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IDENTIFICATION OF COMPONENTS OF PHOSPHOLIPID METABOLISM IN AUTOMATED PHOSPHATE ESTER CHROMATOGRAPHY

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SUMMARY: By means of automated phosphate ester chromatography of rabbit heart ventricles, two compounds, glycerol-3-phosphorylcholine and phosphocholine have been separated and identified from very early eluting material. These two compounds among others (glycerol-3-phosphoethanolamine and phosphoethanolamine for instance) also comprise a significant percentage of the material extracted with perchloric acid from rat adult and embryo hearts and many other tissues as well. Automated phosphate ester chromatography should prove clinically useful in studies of diseases such as hereditary muscular dystrophy as the compounds named are known to be involved.

INTRODUCTION: Although the reactions of the cytidine pathway for the synthesis of phosphatidylcholine and phosphatidylethanolamine were described more than two decades ago (1,2), very little on the regulation *in vivo* of this biosynthetic pathway in mammalian cells has been learned (3). These two compounds are the major phospholipid constituents of plasma and organelle membranes in mammalian cells and play a major role in regulating the biochemical and physicochemical properties of such membranes as well as the activity of many enzymes and enzyme systems. Of great interest also are the associated components or metabolites of these phospholipids: glycerol-3-phosphorylcholine (GPC), glycerol-3-phosphoryl ethanolamine (GPE), phosphocholine (PC) and phosphoethanolamine (PE).

In order to be able to attack problems involving the cytidine pathway, we have been separating and quantifying the phosphate esters of intermediary metabolism from many tissues treated under selected conditions and then subjected to chromatography with the automated phosphate analyzer developed in our laboratory (4,5,6,7,8). One such study has involved changes in fetal rat heart metabolites (13-day embryos) from mothers treated with ethynylestradiol and norethindrone acetate. In a typical chromatogram (Fig. 1) we observed a region of complex peaks (Groups A & B) that were eluted partly in the water used to apply sample to the

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column and partly in the weakly basic, dilute starting buffer. These peaks were eluted before the known phosphate monoesters: creatine phosphate, glucose-1-phosphate or inorganic phosphate (P_i), glycerol-3-phosphate (GP), etc.

With authentic compounds used in cochromatography and by methods detailed below we have found that the first group contains GPC and GPE; the second comprises at least some PC and PE. In the whole adult rat heart we find smaller quantities of these materials than in the embryo yet the composition of the peaks is still complex. On the other hand, adult rabbit heart ventricles we found are quite simple in that there are apparently only two major peaks eluted early: GPC and PC.

METHODS:

Materials: All reagents were of highest purity obtainable. In addition, Choline kinase (ATP: choline phosphotransferase; E.C. No. 2.7.1.32), ATP, authentic GPC, GPE, PD and PE were products of Sigma Chemical Co. Alamine 336 is tri-n-octylamine from General Mills Chemicals, Inc., and Freon-TF was obtained from Miller-Stephenson Chemical Co., Inc.

Preparation of tissue extracts: Tissues were extracted with perchloric acid after first having been frozen and powdered by grinding in a ceramic mortar in the presence of liquid nitrogen (9). Supernatant solution containing acid soluble, phosphorylated metabolites was decanted from the centrifuged protein pellet and extracted with Alamine-336 (10). The pH value of the extract was brought to neutrality with the use of bromothymol blue indicator and a small amount of potassium bicarbonate. In treating the 13-day rat embryo hearts, extractions were carried out directly with ice cold 0.6M $HClO_4$ after the tissues had been frozen with liquid nitrogen and powdered in a stainless steel impact mortar. Perchloric acid was removed from the extract by neutralizing it with $KHCO_3$ and the supernatant solution was decanted from the $KClO_4$ precipitate. Centrifuged tissue pellets were saved for protein determinations (11).

Chromatography of extracts: Neutralized extracts were placed directly on a high efficiency anion exchange system in the automatic phosphate analyzer developed in our laboratory (4,5,12)(Instrument available from Alsab Scientific Products, Inc.).

Glycerol-3-phosphorylcholine, GPE, PC and PE were also isolated by passing a 1-ml aliquot of neutralized extract, for example, through a short column of about 0.5 ml of AGMP-1 (BioRad Laboratories) anion exchange resin (200-400 mesh chloride form) packed in a Pasteur pipet. The compounds of interest were washed through with water while all other phosphorylated compounds remained on the resin.

Cochromatography with authentic GPC, GPE, PC and PE was used in order to establish the probable identity of the compounds of interest in the tissue extract.

