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THE APPLICATION OF CHROMATOGRAPHIC METHODS TO STUDY THE INCORPORATION OF ^{32}P-LABELED ORTHOPHOSPHATE INTO THE PHOSPHATIDES OF RAT LIVER HOMOGENATES*

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In light of the newer paper and column chromatographic techniques for lipide fractionation[1-4] the study of the incorporation of labeled orthophosphate into the phosphatides of rat liver was undertaken. The results presented in this paper show that liver homogenates can carry out this latter process to a small extent and that the lipides which become most actively labeled resemble but are not identical to phosphatidic acids.[5] In contrast to *in vivo* studies[1,3,6], lecithin and phosphatidyl ethanolamine show very little incorporation in the *in vitro* system.

EXPERIMENTAL

Methods and reagents

Methanol, petroleum ether (b.p. 35°–60°), and chloroform which were used for lipide extraction and fractionation were Mallinckrodt 'analytical' reagents. Total lipide P was determined by a modified*** method of HARRIS AND POPAT[7] and ester analyses by the method of RAPPORT AND

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*** The method was modified by using half the amount of perchloric acid, molybdate and elon reagents and bringing the solution to a final volume of 10 ml.

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ALONZO[8]. ^{32}P activity was determined with a Radiation Instrument Development Laboratory Scaler, Model 200, by plating aliquots on aluminum planchets, or by plating on filter paper. Mallinckrodt C.P. silicic acid, 100 mesh, was used for column chromatography of the lipides. Paper chromatographic analyses of the phosphatides were carried out as described previously[1-3].

Liver homogenates

Rat liver (7.93 g wet weight) was homogenized in a ground glass homogenizer for 5 minutes in 12 ml of 0.01 *M* phosphate buffer pH 7.4 and strained once through cheesecloth. Seven ml of the filtered suspension were added to each of two 125 ml Erlenmeyer flasks and to each were added the following: 1.0 ml of 0.1 *M* glucose, 1.0 ml of 0.1 *M* succinate, 1.0 ml of 0.1 *M* glycerol, 1.0 ml of 0.01 *M* ATP (Pabst), and 0.1 ml (130 μc) of ^{32}P-labeled orthophosphate. To one flask, reaction B, was added 1.0 ml of 0.1 *M* NaCN and to the other flask reaction R, was added 1.0 ml of water. Cyanide, ATP, and succinate were neutralized to pH 7.4 before use. The flask containing cyanide was flushed with nitrogen and stoppered. Both flasks were then shaken in a water bath at 37° for 45 minutes.

Lipide extraction

After the incubation period, the contents of each flask were treated with 30 ml of methanol and heated at 60° for 10 minutes. After addition of 15 ml of chloroform, the suspensions were heated again for 10 minutes, cooled, and centrifuged. The supernatant fluids were transferred to appropriate flasks, the residues extracted once more with chloroform-methanol 1:2 and centrifuged. The respective supernatant fluids were combined and evaporated just to dryness *in vacuo* under nitrogen. The residues thus obtained were treated successively with one 20 ml and two 10 ml portions of warm chloroform to selectively extract the total lipides. Each solution was brought to 50 ml with chloroform in a volumetric flask. Reactions R and B yielded 126 and 130 mg of total lipides and 4.6 and 4.5 mg of total P respectively.

