Differential distribution of orthophosphate-^{32}P and glycerol-^{14}C among molecular species of phosphatidylinositols of rat liver in vivo

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ABSTRACT The incorporation of orthophosphate-^{32}P and glycerol-2-^{14}C into the various species of rat liver phosphatidylinositols as a function of time was determined in vivo. ^{32}P was administered intraperitoneally and glycerol-^{14}C was given intravenously. The phosphatidylinositols were resolved intact according to degree of unsaturation. 1–3 hr after injection of the labeled phosphate, the relative specific activity of the linoleoyl dienes exceeded that of the arachidonoyl tetraenes about 17-fold, and that of the trienes and polyenes about 8-fold. The relative specific activities of all the fractions became about equal 2–3 days after administration of ^{32}P.

The labeling patterns obtained with glycerol were comparable to those seen for the phosphate. As early as 5 min about 65% of the activity was localized in the monoenes plus dienes, while only 17% was found in the tetraenes, although the mass proportions of these fractions were 7.1 and 77.0% of the total phosphatidylinositols, respectively. The recovery of the total radioactivity in the monoenes and dienes decreased continuously with time to about 15% at 9 hr, while that recovered in the tetraenes rose steadily to about 70%. The present data are consistent with an active synthesis of the monoenoic and dienoic phosphatidylinositols by way of the phosphatidate, followed by a deacylation–reacylation cycle involving arachidonic acid, as claimed for other rat liver glycerophosphatides.

SUPPLEMENTARY KEY WORDS arachidonoyl · de novo synthesis · transacylation

RECENT WORK on the metabolism of phosphatidylcholine and phosphatidylethanolamine has shown (1, 2) that the monoenoic and dienoic species are preferentially formed de novo by way of 1,2-diglyceride intermediates derived from the corresponding phosphatidic acids. The pathways of synthesis of the polyunsaturated species have not yet been clearly established, although there is evidence that acyltransferases may play a major role in this process.

Since the phosphatidylinositols are presumably formed from phosphatidate without passing through a free diglyceride intermediate (3), an especially close relationship would be expected between the phosphatidate precursors and the inositide end products. In view of the large proportion of 1-stearoyl,2-arachidonoyl species, however, it could be anticipated that much of the phosphatidylinositol is also derived by acyl transfer. This possibility is supported by the demonstration of lysophosphatidylinositol acyl CoA transferases in pigeon tissues (4). Furthermore, it has been shown in vitro that *sn*-glycerol-3-phosphate is a much more effective acceptor for inositol than dipalmitoyl phosphatidate (3) and that the phosphatidates prepared from beef spinal cord are superior to synthetic substrates (5). Thus far no studies have been reported with individual phosphatidates or CDP diacylglycerols of fatty acid compositions like those found in natural inositides. The present work shows that the rat liver phosphatidylinositols have a heterogeneous metabolism in vivo and that more than one mechanism must contribute to their biosynthesis and degradation.

MATERIALS AND METHODS

Radioactive Materials, and Animals

Radioactive orthophosphate ($Na_2H^{32}PO_4$, 200 mCi/mmole) and high purity (>98.5%) glycerol-2-^{14}C (14 mCi/mmole) were purchased from New England Nuclear Corp., Boston, Mass. For injection, the glycerol and

phosphate were diluted in 0.9% NaCl to give 100 and 800 μCi/ml of solution, respectively.

The animals were male Wistar rats which had been kept on a Purina Chow diet during the 10 days prior to the experiment. For work with labeled phosphate, rats weighing 150–170 g were injected intraperitoneally with 400 μCi of the radioactive orthophosphate. The animals were deprived of food for 12 hr prior to killing; there were three animals in each group. For studies with labeled glycerol, rats weighing 310–330 g were injected with 50 μCi of ^{14}C-labeled glycerol via the jugular vein under light ether anesthesia. The animals were allowed to recover and had access to food until they were killed in pairs at various time intervals. The livers were rapidly excised and total lipid extracts were prepared.

Isolation of Phosphatidylinositol

Liver lipids were extracted by the method of Folch, Lees, and Sloane Stanley (6). Phosphatidylinositol was obtained by first applying the total lipid extract in chloroform–methanol 1:1 to a column of alumina (1 mg P/g of alumina) and subsequently eluting with chloroform–methanol 1:1, and ethanol–chloroform–water 5:2:2, as described elsewhere (7). The fraction eluted by the latter solvent was rich in monophosphoinositide; it was purified further by means of thin-layer chromatography, and its purity was established as previously described (8).