Hydrolysis: Choline was hydrolyzed from GPC or GPE by acidifying the solution obtained from the short, preparative AGMP-1 column to about 1M with 70% $HClO_4$ and heating for 15 min. in a boiling water bath. This treatment converted all the GPC and GPE to choline and ethanolamine plus P_i and GP. In another experi-

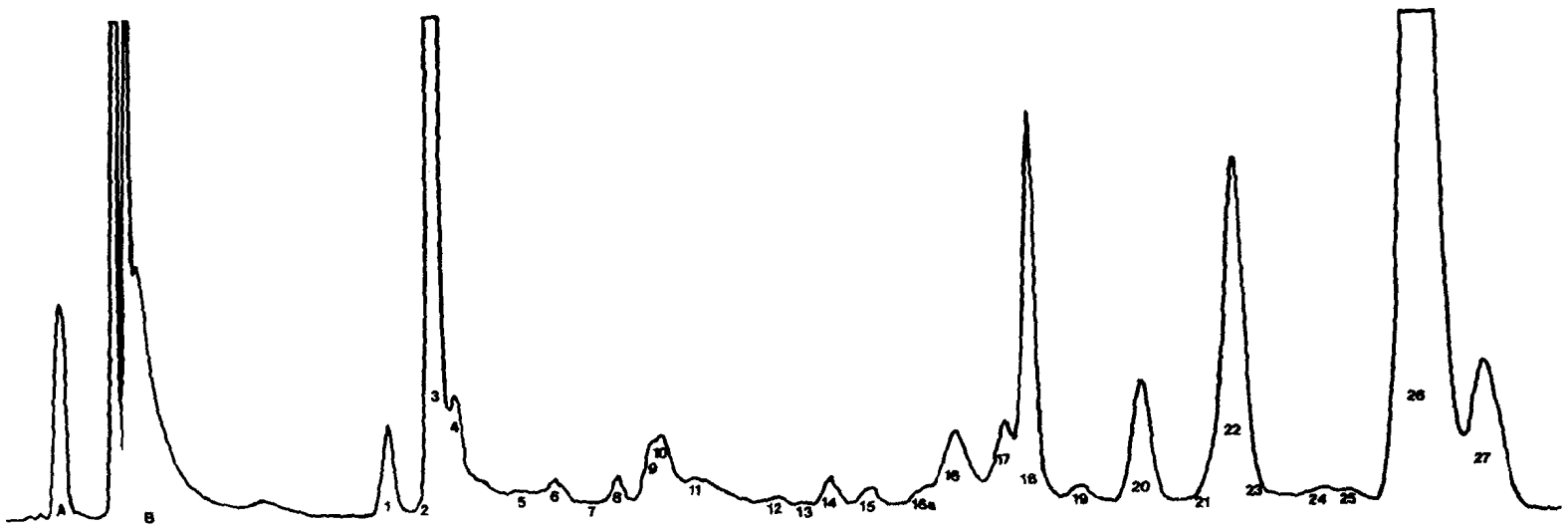

Fig. 1. Phosphorylated metabolic intermediates chromatographed from 13-day old rat embryo heart (∿ 20 mg of tissue). Methods outlined in Materials and Methods section.

Peak Identification: A. Phospholipids - Grp. A, B. Phospholipids - Grp. B, 1. Creatine Phosphate, 2. Glucose-1-Phosphate, 3. Inorganic Phosphate, 4. α-Glycerophosphate, 5. Dihydroxy Acetone Phosphate, 6. Mannose-6-Phosphate, 7. Glyceraldehyde-3-Phosphate,8. Fructose-6-Phosphate, 9. Sedoheptulose-7-Phosphate, 10. Glucose-6-Phosphate, 11. Nicotinamide Adenine Dinucleotide, 12. Pyrophosphate, 13. 3-Phosphoglyceric Acid, 14. Uridine-5'-Monophosphate, 15. Glucose 1,6 Diphosphate,16a. X_1, 16. Adenosine-5'-Monophosphate, 17. Fructose 1,6 Diphosphate, 18. 2,3-Diphosphoglyceric Acid, 19. Uridine-5'-Diphosphate & Inosine-5-Monophosphate, 20. Cytidine-5-Triphosphate, 21. X_2, 22. Adenosine-5-Diphosphate, 23. Guanosine-5-Monophosphate, 24. Guanosine-5-Diphosphate, 25. X_3, 26. Adenosine-5-Triphosphate & reduced Nicotinamide Adenine Dinucleotide, 27. Guanosine-5-Triphosphate.

ment a ~0.3M $HClO_4$ solution seemed less destructive to PC and PE but only about 85% GPC was hydrolyzed in 30 min.

