35%.

influences gradient formation in the column; phospholipid separation is thereby impaired. This effect can be minimized by choosing an adequate flow-rate, *i.e.*, at least 2 ml/min.

The optimal-flow rate in the column for the separation of a phospholipid mixture was found to be less than 1 ml/min. In order to keep the total flow-rate at 2 ml/min and the flow-rate in the column at *ca.* 0.6 ml/min, the chromatographic system was combined with an overflow drain. In this way the flow-rate in the column remained within the range required for adequate separation. The drain consisted of a pressure regulator (Texas Corporation), a manometer (Haenni 12131) and a micrometer valve (Whithey SS21 RS4). The radioactivity in the eluent was measured with a bench-top liquid scintillation counter (Searle Model 4539) using the flow cell shown in Fig. 2. The composition of the eluent (chloroform–propanol–acetic acid–water) was established in agreement with the results of thin-layer chromatography (TLC) of phospholipids. In the eluent for the HPLC analysis the ratio chloroform:propanol (50:55) is kept constant, while the polarity of the solvent was varied by changing the amount of water and acetic acid respectively (Fig. 3). The eluting solvents were carefully degassed prior to use. Eluents: A = chloroform–propanol–acetic acid–water (50:55:2.5:5); B = chloroform–propanol–acetic acid–water (50:55:2.5:8.75); C = chloroform–propanol–acetic acid–water (50:55:5:10).

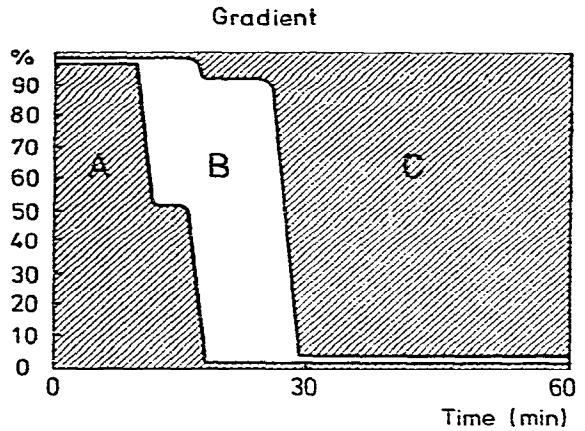

Fig. 3. Gradient pattern for phospholipid elution. Solvents: A = chloroform–propanol–acetic acid–water (50:55:2.5:5); B = chloroform–propanol–acetic acid–water (50:55:2.5:8.75); C = chloroform–propanol–acetic acid–water (50:55:5:10).

RESULTS AND DISCUSSION

The relative specific activities of various phospholipids were established from paper chromatograms. The relative specific activity was defined as:

$$\text{Relative specific activity} = \frac{\%\text{ of total } ^{32}\text{P}}{\%\text{ of total lipid P}} \cdot 100$$

The radioactivity (cpm) in a given phospholipid spot, as a percentage of the total radioactivity of all the spots, is divided by the amount of phosphorus in the spot as a percentage of the total phosphate in the phospholipids. In Table I the following abbreviations are used: PA = phosphatidic acid; DPG = diphosphatidylglycerol; PE = phosphatidylethanolamine; PG = phosphatidylglycerol; PS = phosphatidylserine, PI = phosphatidylinositol; PC = phosphatidylcholine; Sph = sphingomyelin; DPI = diphosphatidylinositol; and LPE = lysophosphatidylethanolamine.

Table I clearly demonstrates the different turnover rates of the various phospholipids. Notable are the high relative specific activity of PG and the different turnover rates of the incorporation of [^{32}P]orthophosphate in PS and PC of leukocytes, erythrocytes and yeast cells. The high relative specific activity of the minor phospholipids enabled us to detect these compounds in HPLC eluates. Compounds with a relatively low specific activity, such as PC, can be recognized if present in sufficient quantities.