The chloroform solution of the total liver lipides was found to contain trace amounts of water-soluble phosphate esters and orthophosphate having a high specific activity. The removal of these contaminants by washing the chloroform extract with aqueous solutions of unlabeled phosphate was avoided, since this treatment gave rise to emulsions which were difficult to separate and invariably led to a loss of significant amounts of phosphatides which accumulated at the chloroform–water interface. The use of 10 per cent trichloracetic acid to precipitate the lipides and proteins and washing this precipitate with 5 per cent trichloracetic acid and then with water, was found to be more satisactory since the lipides extracted in the usual manner from this washed residue contained only barely detectable amounts of the aforementioned contaminants. Some lipide P is lost during this procedure, but paper chromatographic analysis showed that all the phosphatides found in the usual lipide extract also were found in the lipide extract of the washed trichloracetic acid precipitate. The use of perchloric acid to precipitate the lipides was not satisfactory, since this reagent altered the phosphatides so as to render them unsuitable for paper chromatography. A simple yet effective method for counting the radioactivity of a total lipide extract was ultimately found to be as follows: A known amount of lipide extract (25–50 μl) is pipetted on acetic acid washed Whatman no. 1 filter paper and the radioactivity determined before and after washing with water for one hour. The water washing removes all the water soluble contaminants and leaves only the lipides on the paper. This method also gives a more uniform distribution of the lipides and more reproducible results than can be obtained by pipetting on the metal surface of planchets.

Paper chromatographic analysis

A 5 ml aliquot of lipide extract R and B was evaporated to dryness and 0.60 ml of *iso*amyl alcohol–benzene 1:1 added to each. Ten μl of each solution* were employed for paper chromatographic analysis. Di*iso*butyl ketone–acetic acid–water 40:30:7 was used with silicic acid impregnated paper[1], whereas 2-octanol–lutidine–acetic acid 90:5:5[2] and di*iso*butyl ketone–acetic acid 30:5[3] were employed with non-impregnated paper. Autoradiograms were prepared of the water washed (1 hr washing) chromatograms on Kodak no-screen X-ray film (4 weeks exposure). The detection methods and chromatographic technique used are reported elsewhere[1,2].

In order to obtain quantitative information on the lipide spots obtained by paper chromatography, forty four 10 μl spots* of reaction R were placed on nine silicic acid impregnated papers (12 × 42 cm) and the chromatograms developed with the di*iso*butyl ketone-acetic acid-water solvent. The lipide spots were detected with Rhodamine 6G (see Table I) and eluted under reflux for 10 minutes with chloroform methanol 1:1 and then with methanol alone. The extracts were made to a known volume and aliquots used for total P analysis and ^{32}P activity. The results of these experiments are summarized in Table I.

* Ten μl of sample R contained 210 μg of total lipides and 7.67 μg of total P.

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Column fractionation of the total lipides

A 50 ml burette was packed with 10 grams of silicic acid which was suspended in 30 ml of petroleum ether. The column was washed successively with 20 ml of petroleum ether, 20 ml of chloroform-methanol 1:1, and 20 ml of petroleum ether. A 10 ml aliquot of the chloroform solution from reaction R containing 25.2 mg of total lipides was put on the column and eluted with the following solvents in the order given: 50 ml of chloroform, 100 ml of chloroform methanol 4:1, 100 ml of chloroform methanol 1:1, and 150 ml of methanol. Eighty-two 5 ml fractions were collected and aliquots of each were taken for ester and total P analysis and for ^{32}P-activity. The ester and P distribution curves are given in Figs. 1 and 2. In addition, each fraction was subjected to paper chromatographic analysis (Fig. 3) and autoradiograms made of the resulting chromatograms. Aliquots of the peak fractions were also hydrolyzed with 1N HCl and the products obtained analyzed chromatographically for choline, serine, and ethanolamine.

RESULTS

By the combined paper and column chromatographic methods for lipide fractionation it has been demonstrated in the present experiments that rat liver homogenates are able to incorporate labeled orthophosphate to a small extent into several different phosphatides, the most active of which are unidentified lipides having the chromatographic properties[1,2] of phosphatidic acids. The other phosphatides such as lecithin, phosphatidyl ethanolamine, phosphatidyl serine, inositol phosphatide, and sphingomyelin show very little or no labeling. The incorporation into the phosphatides is completely abolished by cyanide and is therefore an aerobic-dependent process. FRIEDKIN AND LEHNINGER[9] observed a similar phenomenon with rat liver particulates.

