TO MY PARENTS

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SUMMARY

The pathways in the biosynthesis of phospholipids in spinach leaves have been investigated both in *in vivo* and *in vitro* studies. Cell free preparations were found to catalyze the formation of PG from CDP-diglyceride and *sn*-[1,3-^{14}C]glycerol-3-phosphate. The intermediate formation of PGP in this pathway was demonstrated by the incorporation of both ^{32}P and ^{14}C into PGP, but only ^{14}C into PG, when [^{14}C+^{32}P]GP was used as substrate. The PGP synthetase required Mg^{2+} or Mn^{2+} for activity, and had a Km for GP of 0.25 mM; the PGP phosphatase was not inhibited by sulfhydryl reagents. Evidence was also obtained for a phosphatidyltransferase which catalyzes the Mn^{2+} - dependent synthesis of PI from CDP-diglyceride and [^{14}C]*myo*-inositol; the transferase can also synthesize PI from inositol by a Mn^{2+} dependent exchange reaction.

Concerning nitrogenous phospholipids, the presence of CDP-diglyceride:serine phosphatidyltransferase for the biosynthesis of PS was suggested by the labelling of PS with ^{14}C from [U-^{14}C]serine, both *in vivo* by leaf slices and *in vitro* by cell free preparations in the presence of CDP-diglyceride. PE was shown to be biosynthesized both by the decarboxylation of PS, and from ethanolamine via the intermediate formation of CDP-ethanolamine. The former pathway was demonstrated *in vivo* by the incorporation of ^{14}C from [U-^{14}C]serine into PS and PE by whole leaves, and *in vitro*

by the decarboxylation of [^{14}C]PS to [^{14}C]PE. Concerning the CDP-ethanolamine pathway, PE was labelled from [^{14}C] ethanolamine by whole leaves and leaf slices. The presence of ethanolaminekinase catalyzing the first step of the pathway was shown *in vitro* by the ATP-dependent formation of phosphorylethanolamine from [^{14}C]ethanolamine, and the phosphorylethanolaminetransferase catalyzing the third step was shown by the diglyceride-stimulated formation of PE from CDP-[^{14}C]ethanolamine. The cytidyltransferase catalyzing the second step could not however be demonstrated *in vitro*, but is presumed to be present from the *in vivo* results.

Evidence for the biosynthesis of PC both by the step-wise methylation of PE by SAM, and by the nucleotide pathway from CDP-choline and diglyceride was obtained. In whole leaves, the methylation pathway was demonstrated by the incorporation of ^{14}C into PC both from [Me-^{14}C]methionine and from [^{14}C]ethanolamine, and into PE-Me from [^{14}C]ethanolamine. The methylation of PE-Me to PE-diMe and of the latter to PC by SAM was demonstrated *in vitro*. The methylation of PE, however, was not observed *in vitro*, possibly as a result of inactivation of the PE:SAM methyltransferase during cell fractionation. Operation of the nucleotide pathway was demonstrated by the labelling of PC, *in vivo* from [^{14}C]choline by leaf slices, and *in vitro* from CDP-[^{14}C]choline by cell-free extracts in the presence of diglyceride.

The synthesis of PE and PC by Ca^{2+}-stimulated

exchange reactions from ^{14}C-labelled ethanolamine and choline respectively, was also observed, but whether these reactions were catalyzed by an independent exchange enzyme or by phospholipase D was not determined. Synthesis of PS by an exchange pathway was not observed.

Evidence for the presence in spinach leaves of several phospholipid degradative enzymes and enzymes involved in the biosynthesis of some non-phospholipids was obtained. Phospholipase A activity towards PE and PC was detected in a microsomal fraction and leaf slices, but not in whole leaves. Also, an acyltransferase in cell free extracts catalyzing the direct transfer of acyl groups from PE or PS to an acyl acceptor, possibly MGD, was detected. The presence of enzymes catalyzing the formation of cyclopropane acids utilizing microsomal-bound phospholipid as substrate was suggested by the labelling of the fatty acid moieties of PE and PC, both from [^{14}C]SAM by a microsomal fraction and from [Me-^{14}C]methionine by whole leaves. [^{14}C]SAM was also shown by *in vitro* and *in vivo* studies to be involved in the biosynthesis of four neutral lipids, one of which was fatty acid [^{14}C]methyl ester probably synthesized by direct methylation of fatty acid.

The subcellular localization of the phospholipid biosynthesizing enzymes was investigated. The PG synthesizing system, and the enzyme systems for the biosynthesis of PC by the nucleotide and methylation pathways, and of PE by the

nucleotide pathway, were found to be localized primarily in the microsomal cell fraction. Activity from these enzymes was also detected in the chloroplast and mitochondrial fractions, but these may have been due to microsomal contamination. PS decarboxylase was localized primarily in the 100,000xg supernatant fraction, but significant activity was also found in the mitochondrial fraction suggesting that the enzyme may have been solubilized from mitochondria during cell fractionation. The phosphatidyltransferases for the biosynthesis of PS and PI were detected in total particulate fractions, but their subcellular localization was not further investigated. The results obtained suggest that PG and PC are not synthesized *in situ* by chloroplasts and mitochondria, respectively, but are derived from these lipids synthesized by the microsomal membrane. Mitochondrial PE can also be derived from PE synthesized by the microsomal nucleotide system, but may also by synthesized *in situ* by the mitochondrial PS decarboxylase.