Subfractionation of Phosphatidylinositols

The purified phosphatidylinositols were separated into four chemical subclasses according to degree of unsaturation by argentation thin-layer chromatography (8). The fatty acid composition of the fractions was determined by gas–liquid chromatography after transmethylation of the eluted phosphatides (7, 8). The masses of the subfractions were obtained by adding known amounts of methyl heptadecanoate as an internal standard and comparing the peak areas. The specific activity of the ^{32}P-labeled fractions was determined by counting the calculated weights of the phosphatides in 10 ml of toluene containing 0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis-2(4-methyl-5-phenyloxazolyl)benzene per liter. The radioactivity was measured in a Nuclear-Chicago scintillation spectrometer (Mark II series). ^{14}C activity was counted as previously described (9), and the specific activities were estimated as above for the phosphate-labeled fractions.

RESULTS

Table 1 gives the radioactivities recovered in the total liver lipids and in the phosphatidylinositols after injection of the labeled phosphate and glycerol. The use of intravenous injection in the series of experiments with glycerol permitted sampling of radioactive lipids at early time intervals. The incorporation into the total and individual lipids was of the order obtained by other investigators (10–12). The specific activity of the total phosphatidylinositols ranged from 200 to 800 dpm/μmole of P with radioactive glycerol over the 9-hr period. In view of the relatively low specific activity, samples of liver phosphatidylinositol containing at least 1000 dpm were used in the analyses. The orthophosphate-^{32}P was used only for the long-term studies which were not feasible with glycerol-^{14}C due to a significant reutilization of the label for fatty acid synthesis at these times. The intraperitoneal route for the phosphate administration was chosen because previous work had shown (12) that this method gave 5–10 times greater specific activities in the liver lipids of young rats than intravenous infusion of the same precursor. The specific activity of the total phosphatidylinositols labeled in this manner ranged from 9,560 to 24,180 cpm/μmole of P over the 4-day experimental period; this was of the same order of magnitude obtained by others (12).

TABLE 1 INCORPORATION OF GLYCEROL-2-^{14}C AND ^{32}P INTO TOTAL LIPIDS AND PHOSPHATIDYLINOSITOLS OF RAT LIVER

Glycerol-2-^{14}C*			^{32}P†	
Time	dpm in Total Lipids	dpm in Total Phosphatidylinositols	Time	cpm in Total Phosphatidylinositols
min			*hr*	
5	888,900	4,190	1	114,410
30	1,164,200	5,670	3	206,090
60	1,246,260	15,800	24	235,510
300	815,470	16,560	48	289,390
540	601,150	16,900	96	266,650

* Values are averages from two separate experimental animals at each time period.

† Values are means from three separate experimental animals at each time period.

Table 2 gives the fatty acid composition of the total phosphatidylinositols in the two groups of animals; these data have a bearing on the proportions of the molecular species in the two experiments. The inositides from the younger rats were lower in palmitic, oleic, and linoleic acids and consequently had less total monoenoic plus dienoic, and palmitoyl species. Silver nitrate thin-layer chromatographic separation of the intact phosphatidylinositols gave essentially four fractions: monoenes plus dienes, trienes, tetraenes, and polyenes (>4 double bonds). The linoleoyl species comprised about 75% of the monoenoic plus dienoic fraction, while the 1-stearoyl,2-arachidonoyl species made up 94% of the tetraenoic fraction. The fatty acid composition of the fractions obtained has been reported previously (8).

TABLE 2 FATTY ACID COMPOSITION OF RAT LIVER PHOSPHATIDYLINOSITOL*

Fatty Acids	Weights of Rats (g) 150–170	310–330
	mole %	
16:0	2.9 ± 0.7	7.1 ± 1.4
18:0	46.2 ± 1.0	40.1 ± 3.6
18:1	0.8 ± 0.3	3.1 ± 0.1
18:2	1.2 ± 0.3	3.3 ± 0.6
20:2	trace	0.2 ± 0.1
20:3	2.7 ± 0.7	3.2 ± 1.3
20:4	42.7 ± 2.1	39.1 ± 1.8
20:5	0.2 ± 0.1	0.2 ± 0.1
22:3	0.6 ± 0.3	0.5 ± 0.2
22:5	0.8 ± 0.3	1.0 ± 0.2
22:6	1.9 ± 0.7	2.2 ± 1.0

* Animals weighing 150–170 g and 310–330 g were used in experiments with radioactive phosphate and with radioactive glycerol, respectively. Handling of animals in two series is described in text. Values are means ± SD for six (150–170 g) and three (310–330 g) animals, respectively.