Because of the small amount of lipides used for paper chromatography, only by autoradiography was it possible to detect those phosphatides which were very weakly labeled. When multiple chromatograms were run and the individual spots combined and concentrated, or when column chromatography was employed, the activity of the weakly labeled lipides was more conclusively ascertained. Thus the data in Table I show that spot R-8 occurs in small amount as evidenced by the P analysis but has the highest specific activity of all the lipides. The spots R-7 and R-6 corresponding to phosphatidyl ethanolamine and phosphatidyl serine, respectively, contain significant activity, but some of this labeling was shown to be due to trailing from spot R-8*. It is further evident that the lecithin spot R-5 contains very little activity, as do the spots corresponding to inositol phosphatide, acetal phosphatide, and sphingomyelin. The material (spots R-0 and R-1), at or near the origin, represents a mixture of small amounts of unidentified lipides having very little activity, and trace amounts of water-soluble phosphate esters** and inorganic phosphate having very high specific activity. These latter water soluble compounds can to a large measure be washed out of chromatograms prepared on silicic acid impregnated paper as evidenced by the simultaneous preparation of autoradiograms from washed and unwashed chromatograms. However, minute amounts of these compounds still remain on the paper. The phosphatide spots, however, exhibit no diminution in intensity of the darkening of the film,

* When chromatograms were developed on paper sheets measuring 12 × 42 cm using chambers 6" i.d. × 18", the fast moving R-8 trailed into spots R-7 and R-6. This trailing was minimized and complete separations of these lipides were achieved by chromatography on 4 × 42 cm strips and by the use of 1000 ml graduate cylinders.

** The following water-soluble phosphate compounds were tested; glycerophosphate, glycerophosphorylcholine, phosphorylcholine, phosphoglyceric acid, adenosine tri-, di-, and monophosphates, cytidine triphosphate, uridine triphosphate, guanosine triphosphate, and orthophosphate. All except glycerophosphate, phosphoglyceric acid, and orthophosphate remained at the origin when run in the di*iso*butyl ketone–acetic acid–water system and using silicic acid impregnated paper. The latter three compounds moved only slightly (R_F less than 0.05) in this solvent.

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TABLE I

SUMMARY OF DATA OBTAINED FROM COMBINED CHROMATOGRAMS OF PHOSPHATIDES FROM RAT LIVER HOMOGENATES OF REACTION R

Spot No.	R_F *Value*[a]	*Dye Test*[b]	*Ninh.*[c]	*Chol.*[d]	*Lipide*[e] *P*	*Fraction of total P*	*Spec. Act.*	*Phosphatide components*[f]
					γ	*per cent*	*c.p.m./mg P*	
R-0	0.00	b	+m	—	2.02	3.71	6340	unidentified
R-1	0.05	b	—	—	2.30	4.22	2100	unidentified (possibly lysophosphatides)
R-2	0.13	b	—	—	2.22	4.04		
R-3	0.34	b-o	—	+w	7.46	13.65	870	inositol phosphatide
R-4	0.38	p	+w	—				sphingomyelin + acetal phosphatide
R-5	0.44	y	—	+s	25.0	45.7	403	lecithin + phosphatidal choline-like lipide
R-6	0.58	b	+w	—	2.99	5.48	1700	phosphatidyl serine
R-7	0.62	y	+s	+w	8.88	16.30	4100	phosphatidyl ethanolamine
R-8	0.69	b	—	—	2.92	5.34	14,400	unidentified
R-9	0.73	y	—	+vw	0.80	1.46	6100	unidentified

[a] The solvent system used was di*iso*butyl ketone-acetic acid-water 40:30:7. The R_F values varied ± 0.05.

[b] The chromatograms were stained by immersing in 0.001% Rhodamine 6G (Colour index 752, National Aniline Division, Allied Chemical and Dye Corporation) for ten minutes and observed while wet under ultraviolet light. b = blue, b-o = blue orange, p = purple, y = yellow.