In Fig. 4 the results are presented of a gradient elution separation of a total phospholipid extract of rabbit polymorphonuclear leukocytes. The different fractions were collected separately and identified by two-dimensional paper chromatography and TLC. A typical two-dimensional paper chromatogram is shown in Fig. 5. The chromatographic behaviour of compound X was identical with that of cytidine

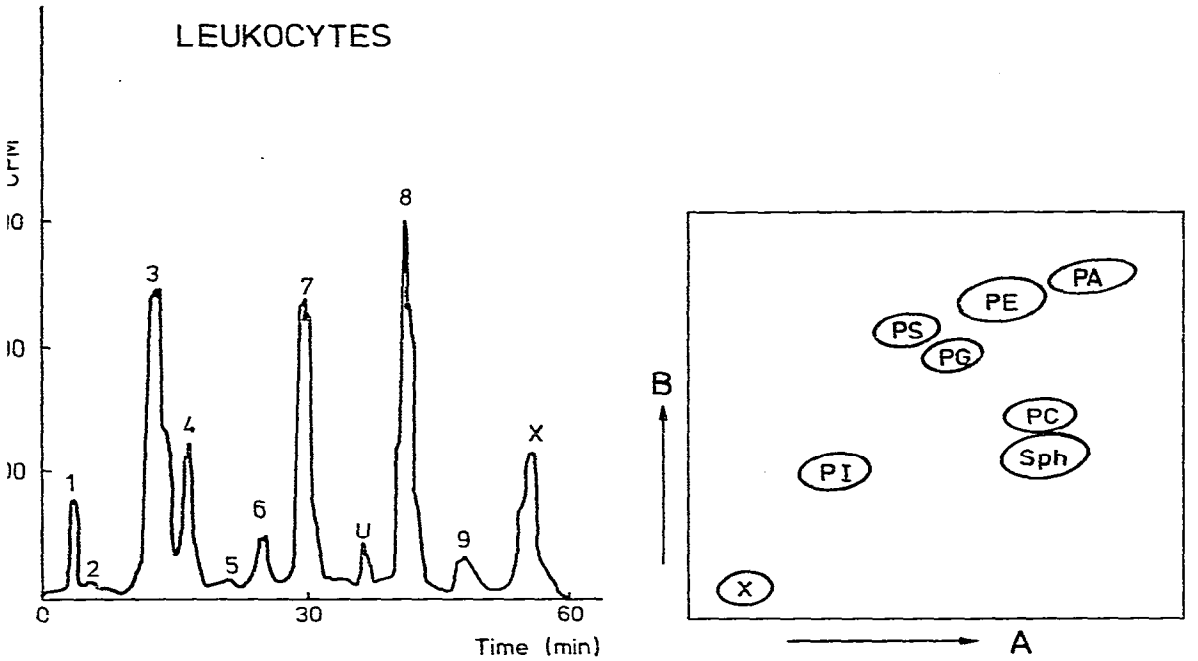

Fig. 4. Chromatogram obtained of a total phospholipid extract of ^{32}P-labelled polymorphonuclear leukocytes (rabbit). Conditions: LiChrosorb column Si 60-10; sample load, 100 μg in 100 μl; gradient of Fig. 3; flow-rate 0.6 ml/min. Substances identified: 1 = PA; 2 = DPG; 3 = PE; 4 = PG; 5 = LPE; 6 = PS; 7 = PI; 8 = PC; 9 = Sph; U = Unidentified. For abbreviations see Table I and text. CPM = Counts per minute.

Fig. 5. Two-dimensional paper chromatogram of phospholipids of polymorphonuclear leukocytes (rabbit). A = First direction on formaldehyde-treated paper; B = second direction on silicic acid paper. For experimental conditions and abbreviations, see text.

diphosphate diglyceride. The amount obtained of this compound was too small for further analysis. Compounds 2 and 5 were not detectable on the paper chromatogram. After combining the corresponding fractions from different runs, compounds 2 and 5 were identified by TLC as diphosphatidylglycerol and lysophosphatidylethanolamine. Peak 8 contains a small quantity of sphingomyelin; the difficulty of separating PC and sphingomyelin has also been noted by Geurts van Kessel *et al.*[4].

Figs. 6 and 7 illustrate the separation of total phospholipids of human erythrocytes and yeast cells respectively. The chromatographic system gives a satisfactory resolution. With the exception of a small amount of Sph in the PC fraction, all the phospholipids are adequately separated. PC and Sph can be separated with acetonitrile–methanol–water as shown by Jungalawa *et al.*[3].

