Metabolism of glycerolipids: V. Metabolism of phosphatidic acid

WILLIAM E. M. LANDS and PRISCILLA HART

Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan

SUMMARY Monoacyl glycerol phosphate is acylated more rapidly than glycerol-3-phosphate by microsomal preparations from rat or guinea pig liver.

Specifically labeled diacyl glycerol phosphate derivatives were prepared. Only the 2-labeled derivative produced radioactive fatty acids when treated with *Crotalus adamanteus* venom, indicating that the phospholipase can hydrolyze diacyl glycerol phosphate and that the reaction is specific for the 2-ester.

When glycerol phosphate was acylated, linoleate and stearate were incorporated at both the 1- and 2-positions. The results suggest that the specificities of the acyl transferase reactions leading to diacyl glycerol phosphate are not adequate to provide the pattern of fatty acids that is known to occur in glycerolipids in nature.

At the time of the early studies on the biosynthesis of phosphatidic acid (1, 2), not enough evidence was available to demonstrate its role as an intermediate in glycerolipid synthesis. Subsequent work has shown that the glycerol and perhaps the fatty acid, although often not the phosphate, portions of the phosphatidic acid are found in the resultant lipids (3). The amount of diacyl GP[1] in a tissue at a given time is quite small and relatively little has been done so far toward determining its fatty acid composition (5). Nevertheless, suggestions such as that of Savary and Desnuelle (6) have been made concerning the number and specificity of the enzymes catalyzing fatty acid esterification during the synthesis of diacyl GP. In their outstanding study of diacyl GP formation, Kornberg and Pricer (1) reported specificities for the incorporation of different acids, but their experiments were not designed to indicate whether a given acid (e.g., stearic) may be esterified more rapidly at the 1- or 2-position of glycerol-3-phosphate. The following experiments[2] were designed to study the positional specificity of fatty acid incorporation into diacyl GP.

EXPERIMENTAL METHODS

Diacyl GP was prepared as the ethanol-insoluble sodium salt produced from purified egg lecithin (8) by treatment with cabbage phospholipase D (9) (ester: phosphorus = 2:1). Monoacyl GP was obtained by phosphorylating monoolein with $POCl_3$ in chloroform (10) and isolating the ethanol-insoluble sodium salt (ester:phosphorus = 1:1).

Silicic acid (Mallinckrodt analytical reagent, 100 mesh) was passed through a 200-mesh sieve. The 100–200 mesh particles were set aside for column chromatography, and the material finer than 200 mesh was used for thin-layer chromatography without further treatment. No $CaSO_4$ or other salt was added to the silicic acid.

Thin-Layer Chromatography (TLC). An adjustable Stahl Model S spreader (Brinkmann Instruments, Inc., Great Neck, Long Island, New York) was used to coat 20 × 20 cm glass plates with silicic acid. Sixty grams of silicic acid (200 mesh), shaken vigorously in a stoppered Erlenmeyer flask with 100 ml of water and 0.5 ml of methanol, was sufficient to coat six plates to a thickness

[1] The unequivocal stereospecific rule described by Hirschmann (4) is used to indicate the stereochemistry of the glycerol derivatives described in this paper. Thus the "parent" compound of the naturally occurring phosphoglycerides is glycerol-3-phosphate and D- and L- prefixes are not needed. For convenience, the following abbreviations are used: GP, glycerol-3-phosphate; GPC, glycerol-3-phosphorylcholine; GPE, glycerol-3-phosphorylethanolamine: GPG, glycerol-3-phosphorylglycerol; GPI, glycerol-3-phosphorylinositol; CDP, cytidine diphosphate. In this way, the product of hydrolysis of egg lecithin (diacyl GPC) catalyzed by snake venom phospholipase A would be named 1-acyl GPC rather than lysolecithin and may be distinguished from its other isomer, 2-acyl GPC, which can be prepared from plasmalogens by mild cleavage of the alkenyl ether. When acylated with radioactive acids, the above monoacyl GPC isomers would yield 2- and 1-labeled diacyl GPC derivatives respectively.

[2] A preliminary report has been given (7).

of 0.25 mm. Methanol was found to reduce the surface tension and produce a more even spreading of the silicic acid. The particles of silicic acid were settled by rapping the plates lightly against the desk, and the plates were allowed to dry at ambient temperature and humidity. No attempt was made to reduce the moisture in the plates below the level of the humidity in the lab since several tests with "heat-activated" plates showed no appreciable difference in separating the phosphatides.