Incorporation of Glycerol-2-^{14}C

Fig. 1 gives the percentage distribution of radioactive glycerol among the various molecular species of rat liver phosphatidylinositols. At 5 min the monoenes plus dienes, trienes, tetraenes, and polyenes contained an average of 65, 7, 17, and 11% of the total incorporated radioactivity, although the mass distribution of the species was 7.1, 5.5, 77.0, and 10.4%, respectively. The proportion of total activity recovered in the monoenes plus dienes decreased continuously with time to about 15% after 9 hr, while that of the tetraenes rose steadily and had reached 70% at the end of the experiment. A crossover point was estimated to have occurred soon after the first hour following injection of the labeled precursor. The time course of labeling of the trienes and polyenes was intermediate between that of the dienes and tetraenes.

Table 3 gives the relative specific activities of the various classes of the phosphatidylinositols after glycerol labeling. At the early times, the monoenes plus dienes showed specific activities some 40–50 times higher than

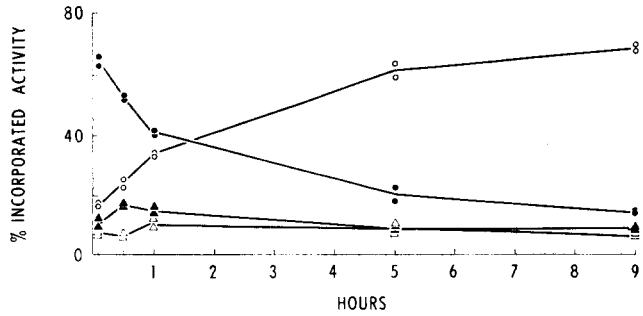

FIG. 1. Percentage distribution of glycerol-2-^{14}C among classes of rat liver phosphatidylinositols after intravenous injection. ●, Monoenes + dienes; △, trienes; ○, tetraenes; ▲, polyenes. Each point represents a separate animal.

those of the tetraenes. Over the 9-hr period of the experiment, it was observed that the relative specific activity of the monoene plus diene fraction decreased about fourfold, while that of the tetraenes showed a fourfold increase. The relative specific activities of the trienes and polyenes showed minor fluctuations with time.

There are no in vivo data in the literature with which to compare our results. A low incorporation of labeled glycerol into the arachidonoyl fraction of phosphatidylinositol in comparison with the less unsaturated species during de novo synthesis in vitro has been observed (13), which is in agreement with earlier studies on phosphatidic acid and other glycerophosphatides (1, 14).

Incorporation of Inorganic ^{32}P

Fig. 2 shows the course of incorporation of orthophosphate-^{32}P into the various molecular species of phosphatidylinositol of rat liver over the period of 1 to 96 hr. During this time, the distribution of radioactivity in the tetraenes steadily increased and then gradually leveled off, while the radioactivity of the monoenes plus dienes steadily decreased; this was similar to the appearance and disappearance of labeled glycerol in these species over a similar period of time (Fig. 1). The general shape of the curves, however, suggests that the incorporation of phosphate also must have been higher in the monoene

TABLE 3 RELATIVE SPECIFIC ACTIVITIES OF CLASSES OF RAT LIVER PHOSPHATIDYLINOSITOLS AT VARIOUS TIMES AFTER INJECTION OF GLYCEROL-2-^{14}C

Chemical Classes	Relative Specific Activities* 5 min 1	5 min 2	30 min 1	30 min 2	60 min 1	60 min 2	300 min 1	300 min 2	540 Min 1	540 Min 2
Monoenes + dienes	8.96	9.39	7.55	7.46	5.98	5.66	3.32	2.54	2.08	2.14
Trienes	1.33	1.42	1.26	1.17	1.68	2.33	1.31	2.02	1.40	1.09
Tetraenes	0.22	0.21	0.29	0.33	0.43	0.44	0.77	0.83	0.89	0.91
Polyenes	1.21	0.90	1.66	1.51	1.51	1.30	0.96	0.65	0.87	0.84

* The relative specific activity of the various classes is defined as: specific activity of the class/specific activity of the unfractionated total phosphatidylinositols. Experiments 1 and 2 at each time interval represent separate animals.