[c] The chromatograms were sprayed with 0.3% ninhydrin in acetone–lutidine 9:1. w = weak, m = moderate, s = strong, vw = very weak. — = negative.

[d] Phosphomolybdate–$SnCl_2$ was used as described previously[2]. The letters w, vw, and s have the same meaning with respect to the intensity of the test as given in footnote c.

[e] Represents lipide material from eight 10 μl spots containing 1.68 mg of total lipide and 61.3 μg of total lipide P. The per cent recovery of the P put on the paper was 89%. The filter paper blank for the amount used in these determinations was between 0.1–0.8 γ. The value for spot R-9 is thus of questionable significance. The specific activity of this spot is probably much higher than shown.

[f] The identification of inositol phosphatide and acetal phosphatide is tentative.

whereas the spots due to the phosphate compounds previously mentioned show a marked decrease in intensity. This study was confirmed with model experiments by the use of ^{32}P-labeled orthophosphate and glycerophosphate either alone or mixed with unlabeled lipides. Spot R-9 contains appreciable activity but was not identified.

From the data in Table I it can be seen that lecithin, phosphatidyl ethanolamine, phosphatidyl serine, acetal phosphatide, inositol phosphatide, and sphingomyelin constitute 81 % of the total lipide P. These results were supported by column chromatographic studies (Fig. 1). The finding that only 89% of the P applied to the paper was recovered by the elution method used is believed due to the fact that the water washing of the chromatograms prior to eluting the lipides removes some water-soluble phosphate esters and orthophosphate. Furthermore, small amounts of phosphatides are firmly bound to the filter paper and are very refractory to elution with organic solvents. These studies were confirmed by the use of pure phosphatides. It has subsequently been found that repeated extraction with hot 0.5 *N* HCl in redistilled methanol can raise the P recovery yield up to 95%.

The experimental data on the fractions obtained by column chromatography are given in Figs. 1–3. It was found that fractions 14–18 contained the fast moving lipides

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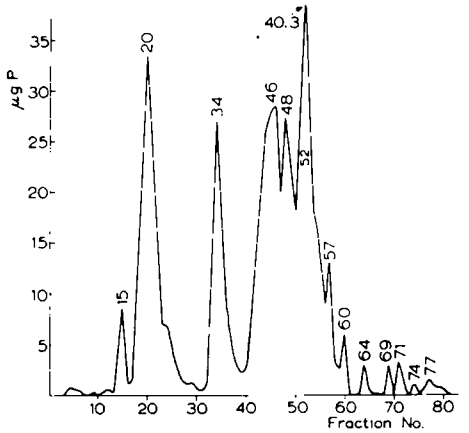

Fig. 1. Elution curve of the total lipides extracted from rat liver homogenates showing the distribution of lipide P. The experimental details are given in the text. The amount of P in μg and the per cent of the total P represented by the major peaks are as follows: 10–16, 15 μg, 2.4%; 17–31, 140 μg, 22.8%; 32–39, 74 μg, 12.1%; 40–50, 214 μg, 34.8%; 51–55, 118 μg, 19.3%; and 56–58, 25 μg, 4.1%. The significance of the small peaks 64 77 is uncertain. Most of the radioactivity occurred in fractions 14–18.

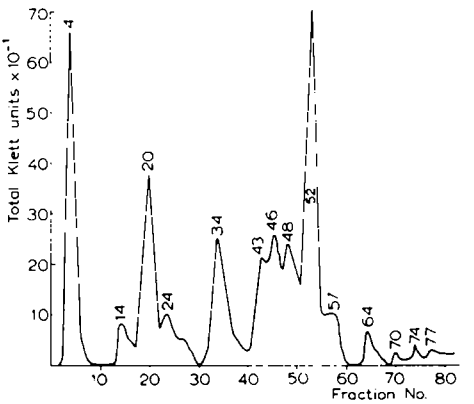

Fig. 2. Elution curve of the total lipides extracted from rat liver homogenates showing the distribution of lipide ester. The experimental details are given in the text. The significance of the minor peaks 60 77 is uncertain. The ester/P molar ratios of the peak fractions were as follows: 3–5 ∞; 14–18, 3.58; 19–23, 2.18; 33–37-varied 0.55 to 1.42; 46 50, 0.97 and 51–59, 1.88.