Vertical channels were produced on the TLC plates by scoring the adsorbent layer the full height of the plate with a sharp blade. No channel was closer than 2 cm to the outer edges of the plate, and the number of channels varied from 4 to 8 depending on the number of different samples that were to be applied. A gentle stream of air was used to remove the silicic acid particles from between the channels, thus insuring that no mixing of separate samples could occur as the solvent ascended.

To apply the sample to the adsorbent with as little "flaking" as possible, two small holes about the same diameter as the tip of a disposable Pasteur pipette were placed in the adsorbent layer at the origin, before the sample was applied. The sample, dissolved in a relatively volatile solvent when possible (methanol–chloroform 1:2 (v/v) was usually employed), was applied with a Pasteur pipette by touching the tip to the surface of the glass and allowing the solvent to spread into the adsorbent layer. Several portions of fresh solvent were used to rinse the glassware and provide a quantitative transfer of the material to the silicic acid.

The plates were developed in air-tight solvent jars until the solvent front was about 3 cm from the top of the plate. The plates were removed from the jar, allowed to dry for about 5 min, and then placed in an iodine chamber for about 15 min. The approximate regions of the diacyl GP, monoacyl GP, and GPC derivatives, as evidenced by the yellow color of complexed I_2, were noted. The plates were placed in the hood until most of the yellow color disappeared and then sprayed with Neatan (Brinkmann Instruments, Inc., Great Neck, Long Island, New York), until the silicic acid was thoroughly moistened. While the Neatan was still moist, the individual channels were divided horizontally at 10- to 15-mm intervals with a sharp blade so that a series of uniform rectangular blocks of adsorbent were defined. The Neatan-impregnated plates were placed in an oven at 80° until thoroughly dried (about 20 min). After the plate had cooled to room temperature, it was moistened by spraying it with water and the blocks of adsorbent were easily lifted off the glass with a spatula and placed into 20-ml low-potassium glass vials. The vials were heated in the 80° oven for about 45 min to insure complete removal of water from the silicic acid samples before the scintillation liquid was added. The scintillator solution was usually added while the vials were still warm to get a more facile dispersion of the silicic acid particles.

A thixotropic gel of 3% (w/v) of Cab-O-Sil (Cabot Corp., Boston, Massachusetts), in a toluene scintillator solution containing 0.4% 2,5-diphenyloxazole (PPO) and 0.01% 1,4 - bis - 2 - (5 - phenyloxazolyl) - benzene (POPOP) was used in an attempt to keep the silicic acid particles in suspension. A corresponding solution without Cab-O-Sil was also used. Using a Tri-Carb liquid scintillation spectrometer, the counting efficiency was comparable in the two systems and did not seem to be affected by the degree of suspension of the silicic acid. Counting efficiency, however, depended upon the type of compound in which the radioactivity occurred, since radioactivity in fatty acid was counted more efficiently than that incorporated in phospholipids. Apparently the phospholipids were more tightly bound to the silicic acid particles so that there was appreciable self-absorption of the beta radiation. A correction curve was formulated by plotting the ratio of the counts obtained from two channels with discriminator settings at 10–50 and 10–100 against the relative counting efficiency (11).

Solvent Systems for TLC. Methanol–chloroform 13:87 (v/v) containing 0.5 ml of concentrated HCl per 100 ml (solvent A) was found to give the sharpest resolution between the monoacyl GP and diacyl GP. This system moved fatty acids to the solvent front, followed by diacyl GP; monoacyl GP remained at the origin or moved slightly above it. When separation of the free fatty acids from the phospholipids was the only result desired, 100% diethyl ether was found to be a satisfactory solvent. A solution of diethyl ether–ethanol–formic acid–concentrated HCl 150:1:2:0.5 (solvent B) was used to separate fatty acids from diacyl GP and monacyl GP. In this solvent, fatty acids moved to the front, diacyl GP moved more slowly, and monacyl GP stayed at or near the origin. The R_F of the diacyl GP could be controlled to some extent by first running the plate in 100% diethyl ether and then transferring it to the acidic ether (solvent B) tank. The longer the plate was allowed to dry between chromatographic runs, the greater was the resulting R_F of the diacyl GP. The most desirable time of drying for our purposes was until all visible signs of ether had just evaporated from the adsorbent layer. To further insure separation of free fatty acids from diacyl GP, the development in 100% ether, which moves the free fatty acids with the solvent front, was allowed to proceed to 17 cm, while development in solvent B was stopped at 15 cm. In all systems, a disconcerting number of counts remained at the origin, regardless of the form in which the radioactivity was present when applied. This problem was less serious with solvent A.