TABLE 4 RELATIVE SPECIFIC ACTIVITIES OF CLASSES OF RAT LIVER PHOSPHATIDYLINOSITOLS AT VARIOUS TIMES AFTER ADMINISTRATION OF ^{32}P

Chemical Classes	Relative Specific Activities*				
	1 hr	3 hr	1 day	2 days	4 days
Monoenes + dienes	10.74 ± 0.99	9.31 ± 1.11	5.10 ± 0.77	1.95 ± 0.46	1.17 ± 0.15
Trienes	1.23 ± 0.36	0.86 ± 0.26	0.52 ± 0.02	1.00 ± 0.21	0.98 ± 0.27
Tetraenes	0.62 ± 0.10	0.71 ± 0.04	0.91 ± 0.02	0.97 ± 0.03	1.00 ± 0.05
Polyenes	1.43 ± 0.45	1.77 ± 0.30	0.96 ± 0.04	1.03 ± 0.10	1.01 ± 0.37

* See legend to Table 3. The values are means ± SD for three animals at each time interval.

plus diene fraction and lower in the tetraene fraction at the early times, with a possible crossover point sometime prior to 1 hr after injection. Such a transient appearance of the radioactive phosphate in the monoene plus diene fraction during the early times is supported by the specific activity data plotted in Fig. 3. At 1 and 3 hr the monoenes plus dienes had the highest specific activity, and the tetraene fraction the lowest. As the time progressed (24–96 hr) the specific activities of the monoenes plus dienes decreased drastically, while those of the tetraenes gradually increased. An active incorporation of ^{32}P at early times was also noted for the polyenes, while the trienes had specific activities intermediate between or less than those of the polyenes and tetraenes at all times. By 96 hr all four fractions had approximately equal specific activities.

Over the later times in studies with both labeled phosphate and glycerol, the decrease in specific activity of the monoenes plus dienes was invariably accompanied by an increase in the specific activity of the tetraenes. Interestingly, the loss in activity from some fractions was of the order of gain by others, although it may not be immediately obvious because of the differences in their masses. Thus, multiplication of the counts in the total inositides by the percentage of this activity localized in the designated species based on the glycerol data reveals that the apparent net increase in the radioactivity of the total tetraenoic pool obtained experimentally was 4980 dpm (= 61.7% × 16,570 − 33.3% × 15,790), while the net decrease in the radioactivity from breakdown of the monoenes and dienes was 3070 dpm (= 41.1% × 15,790 − 20.6% × 16,570), over the period of 60–300

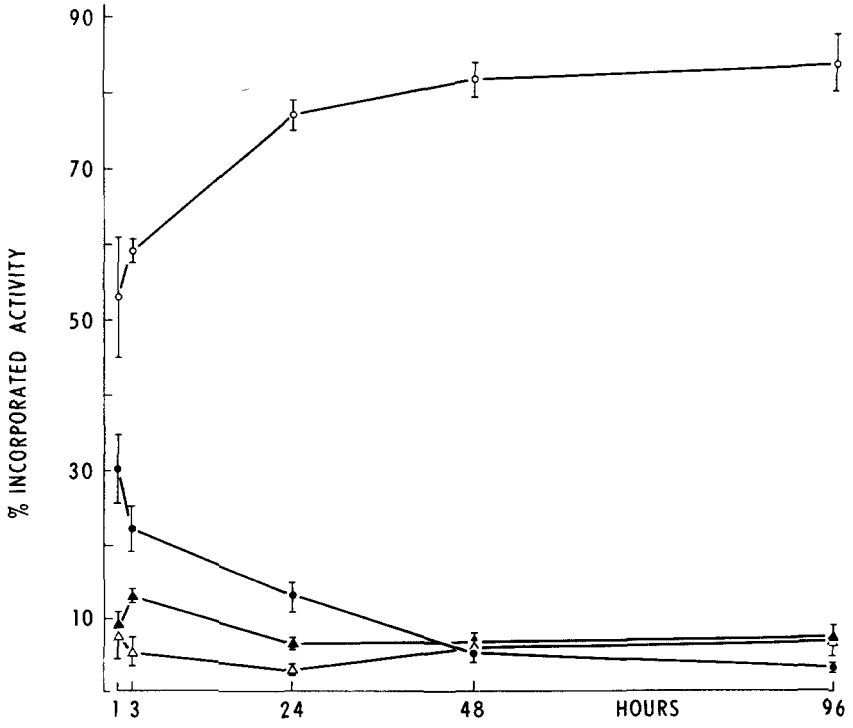

FIG. 2. Percentage distribution of ^{32}P among classes of rat liver phosphatidylinositols after intraperitoneal administration. Symbols for classes are as given in legend to Fig. 1. Each point represents the mean ± SD for three animals.