R-9 and R-8 (see Table I); fractions 19–23 contained mainly phosphatidyl ethanolamine (R-7) and a small amount of phosphatidyl serine (R-6); fractions 33–37 contained a mixture of lipides corresponding to acetal phosphatide (R-4), inositol phosphatide (R-3), and smaller amounts of phosphatidyl serine (R-6) and the unidentified phosphatides R-2 and R-1; fractions 46–50 contained lipides resembling phosphatidal cholines*[10]; fractions 51–54 contained mainly lecithin (R-5); and fractions 55–59

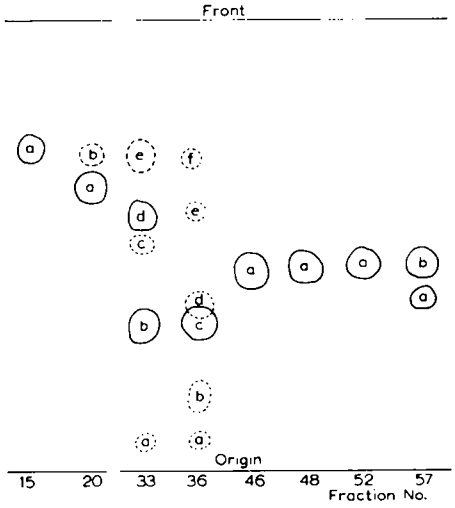

Fig. 3. Tracings of paper chromatograms of the peak lipide fractions obtained by column chromatography. The solvent system used for paper chromatography was di*iso*butyl ketone-acetic acid-water 40:30:7[1]. The lipides were detected with Rhodamine 6G. The spots shown in broken lines occurred in very small amount. The identity of the lipide spots is as follows: 15a-unidentified; 20a-phosphatidyl ethanolamine; 33d-, 36e-phosphatidyl serine (tentative); 33b-, 36c-inositol phosphatide (tentative); 36d-acetal phosphatide (tentative); 46a-, 48a-phosphatidal choline-like lipide; 52a-, 57b-lecithin; 57a-sphingomyelin; others—not identified. A positive choline test was given by spots 20a, 46a, 48a, 52a, 57a, and 57b. The positive test given by 20a is a non-specific reaction due to unsaturated phosphatidyl ethanol-amine. A positive ninhydrin test was given by spots 20a, 33d, 36d and 36e. Details of the experimental procedure are given in the text.

* In agreement with the structure of phosphatidal choline[10] it was found that the ester/P molar ratio for fractions 46 and 49 was 0.97 and that of lecithin (fraction 52) was 1.88, corresponding to a monoester and a diester respectively.

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contained nearly equal amounts of lecithin and sphingomyelin. Very few or no phosphatides were detected in the other fractions, either because none was present or because they occurred in very small amount. The non-phosphatides were found in fractions 2–7 (Fig. 2). A further characterization of the phosphatides present in the peak fractions was made by acid hydrolysis studies, which revealed that the highest amount of ethanolamine occurred in fraction 20 and a small amount in fraction 34; serine was present in small amount in fraction 20 and 34; and choline was present in fractions 46, 49, 52, and 57.

From the combined ester and P analyses it was shown that the ester peak at fraction 4 (Fig. 2) contained essentially only non-phosphatides such as cholesterol esters and triglycerides. These latter lipides were separated and identified by paper chromatographic analysis on silicic acid impregnated paper using isooctane-acetone-acetic acid 95:4:1 as solvent. Free cholesterol also occurred in this fraction. This latter solvent system, which was developed in this laboratory, separated the major types of non-phosphatides such as cholesterol, cholesterol ester, glycerides, and fatty acids.