Preparation of Radioactive Diacyl GPC. Samples of 1- and 2-labeled diacyl GP were obtained from 1- and 2-labeled diacyl GPC, respectively. The latter compounds were prepared in a manner similar to that described by Robertson and Lands (12), using either 15 μmoles of 2-acyl GPC with 20 μmoles of stearic acid-C^{14} or 15 μmoles of 1-acyl GPC with 20 μmoles of linoleic acid-C^{14} taken up in 15 ml of potassium phosphate buffer (pH 7.3). Sand was used with the 2-acyl derivative and stearic acid to help disperse the less soluble materials. Fifty micromoles of $MgCl_2$, 1 μmole of CoA, 40 μmoles of ATP, and 1.0 ml of a rat liver microsome preparation were included in each incubation mixture. At intervals of 30 and 60 min, additional CoA, ATP, and microsomes were added in amounts equal to those originally added. The incubation mixtures were allowed to react for 1.5 hr at room temperature, with intermittent swirling of the flasks. The reaction was stopped by the addition of 50 ml of methanol–chloroform 4:1. Methanol–chloroform 1:4 (150 ml) was added and the mixture was washed twice with water. Chloroform was used to extract any lipids from the first water wash. It was then added to the main chloroform–methanol solution and this solution was evaporated to dryness. The lipids were then dissolved in ether–benzene 1:1, placed on a 4-g silicic acid column, and eluted with successive 40 ml portions of ether–benzene 1:1, ethanol–ether 7:93, methanol, and chloroform–methanol–water 65:40:5.

Preparation of Radioactive Diacyl GP. The eluate obtained with methanol (diacyl GPC) was evaporated and treated with 5 mg of cabbage phospholipase D (California Corporation for Biochemical Research, Los Angeles, California) in 10 ml of acetate buffer (0.05 M, pH 5.6) containing 500 μmoles of $CaCl_2$. This mixture was shaken vigorously, and then 4 ml of ether was added and the shaking was repeated. After incubation overnight at room temperature, the reaction was stopped with 0.3 ml of 70% $HClO_4$. The water layer was separated from the ether layer and washed several times with fresh ether. The ether layers were combined and washed with water. Sodium bicarbonate was added to the wet ether to assure that all the diacyl GP would be present as the disodium salt. The ether was evaporated and the lipid was taken up again in a small volume of dry ether. The $NaHCO_3$ was removed from the ether solution by centrifugation. A portion of benzene equal to the volume of ether was added to the ether solution and the resulting solution of lipids was placed on a 4-g silicic acid column. The column was eluted with successive 40-ml portions of ether–benzene 1:1, ether, ethanol–ether 7:93, methanol–ethanol 1:9, methanol, and chloroform–methanol–water 65:40:5. The eluate obtained with methanol–ethanol 1:9 contained the radioactive diacyl GP. This eluate was evaporated and the residue was dissolved in 10 ml of ether.

Hydrolysis of Diacyl GP by Phospholipase A. Appropriate amounts of either 1- or 2-labeled diacyl GP dissolved in ether (7–12 × 10^3 cpm) were added to 20-ml vials containing 1.2 μmoles of unlabeled diacyl GPC. The unlabeled diacyl GPC was added to serve as an emulsifier. The liquid volume was adjusted to 5 ml with ether and 0.05 ml of *Crotalus adamanteus* venom (Ross Allen's Reptile Institute, Silver Springs, Florida) solution (25 mg of venom and 20 μmoles of $CaCl_2$ in 5 ml of 0.1 M tris chloride buffer, pH 7.5) was added. The mixture was shaken vigorously and allowed to react for at least 3 hr. Identical vials containing tris chloride buffer and $CaCl_2$ but no venom were set up for controls. The reactions were stopped by adding 0.1 ml of 0.025 M EDTA and 0.5 ml of methanol, mixing well, and evaporating. The samples were dissolved in methanol–chloroform 1:2 and applied to a channeled TLC plate, as described earlier. The vials were rinsed several times with methanol–chloroform in order to transfer as much of the material as possible to the TLC plate, which was then developed in solvent A.