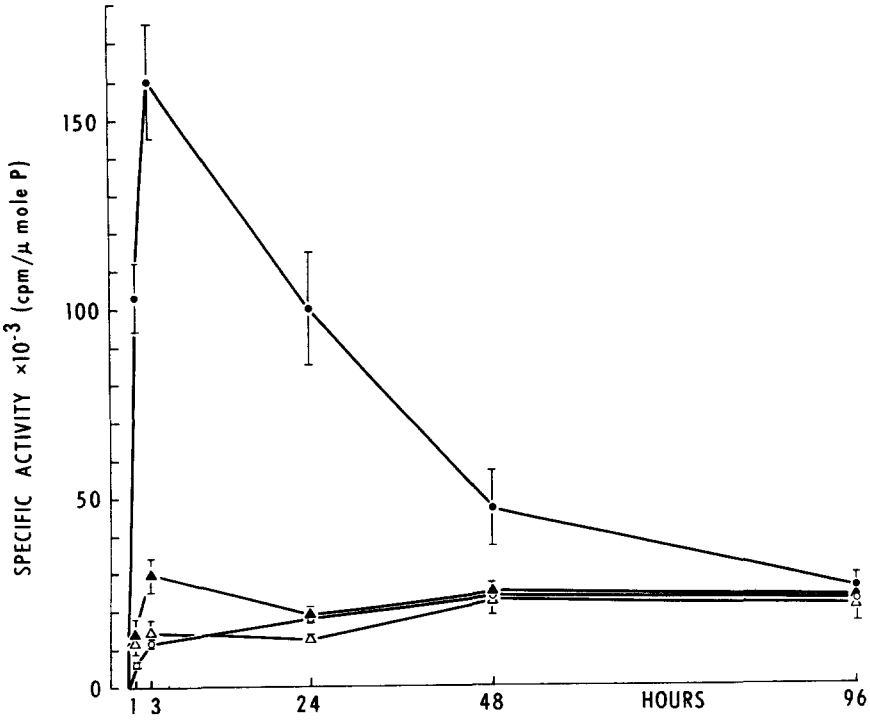

FIG. 3. Specific radioactivities of the classes of phosphatidylinositols of rat liver after intraperitoneal administration of ^{32}P. Symbols for classes are as given in legend to Fig. 1. Each point represents the mean ± SD for three animals.

min. If we assume that all inositides are degraded to the same extent as the monoenoic plus dienoic species, which is 47.3% (= 3070/6490 × 100), then the expected loss of activity from the total phosphatidylinositol pool amounts to 7470 dpm (= 47.3% × 15,790) over this time period if no reacylation occurs. The gross total increase in the activity of the inositides in the same time interval is calculated to be 8250 (= 16,570 − [15,790 − 7470]) over that reached by breakdown alone, of which 90% (= [10,230 − 2770]/8250 × 100) is in the tetraenes, when 2770 (= 5250 − [47.3% × 5250]) is that after breakdown of tetraenes. That is, the specific activity of the total phosphatidylinositols showed little change, although redistribution of activity among the species was considerable.

Table 4 gives the relative specific activities of the various classes of the liver phosphatidylinositols. The monoenes plus dienes had specific activities approximately 11 times higher than those of the unfractionated phosphatidylinositols at 1 hr. In contrast, the tetraenes had specific activities which were only about one-half those of the total inositides. Although the specific activities of the monoenes plus dienes were initially about 17 times higher than those of the tetraenes, by the fourth day the relative specific activities were nearly equal for all species. The general pattern of ^{32}P incorporation into the rat liver phosphatidylinositols at the 1- and 3-hr time periods is therefore generally similar to that noted previously for phosphatidylcholine and phosphatidylethanolamine of rat liver (15, 16), although no data were presented for longer time periods.

DISCUSSION

The present investigation demonstrates as yet unrecognized metabolic heterogeneity among the various molecular species of rat liver phosphatidylinositols with regard to rates of biosynthesis in vivo. It confirms and extends the findings of Akino and Shimojo (13), who showed in in vitro experiments that the tetraenoic phosphatidylinositols are formed by a mechanism independent of de novo synthesis via phosphatidic acid.