By appropriate choice of the solvent parameters, all the different classes of phospholipids can be separated. To collect PG as a separate fraction we had to enhance the resolution of the column for PE and PG. For this purpose the solvents should have the following composition: A = toluene–propanol–acetic acid–water (3:4:0.2:0.3); B = toluene–propanol–acetic acid–water (3:4:0.5:0.3); C = toluene–propanol–acetic acid–water (3:4:0.5:0.65). The polarity of the eluent is increased first by acetic acid and then by water; the gradient pattern remains the same as that

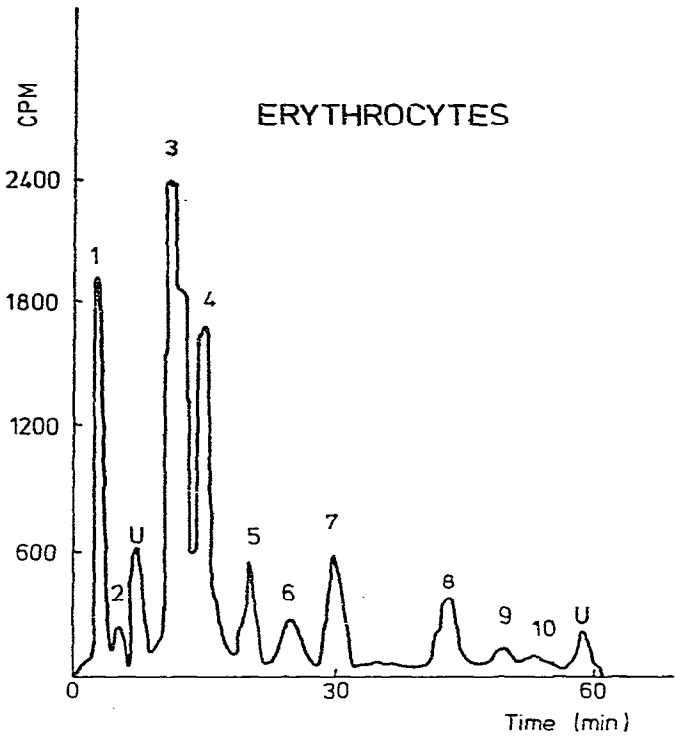

Fig. 6. Chromatogram obtained of a total phospholipid extract of ^{32}P-labelled human erythrocytes. Conditions and symbols: see legend of Fig. 4. Compound 10 identified as DPI.

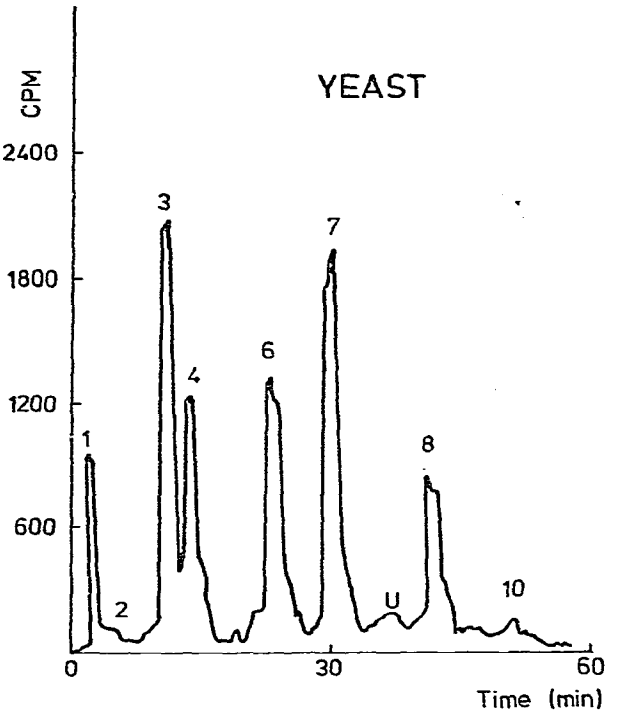

Fig. 7. Chromatogram obtained of a total phospholipid extract of ^{32}P-labelled bakers' yeast cells (Delft I). Conditions and symbols: see legend of Fig. 4.

shown in Fig. 3. This eluent increases the retention time for PE and decreases that for PG, which results in the complete separation of PG and PE. However, with this eluent PE and PS are not well-separated, due to a decrease of the retention time for PS; an additional disadvantage is the peak broadening of PC.

Our results indicate that HPLC yields better phospholipid separation than conventional silicic acid column chromatography. A critical factor is the flow-rate in the column, which should be high enough to establish an effective gradient but not so high that resolution is impaired. The drain we introduced in the system for HPLC of lipids was designed to meet this requirement. The choice of ^{32}P as a marker made it possible to detect minor compounds in a total phospholipid extract which are not detectable with conventional detection systems.

By using HPLC in a semi-preparative way, unidentified phospholipids can be separated and collected for further investigations. This is an essential step for the elucidation of the phospholipid biosynthesis in the cell.

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