Comparative Acyl Transferase Studies. Oleyl-l-C^{14} CoA was allowed to react with a variety of substrates in the presence of subcellular particles from the livers of rats and guinea pigs. A typical incubation mixture contained 40 mμmoles of oleyl-l-C^{14}-CoA, 200 mμmoles of monoacyl-GP (200 mμmole amounts of other substrates were used as indicated in Table 2), 1.5 mg of microsomal protein, and 50 μmoles of potassium phosphate buffer (pH 7.4) in a total volume of 1 ml. The reaction was allowed to proceed for 10 min and then stopped by the addition of 20 ml of methanol-chloroform 1:2. The mixture was washed twice with water and evaporated to dryness. The lipids were dissolved in ether, applied to a 4-g silicic acid column, and eluted with the following solvents: fraction 1 and 2, 20 ml of ether–petroleum ether 17:3; 3, 15 ml of ether; 4 and 5, 20 ml of ethanol–ether 1:4; 6 and 7, methanol–ethanol 1:9; 8, 9, and 10 contained 10, 50, and 10 ml of methanol, respectively.

Acylation of Monoacyl GP. Monoacyl GP, produced by phosphorylating monoolein, was used as an acceptor in the microsomal acylating system containing ATP, $MgCl_2$, CoA, and C^{14}-fatty acid. Optimal acylation occurred at pH 8.7 (tris chloride buffer). Guinea pig liver microsomes were routinely used instead of rat liver preparations. The reaction was stopped after 30 min by the addition of 4 ml of methanol–chloroform 4:1 and 12 ml of methanol–chloroform 1:4. To minimize the loss of diacyl GP from the chloroform extract in the process of removing the water-soluble impurities, the extract was washed with 2 ml of 0.025 M HCl containing 2% acetic acid. After the aqueous layer was removed, the

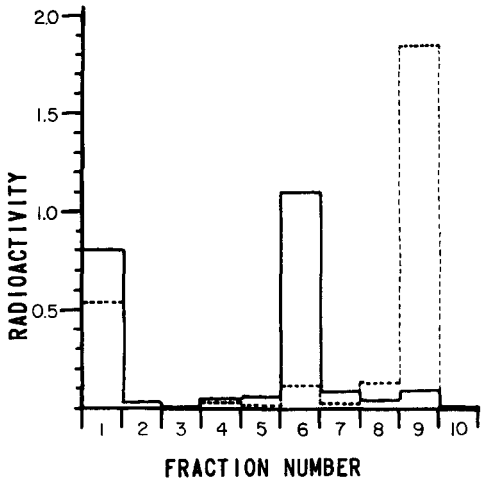


FIG. 1. Separation of diacyl GP and diacyl GPC by column chromatography. The two curves show the distribution of radioactivity (expressed as counts per minute per fraction $\times 10^{-3}$) in the eluates from a silicic acid column treated in the manner described under Experimental Methods. The radioactive lipids were produced by a 10-min incubation of C^{14}-oleyl CoA with approximately 1.5 mg of microsomal protein to which either acyl GP (solid line) or acyl GPC (dotted line) was added as an acceptor. The free fatty acids are eluted with ether–petroleum ether 17:3 (fraction 1), the diacyl GP is eluted with methanol–ethanol 1:9 (fraction 6), and the diacyl GPC is eluted with methanol (fraction 9).

organic phase was washed again with 4 ml of water, and the cloudy organic phase was cleared with methanol. A convenient portion of the chloroform solution was evaporated to dryness. The residue was dissolved in methanol–chloroform 1:2 and was applied to a TLC plate. The plate was developed in the acidic ether system (solvent B), and the resulting distribution pattern of radioactive products was analyzed by scintillation counting.

Acylation of Glycerol-3-phosphate. A sample of linoleic acid-C^{14} (76 mμmoles, 4.7 $\times$ 10^5 cpm per μmole) and approximately twice that amount of non-radioactive stearic acid were dried on sand in a test tube. The sand was shaken with 0.5 ml of tris chloride buffer (pH 8.7, 0.1 M) to disperse the fatty acids, and an aqueous solution of GP (2 μmoles) was added. The mixture was incubated with 10 μmoles of ATP, 0.2 μmoles of CoA, 10 μmoles of $MgCl_2$, and guinea pig microsomes for 1.5 hr. The reaction was stopped with chloroform and methanol, and the water-soluble impurities were removed in the manner described for the reaction of acyl GP. The organic phase was evaporated to dryness, and the lipids were dissolved in ether–benzene 1:1 and placed on a 4-g silicic acid column. The column was eluted with several column volumes each of ether–benzene 1:1, ether, ethanol–ether 7:93, methanol–ethanol 1:9, and methanol. The radioactive diacyl GP obtained from the methanol–ethanol 1:9 eluate was treated with either *Crotalus adamanteus* venom (for both 0 and 1.5 hr) or control buffer without venom as explained earlier. Analysis of the resulting products was made by TLC with solvent A. A similar preparation, using radioactive stearic acid and non-radioactive linoleic acid, was carried through the same treatments and analyses.