Use of Choline Kinase: This enzyme was used as a reagent to convert choline into choline phosphate in order to prove that GPC indeed was present and could be determined quantitatively as well as qualitatively by cochromatography. A 500-µl sample of hydrolyzed heart extract was slightly alkalinized with $KHCO_3$ (pH 8 to 8.5) and $MgCl_2$ (~5mM) and ATP (~5mM) were added together with about 0.1 unit of enzyme. After standing at room temperature for 30 min. to an hour, the solution was acidified with $HClO_4$ (to ~0.3M) and again extracted with Alamine and neutralized. It was then ready for chromatography.

RESULTS AND DISCUSSION:

A typical chromatogram of pooled 13-day embryo hearts is illustrated in Fig. 1. From chromatograms of the adult rat heart a calculation revealed (Table I) that the adult contains quantitatively smaller amounts of compounds in peak groups A and B than the embryo. Since these are phospholipid metabolites and probably precursor materials this is not surprising in that in the very young tissues one expects accelerated membrane synthesis and growth more than merely maintenance. The compounds GPC and GPE cochromatographed with group A (Fig. 1) and together provide a somewhat broadened peak, not sharp. Group B peaks probably contain some PC and PE since these two compounds eluted in the very early portion of this region as shown by cochromatography. The adult heart contains about twice as much of Group B compounds as Group A, while in the embryo there is more than 5 to 10 times as much. The embryo contains a total of more than ~6 times

TABLE I

COMPARISON OF ADULT AND FETAL RAT HEART
ACID SOLUBLE PHOSPHOLIPID METABOLITES

Heart	Group A: GPC + GPE (nmoles/mg protein)	Group B: PC + PE + Unknowns (nmoles/mg protein)	% of Total PO_4
Adult	1.97	4.3	7.7
13-Day Embryos	4.9	27.3	20
	3.5	41.5	27
	3.2	31.7	20

Details of extraction and chromatography are given in Materials and Methods Section. Amount of tissue necessary for one chromatogram: ~4 mg of protein (equivalent to about 20 mg of wet weight tissue or ~17 to 25 embryo hearts).

as much of all these compounds taken together as does the adult. The A and B group compounds make up a relatively larger proportion of the total phosphate ester material extractable with acid from the embryo than from the adult.

The automated phosphate analyzer permits us to analyze very small amounts of tissue (Table I) and therefore can be used in clinical biopsy procedures.

We had suspected that the very early peaks in our chromatrogram contained phosphodiester material and the cochromatography experiments have shown that this is probably the case. A perusal of the literature revealed that the nuclear magnetic resonance technique has been used also to discover the presence of phosphodiester material in various tissues (13,14,15 for instance). The procedure for isolation of the suspected material was, however, more complicated than that which we have used (see Methods Section and Ref. 13,14).

Chromatography can be a very powerful method for determining the composition of certain compounds when used in conjunction with chemical methods that move the peaks to different locations (i.e. alter their retention times) by simple chemical treatments such as hydrolysis or enzymic reaction, etc., followed by rechromatography of the treated extract. Accordingly we used the following approach to prove that the GPC is truly a component of the Group A material. Rabbit heart ventricles were prepared as described in the Methods Section. When chromatographed, only two very sharp peaks were found in the Group A and Group B regions. We first separated peaks "A and B" from all other phosphate esters by washing them through a short anion exchange column prepared in the chloride form. As detailed in Table II, acid hydrolysis converted GPC to GP which elutes near P_i upon chromatographing the hydrolyzate. The hydrolyzed extract also contained an amount of choline equivalent to [GP] which was then treated separately in another portion of the hydrolyzate with choline kinase. Upon rechromatography, choline was quantitatively converted to PC and recovered from the solution, eluting in the Group B position identical to authentic PC. We interpreted these results to mean that the two peaks of rabbit heart ventricle are indeed GPC and PC. The final amount of PC recovered (29.3 nmoles) is about 85% of the total

TABLE II

EXAMPLE DETERMINATION OF GPC IN RABBIT HEART EXTRACT

Sample and Treatment	nmoles found			
	GPC	PC	GP + P_i	Total
Extract	24.7	11.8	0	36.5
Hydrolyzed Extract	0	12.8	21.8	34.6
Choline kinase treated	0	29.3	19.9	

Details are given in Materials and Methods.

value of 34.6 in the hydrolyzate, a fairly good recovery when working with nmole quantities: 12.8 nmoles of PC + 21.8 nmoles of GP (equivalent to choline split from GPC) = 34.6 nmoles total PC that should have been found with choline kinase treatment.

Recent observations in our laboratory indicate that the group A and B regions are highly susceptible to changes depending upon the specific tissue examined and conditions of treatment. Transformed cells from tissue culture are qualitatively and quantitatively different from the normal. A report on this subject is in preparation and experiments with radioactive phosphate will reveal the metabolic flux through the complex phospholipid metabolites. In addition, studies on dystrophic muscle with techniques such as we have outlined in this report may provide important insights into the nature of the disease (see also Ref. 13).

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