The studies of Trewhella and Collins (16), Arvidson (15), and Balint et al. (17), however, have suggested a corresponding metabolic complexity for rat liver phosphatidylcholines and phosphatidylethanolamines at early time intervals after administration of isotopic precursors. Unlike lecithin, which is a secreted phospholipid and a major component of plasma and biliary phosphatides (17, 18), the phosphatidylinositol remains largely in the liver. It is therefore possible to follow its transformation over an extended period of time with a remarkable conservation of the label. It was hoped that such a study would reveal the biochemical basis of the slow turnover of the hepatic phosphatidylinositols in vivo (12).

A comparison of the percentage incorporation of radioactivity from glycerol and inorganic phosphate into the dienoic and tetraenoic phosphatidylinositols reveals close similarities; this is consistent with phosphatidic acid serving as the initial precursor in their biosynthesis (3). Minor discrepancies may have been due in part to the use of rats of different ages and weights in these studies as well as to differences in the route of administration of the label. The quantitative significance of these results was thought not to be influenced by the small amount of radioactivity appearing in the fatty acids 5–9 hr after giving glycerol-2-^{14}C, for which no correction was made. 2.5–4.0% of the total radioactivity of the phosphoinositides was present in the acyl moieties, with the remainder present in the glycerol backbone.

Aside from confirming the anticipated similar metabolism of organic phosphate and glycerol in the phosphatidylinositols, the present study suggests that there is a transformation of the glycerol–phosphate backbone, associated originally with the dienoic fraction, into the tetraenoic species with time. This possibility would be of great metabolic interest and requires close examination. Assuming that the phosphatidate itself enters into the phosphatidylinositol (3), it is difficult to explain the formation of hepatic phosphoinositides containing 73% 1-stearoyl,2-arachidonoyl species when such comprise only a minor fraction of the precursor phosphatidates (19, 20). It is possible, however, that a preferential selection of certain phosphatidate species for phosphatidylinositol synthesis has taken place via the corresponding CDP diglycerides. In this regard, Thompson, Strickland, and Rossiter (5) observed that distearoyl and dioleoyl phosphatides were less effective than a mixture of natural phosphatidates prepared from ox spinal-cord lecithin in stimulating phosphatidylinositol synthesis in rat brain preparations. Although no similar in vitro studies with heterogeneous substrates have been reported in rat liver, recent work (21, 22) has shown little or no specificity for the incorporation of various unsaturated diglycerides into hepatic phosphatidylcholines and ethanolamines. In the present study, the distribution of ^{14}C-labeled glycerol among the various classes of the phosphatidylinositols as early as 5 min was generally similar to that found by others in phosphatidic acid (1, 20); this might be expected if these species were randomly incorporated into the phospholipid.

A more likely possibility for the formation of the arachidonoyl phosphoinositides is by acyl transfer to lysophosphatidylinositols, as claimed for the tetraenoic lecithins and cephalins both in vitro (2, 23) and in vivo (9, 24). This possibility is especially attractive, since the occurrence of such transferases has already been demonstrated in selected pigeon (4) and rat (25) tissues. Although the composition of the hepatic lysophosphatidylinositols has not been determined, it is disturbing to note that the monoenoic and dienoic phosphatidylinositols of both rat liver and bovine brain contain considerably more palmitic acid relative to stearic acid than the tetraene species (7, 8). A more elaborate acyl exchange would therefore have to be postulated in order to account for an effective transformation of 1-palmitoyl,2-linoleoyl into 1-stearoyl,2-arachidonoyl species mediated via stereospecific phospholipases and acyltransferases. Conceivably, this retailoring could be brought about by chain elongation and desaturation within intact phosphatide molecules as suggested for green algae (26), but no evidence for such a mechanism has yet been presented in mammalian systems.

An alternate mechanism for the formation of phosphatidylinositols of as yet undefined acyl composition and stereospecificity is suggested on the basis of the observation (3) that *sn*-glycerol-3-phosphate apparently is a more effective precursor of phosphoinositide than is phosphatidic acid. However, the failure of added phosphatidate to be effectively incorporated into phosphatidylinositol may be explained partly by the physical state of the substrate. It has also been shown that glycerylphosphorylinositol is not acylated by the common acyltransferases (4), nor are glycerylphosphorylcholine and glycerylphosphorylethanolamine (25, 27). More extensive studies with presursors of naturally occurring fatty acid compositions are definitely necessary.

The present work strongly suggests that the polyunsaturated phosphatidylinositols are formed by acylation of the lysophosphatidylinositols, which are generated largely from the monoenoic and dienoic species of monophosphoinositides. The latter were the major products of the phosphatidic acid pathway in vivo.

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