RESULTS

Hydrolysis of the choline from the 1- and 2-labeled diacyl GPC with cabbage phospholipase D would be expected to form the corresponding specifically labeled diacyl GP. The radioactive product of this hydrolysis is eluted from silicic acid with methanol-ethanol 1:9, as is the sample of diacyl GP derived from egg lecithin. It also moves on TLC plates with the same R_F as diacyl GP. The data in Fig. 1 illustrate the effectiveness of silicic acid columns in separating diacyl GP from diacyl GPC. The diacyl GP that was derived from 1-labeled diacyl GPC yielded on treatment with snake venom radioactivity in the region of the TLC chromatogram occupied by monoacyl GP (Table 1). In contrast, 2-labeled diacyl GP yielded the greatest portion of radioactivity in the free fatty acid region. The R_F values obtained with TLC varied with different plates and occasionally varied from one side of a plate to the other. For this reason, reference compounds were applied to adjacent channels at both sides of those containing

TABLE 1. THIN-LAYER CHROMATOGRAPHIC SEPARATION OF THE HYDROLYSIS PRODUCTS OF 1- AND 2-LABELED DIACYL GP

Reference Compound	1-Labeled		2-Labeled	
	Control	+ Venom	Control	+ Venom
	37*	55	18	210
Fatty Acid	96	79	27	8,700
	336	26	54	3,670
	302	17	32	845
	321	20	105	169
	1,351	27	915	388
	1,931	54	1,290	359
Diacyl GP	1,421	101	1,755	172
	985	197	6,500	433
	915	239	3,360	311
	880	670	1,730	177
	352	1,090	823	217
Monoacyl GP	297	1,950	447	264
	288	1,495	288	238
	984	1,860	1,870	1,520
	1,995	4,540		

* The numbers indicate the radioactivity in counts per minute (after correction for quenching) of similarly-sized blocks of silicic acid from a TLC plate developed in solvent A and treated as described in the text. The radioactivity is indicated in the table with the same relative position that occurred on the plate; e.g., values at the top were at the solvent front, whereas those at the bottom are from the blocks at the origin.

the radioactive compounds. In this way, the radioactive products could be identified, although in many instances the radioactive compounds migrated with a slightly lower (approx. 10%) R_F than the reference compounds.

Comparative studies of the acyl transferase reaction showed that appreciable activity was present in the microsomal fractions of both rat and guinea pig liver (Table 2). A small amount (1–2 mμmoles) of radioactive acid was found in each fraction from experiments in which microsomes were present, even when no substrate was added. This may be due to small amounts of phospholipid substrate already present in the enzyme preparation, and represents a "control" level that should be subtracted from the results obtained when substrates were added. With this correction, rat liver particles can be seen to be much less effective in acylating GP than were guinea pig particles. In addition, the results, particularly with the rat enzymes, for the GP and GPC derivatives are comparable in that the monoacyl derivatives were better substrates than the non-acylated derivatives.

Although the extent of reaction with monoacyl GP was not great, the pattern of reactivity, linoleic > oleic ≥ palmitic > stearic, was indicated for the free acids (Table 3). In contrast to the acylation of monoacyl GPC, which showed no appreciable pH optimum, the rate of esterification of fatty acids with monoacyl GP appeared to be greatest at pH 8.7.

In most of the systems studied, the fatty acid activating enzymes appeared to produce linoleyl CoA more rapidly than stearyl CoA, probably because of the extreme insolubility of stearic acid in water. In order to equalize the amounts of each acyl CoA available for the acyl transfer reaction with GP, a greater amount of stearic acid was used in the reaction mixtures. Sand was also used to provide greater surface area and mechanical action when stirring.

The product of the acylation reaction was separated from free acids and isolated from a silicic acid column with methanol–ethanol 1 : 9 in a manner similar to that shown in Figure 1. Approximately 20% of the stearate and 60% of the linoleate were incorporated during 1.5 hr incubation. Aliquots of the radioactive diacyl GP fraction were treated with *Crotalus adamanteus* venom to determine the site of acid-C^{14} incorporation. The control samples for "zero time" and without venom showed that no radioactive free fatty acids were present initially. Radioactivity in the resulting monoacyl GP fraction indicates esterification at the 1-position, whereas radioactivity in the free fatty acid fraction indicates that the isotope was incorporated at the 2-position. The results in Table 4 indicate that stearic acid and linoleic acid are esterified at both the 1- and 2-positions, and suggest that there is very little positional selectivity for these fatty acids in the first acylation of GP.