The studies presented in this paper are to be contrasted with the *in vivo* incorporation of ^{32}P-labeled orthophosphate into various tissues of the rat [1,6] in which it was found that lecithin and phosphatidyl ethanolamine and a lipide behaving like inositol phosphatide were heavily labeled, whereas phosphatidic acid could not be detected. The fast moving lipide observed on the autoradiograms from phosphatides of liver homogenates does not correspond to the fast moving lipide observed on autoradiograms of phosphatides from the previous *in vivo* experiments, since they move differently on non-impregnated paper in solvents consisting of octanol-lutidine-acetic acid[2] and di*iso*butyl ketone-acetic acid[3]. The fast moving component which is labeled in the *in vitro* homogenate system migrates like a phosphatidic acid[1,2] whereas the corresponding component labeled *in vivo* does not. However, the active lipide in this present *in vitro* study has not been characterized.

Further studies on the incorporation of labeled orthophosphate by rat liver homogenates showed that glycerol stimulated the incorporation two-fold but that choline, ethanolamine, and serine caused no stimulation. Dinitrophenol had an inhibitory effect similar to that of cyanide. The addition of ceramide and phosphatidic acid to the system caused a slight inhibition of the incorporation rather than enhancing the synthesis of phosphoglycerides and sphingomyelin. As expected, it was also found that the addition of unlabeled phosphate caused a decrease in the incorporation.

DISCUSSION

The results of the present experiments demonstrate the incorporation of labeled orthophosphate by rat liver homogenates into several phosphatides, the most active of which remain unidentified. KORNBERG AND PRICER[11], KENNEDY[12] and DAWSON[13] have indicated that phosphatidic acids are synthesized *in vitro*. The identification of these active lipides is however tentative, and the possibility exists that they are different anionic phosphoglycerides. In the present study, the ester/P molar ratio of this lipide was about twice the theoretical value for a phosphatidic acid. KORNBERG AND PRICER[11] have observed a similar high ratio for their 'phosphatidic acid' fraction. It is noteworthy that these phosphatides constitute only a small fraction of the total lipides of either liver homogenates or mitochondria[5].

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The remarkable difference between *in vivo* and *in vitro* systems is clearly manifested by comparing the results of the present experiments with previous *in vivo* studies from this laboratory[1,6]. The relative inertness of liver homogenates[13,14] and liver mitochondrial suspensions[12] to incorporate orthophosphate into the common and abundantly occurring phosphatides such as lecithin and phosphatidyl ethanolamine might be due to the inactivation of enzymes required for their synthesis or to the accentuation of metabolic pathways leading to the synthesis of the phosphatidic acid-like compounds. In any case the enigma exists that although these latter lipides are heavily labeled *in vitro*, they are completely non-existent or show no labeling in the *in vivo* systems. If they were intermediates in the metabolic route toward lecithin or the other common phosphatides, at least trace amounts of these compounds should have been detected on autoradiograms of rat tissue phosphatides labeled *in vivo*.

The combined paper and column chromatographic methods have now been shown to be very effective techniques for the study of the phosphatides both on a micro and macro scale and may open the way for the elucidation of the metabolism and for the isolation and purification of these compounds. With these techniques it appears that lipides behaving like phosphatidal cholines* are constituents of liver and this extends the work of RAPPORT AND ALONZO[10] who have demonstrated the presence of these compounds in heart muscle.

SUMMARY

The incorporation of ^{32}P-labeled orthophosphate into the phosphatides of rat liver homogenates was studied by paper and column chromatographic methods. The results show that the degree of incorporation is small and that the lipides having the highest activity correspond chromatographically to phosphatidic acids but still remain to be characterized. On the other hand, the common and abundantly occurring phosphatides such as lecithin and phosphatidyl ethanolamine show very little incorporation. The incorporation is an aerobic dependent process and is stimulated by glycerol. By column chromatography it appears that phosphatides behaving like phosphatidal cholines occur in rat liver.

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* See footnote on page 589.