TABLE 2. INCORPORATION OF OLEIC ACID FROM C^{14}-OLEYL COA INTO PHOSPHOLIPIDS

Microsomal Preparation	Substrate Added	Oleic Acid-C^{14} in Eluted Fractions* EtOH-ether 1:4	MeOH-EtOH 1:9	MeOH
Guinea pig liver	Monoacyl GPC	0.7	2.3	28.5
	GPC	1.0	3.0	3.4
	Monoacyl GP	1.7	18.1	1.3
	GP	1.9	8.6	3.5
	None	1.0	3.5	2.2
None	None	0.5	0.1	0.0
Rat liver	Monoacyl GPC	0.3	1.9	27.1
	GPC	3.0	2.6	4.5
	Monoacyl GP	2.6	18.8	2.0
	GP	0.9	2.6	4.2

* The values are given in terms of mμmoles of oleic acid, based upon measurement of the radioactivity per fraction. The main radioactive phosphatide eluted with methanol–ethanol 1 : 9 is the sodium salt of diacyl GP; and with methanol, diacyl GPC. The material eluted with ethanol–ether is tentatively regarded as the acidic form of diacyl GP.

TABLE 3. ESTERIFICATION OF FATTY ACIDS WITH MONOACYL GP TO FORM DIACYL GP

	Fatty Acid-C^{14}	pH	Diacyl GP
			cpm
Expt. 1*	Linoleic	7.3	5,200
	Linoleic	8.2	6,400
	Linoleic	8.6	7,800
	Linoleic	8.7	8,300
	Linoleic	9.2	8,000
	Linoleic	9.4	7,300
Expt. 2	Linoleic	8.7	15,000 (16,000)†
	Oleic	8.7	6,800 (7,400)
	Palmitic	8.7	6,300 (6,800)
	Stearic	8.7	3,500 (4,000)

* The reaction mixtures contained, in addition to the enzyme, 0.15 μmoles of C^{14}-fatty acid (7×10^4 cpm), 5 μmoles of ATP, 0.1 μmoles of CoA, 10μmoles of $MgCl_2$, 50 μmoles of tris chloride, and 0.25 μmoles of acyl GP in a final volume of 1 ml. Experiment 2 contained similar amounts, except that 1 μmole of acyl GP was used.

† The numbers in parentheses are the average of six different incubations.

DISCUSSION

Hanahan (13) and others (14, 15) have presented a wide variety of evidence that the fatty acids in animal lecithins are not distributed between the 1- and 2-positions in a random fashion. The question as to whether the unsaturated acids are at the 2-position and whether

C. adamanteus venom phospholipase is specific for the 1- or 2-position has been adequately answered by the recent results of Hanahan et al. (16), Tattrie (17), and De Haas and Van Deenen (18). The results of these studies led us to consider three questions concerning the control of fatty acid composition. (*a*) Why are the fatty acid compositions different at different positions within a glycerolipid? (*b*) Why are the fatty acid compositions different for the different glycerolipids in the same tissue? (*c*) Why are the fatty acid compositions of the same glycerolipids different in different tissues?

Some of the reasons why the fatty acid composition might be considered important are its possible effects on the solubility of the glycerolipids and the extent of their interaction with lipoproteins, which, in turn, could affect the stability of cellular membranes.

Dittmer and Hanahan have shown (19) that the rates of synthesis and degradation of fatty acid esters at the 1- and 2-positions are different in the phosphatides and

TABLE 4. LOCATION OF STEARIC AND LINOLEIC ACIDS IN DIACYL GP SYNTHESIZED FROM GP

Expt. No.	Stearic Acid-C^{14}		Linoleic Acid-C^{14}	
	1-position	2-position	1-position	2-position
	cpm × 10^{-3}			
1	5.3	3.9	5.0	5.1
2	5.3	3.2	4.9	5.5
3	3.8	2.8	5.1	5.5
4	3.0	1.9	3.0	3.3
Total	17.4	11.7	18.0	19.4
Isotope in 2-position	40%		52%	

suggested different specific functions could exist for phosphatides with different acid compositions. Both Mattson and Volpenhein (20) and Savary et al. (21) have given evidence for a non-random pattern of acids in the triglycerides. They noted, in addition, that part of the esters were left intact during intestinal digestion and absorption. Mattson and Volpenhein (22) estimated that the remaining monoglyceride was acylated in a random fashion to produce "partially random" triglycerides. These results suggest that vegetable oils with high levels of unsaturated acids will produce lymph triglycerides with a predominance of unsaturated acids esterified at the 2-position. In turn, lymph glycerides, following reactions described by Kennedy (23), can lead to diglycerides, and thus phosphatides, containing greater amounts of unsaturated acids at the 2- than the 1-position. This type of process was suggested again recently by Brockerhoff et al. (24) in discussing lipids of marine origin. If this is an important process, we might also shift fatty acid patterns of phosphatides from normal to abnormal by feeding excessive amounts of saturated fats.

Another process that could exert some control over the acid composition of the various glycerides would be the selective reaction of certain diglycerides with the CDP-choline (or-ethanolamine)–diglyceride cholinephosphotransferase. Differences in choline and ethanolamine phosphatides that are now known (14) might have resulted from this type of preferential reaction of specific diglycerides, and Weiss et al. reported a brief study (25) of this possibility in terms of triglyceride and lecithin synthesis from diglycerides.

In addition to the two processes mentioned above, the pattern of acids in a glycerolipid might be controlled by the specificities of various acyl transferases. Using rat liver microsomes, we have found that monoacyl derivatives of GPC, GPE, and GP are converted to the diacyl derivatives in the presence of acyl CoA thiolesters. We have not yet observed a conversion of monoglyceride to diglyceride catalyzed by liver particles, although the reaction is known to occur with intestinal particles (26).

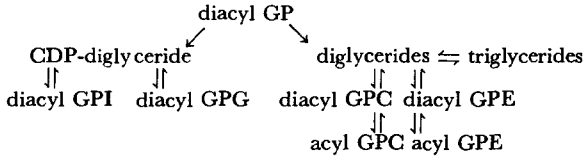
Our results show that the use of venom phospholipase A for selective liberation of the fatty acid esterified at the 2-position of phosphoglycerides can now be extended to diacyl GP (Table 1). This will allow further study of the fatty acid distribution patterns in not only the major phosphoglycerides, diacyl GPC, and diacyl GPE, but also diacyl GP, a likely precursor of these compounds.

Acyl transfer catalyzed by liver microsomes seems to be more effective (Table 2) with the lipid-soluble monoacylated derivatives. This phenomenon may be due in part to enzyme selectivity and perhaps also to an adsorption of the lipoidal acceptor onto the hydrophobic membranes that contain the enzymes. Regardless of the mechanism, one consequence is that monoacylated intermediates would not be expected to accumulate in such a system, but would react further to form diacyl derivatives. This phenomenon could help explain why Kornberg and Pricer were unsuccessful in their attempt to isolate monoacyl GP during their study of GP acylation (1) and why more recent work showed very little monoacyl GP accumulation when guinea pig liver cytoplasmic particles were used (27). Microsomal particles from rat liver were less effective than those from guinea pig liver in acylating GP although they were comparable in their action upon monoacyl GP. These results suggest that the first acylation of GP may be catalyzed by a different enzyme than the second acylation and that the first enzyme may be present at a lower concentration in rat liver than in guinea pig liver.

The fact that linoleate was more effective than stearate in acylating monoacyl GP may be due in part to some relatively indirect rate-limiting aspects such as solubility

and the activity of the fatty acid activating enzymes. The pH optimum may also reflect a number of factors. In any event, these experiments provide a basis for the study of positional specificity summarized in Table 4.

The experiments with two different fatty acids available for diacyl GP synthesis were designed to reproduce in part the situation that might occur in vivo. Differences in specificity for fatty acid esterification at the two positions of GP can be discerned by noting the degree of competition for the sites as indicated by the distribution of radioactive acids (Table 4). In the experiments with radioactive stearate, we can see that the process of incorporating linoleate does not appreciably prevent stearate from being esterified at the 2-position. Similarly, added stearate did not prevent linoleate from being esterified at the 1-position, but the acids seem to be incorporated in an almost random fashion. Thus the results available at present suggest that the specificities of the acyl transferase reactions leading to diacyl GP are not adequate to provide the specific pattern of fatty acids that is known to occur in the tissue glycerolipids. Since the acyl transferases operating on monoacyl GPC and monacyl GPE are selective (28, 29), the fatty acid patterns observed in tissue phosphoglycerides may arise from a redistribution of fatty acids after the nitrogenous base has been attached to the molecule. One consequence of this would be that the phosphoglycerides derived from phosphatidic acid via CDP-diglyceride (e.g., phosphatidyl inositol) would have a more random arrangement of fatty acids (30) than those in equilibrium with the CDP-choline–diglyceride cholinephosphotransferase system. Recent work has indicated, however, that acyl GPI also may be acylated enzymatically to form diacyl GPI (31). In addition, the triglycerides would be expected to obtain a partially ordered pattern as a consequence of equilibration with the diglyceride pool, which, in turn, may be equilibrated with the specifically labeled phosphatides, diacyl GPC and diacyl GPE. Some of the known metabolic reactions that could allow equilibration of the various glycerolipids are indicated below.



The steady-state concentrations and fatty acid compositions of the intermediates in the above reactions are probably different in different tissues and may be affected by dietary factors. Thus, considerably more comparative data regarding these concentrations and compositions in different tissues will be needed to provide the answers to the three questions noted earlier in the discussion.

Work reported here was supported by PHS Research Grant AM-05310 from the National Institutes of Health, U.S. Public Health Service.

Manuscript received August 19, 1963; accepted October 15, 1963.

References

1. Kornberg, A., and W. E. Pricer, Jr. *J. Biol. Chem.* **204:** 345, 1953.
2. Kennedy, E. P. *J. Biol. Chem.* **201:** 399, 1953.
3. Kennedy, E. P. *Federation Proc.* **20:** 934, 1961.
4. Hirschmann, H. *J. Biol. Chem.* **235:** 2762, 1960.
5. Hubscher, G., and B. Clark. *Biochim. Biophys. Acta* **41:** 45, 1960.
6. Savary, P., and P. Desnuelle. In *The Enzymes of Lipid Metabolism*, edited by P. Desnuelle, New York, Pergamon Press, 1961, pp. 138–145.
7. Lands, W. E. M., and P. Hart. *Federation Proc.* **22:** 416, 1963.
8. Hanahan, D. J. In *Biochemical Preparations*, edited by M. J. Coon, New York, John Wiley and Sons, Inc., 1962, Vol. 9, pp. 55–58.
9. Davidson, F. M., and C. Long. *Biochem. J.* **69:** 458, 1958.
10. Baer, E., and A. Kindler. *Biochemistry*, **1:** 518, 1962.
11. Baillie, L. A. *Intern. J. Appl. Radiation Isotopes* **8:** 1, 1960.
12. Robertson, A. F., and W. E. M. Lands. *Biochemistry*, **1:** 804, 1962.
13. Hanahan, D. J. In *Lipid Chemistry*, New York, John Wiley and Sons, Inc., 1960, pp. 71–78.
14. Rhodes, D. N. *Biochem. J.* **68:** 380, 1958.
15. Borgström, B. *Acta Chem. Scand.* **11:** 749, 1957.
16. Hanahan, D. J., H. Brockerhoff, and E. J. Barron. *J. Biol. Chem.* **235:** 1917, 1960.
17. Tattrie, N. H. *J. Lipid Res.* **1:** 60, 1959.
18. De Haas, G. H., and L. L. M. Van Deenen. *Biochim. Biophys. Acta* **48:** 215, 1961.
19. Dittmer, J. C., and D. J. Hanahan. *J. Biol. Chem.* **234:** 1963, 1959.
20. Mattson, F. H., and R. A. Volpenhein. *J. Biol. Chem.* **236:** 1891, 1961.
21. Savary, P., M. J. Constantin, and P. Desnuelle. *Biochim. Biophys. Acta* **48:** 562, 1961.
22. Mattson, F. H., and R. A. Volpenhein. *J. Biol. Chem.* **237:** 53, 1962.
23. Kennedy, E. P. *Ann. Rev. Biochem.* **26:** 119, 1957.
24. Brockerhoff, H., R. G. Ackman, and R. J. Hoyle. *Arch. Biochem. Biophys.* **100:** 9, 1963.
25. Weiss, S. B., E. P. Kennedy, and J. Y. Kiyasu. *J. Biol. Chem.* **235:** 40, 1960.
26. Senior, J. R., and K. J. Isselbacher. *J. Biol. Chem.* **237:** 1454, 1962.
27. Pieringer, R. A., and L. E. Hokin. *J. Biol. Chem.* **237:** 659, 1962.
28. Lands, W. E. M., and I. Merkl. *J. Biol. Chem.* **238:** 898, 1963.
29. Merkl, I., and W. E. M. Lands. *J. Biol. Chem.* **238:** 905, 1963.
30. Brockerhoff, H. *Arch. Biochem. Biophys.* **93:** 641, 1961.
31. Keenan, R. W., and L. E. Hokin. *Biochim. Biophys. Acta* **60:** 428, 1962.