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ABBREVIATIONS

ADP	Adenosinediphosphate
ATP	Adenosinetriphosphate
Ci	Curie
CDP-diglyceride	Cytidinediphosphate diglyceride
CMP	Cytidinemonophosphate
CTP	Cytidinetriphosphate
CL	Cardiolipin
CoA	Coenzyme A
DGD	Digalactosyl diglyceride
GP	Glycerophosphate
GPC	Glycerol phosphorylcholine
GPE	Glycerol phosphorylethanolamine
GPI	Glycerol phosphorylinositol
GPS	Glycerol phosphorylserine
MGD	Monogalactosyl diglyceride
NL	Neutral lipid
Pi	Phosphate
Pi-Pi	Pyrophosphate
PA	Phosphatidic acid
PC	Phosphatidyl choline
PE	Phosphatidyl ethanolamine
PE-Me	Phosphatidyl monomethylethanolamine
PE-diMe	Phosphatidyl dimethylethanolamine
PG	Phosphatidyl glycerol

PGP	Phosphatidyl glycerolphosphate
PI	Phosphatidyl inositol
PS	Phosphatidyl serine
POPOP	1,4-bis-[2-(5-phenyloxazolyl)]-benzene
PPO	2,5-Diphenyloxazole
SAHC	S-adenosylhomocysteine
SAM	S-adenosylmethionine
TLC	Thin layer chromatography
R_f	Relative mobility

A. INTRODUCTION

I. General-Membranes and Lipids

Biological membranes are fundamental to life. In addition to providing a physical barrier which separates cells and subcellular organelles from their respective environments, membranes are intimately involved in essential life processes. In both procaryotic and eucaryotic cells they facilitate and control the transport of metabolites between the two environments they separate. In eucaryotic cells they are involved in oxidative phosphorylation and protein synthesis as well as many other cell functions, while photosynthetic organisms are of special interest among eucaryots in that the molecular machinery for photosynthesis is localized in the lamellae membrane of chloroplasts (Branton, 1969; Korn, 1969; Stoeckenius and Engelman, 1969).

The study of membranes is one of the most exciting fields of modern biochemistry. Of particular interest is the problem of membrane assembly from the individual lipid and protein membrane constituents. Clearly this problem can only be approached when knowledge has been gained concerning, i) the pathways in the biosynthesis of lipid and protein, and ii) the subcellular localization of individual lipids and protein and of the enzymes involved in their biosynthesis. Lipids generally comprise 20-40% by weight of membranes. A major class of lipid are the phospholipids, and during the

last 20 years knowledge of their structure and biosynthesis has grown rapidly. However, studies on the biosynthesis of phospholipids has largely been confined to animals and bacteria, while very few detailed studies have been made with plant tissues and even less with photosynthetic tissue of higher plants. This will become apparent in the following introduction, the first part of which will discuss the interesting structure and subcellular localization of plant phospholipids, while the second and third parts will review the current knowledge concerning the biosynthesis of phospholipids in Nature and the subcellular localization of the enzymes involved.

II. Glycerolipids of Photosynthetic Plant Tissue

1. Composition

In most photosynthetic tissues, phospholipids amount to about 20%, and glycolipids about 40% of total lipid. In leaves and green algae, phospholipids consist mainly of phosphatidyl choline (PC), phosphatidyl glycerol (PG), phosphatidyl ethanolamine (PE) and phosphatidyl inositol (PI), of which PC is the major component (Table 1). However, in photosynthetic bacteria, PC is replaced by PE as the major component, and in blue green algae PC, PE and PI are completely absent (see Table 1, and Kates, 1970). Minor phospholipids occasionally reported to be present in photosynthetic and other plant tissues include phosphatidyl serine (PS),

TABLE 1. PHOSPHOLIPID COMPOSITION OF PHOTOSYNTHETIC TISSUE[a]

Tissue	PG	PC	PE	PS	PI	CL
Leaves						
Spinach, total[b]	26	47	17	-	9	trace
Chloroplast[c]	58	27	trace	-	11	trace
Lamellae[d]	65	24	0	-	11	-
Cauliflower chloroplasts[e]	44	19	13	9	12	3
Runner bean[f]	22	45	17	-	9	-
Tomato[g]	19	50	20	-	4	4
Maidenhair tree[g]	22	48	14	-	6	7
Green algae						
Chlorella vulgaris[h]	27	46	9	-	14	1
Scenedesmus obliquus[i]	41	36	6	1	15	-
Photosynthetic bacteria						
Rhodospirillum rubrum[j]	29	6	57	-	-	8
Rhodopseudomonas spheroides[j]	54	4	36	-	-	6

[a] Values are expressed as percentage of total phospholipids. Dash (-) indicates data unavailable.

[b] Wintermans (1963).

[c] Douce *et al.* (1968).

[d] Allen *et al.* (1966).

[e] Schwertner and Biale (1973).

[f] Kates (1960).

[g] Roughan and Batt (1969).

[h] Sastry and Kates (1965).

[i] Benson and Maruo (1958); Benson and Strickland (1960).

[j] Wood *et al.* (1965); Haverkate *et al.* (1965).

diphosphatidyl glycerol (cardiolipin) and phosphatidic acid (PA). Concerning PS, Hutt and co-workers (1950) and Kates and Eberhardt (1957) isolated small amounts of serine from the hydrolysate of peanut and bean leaf phospholipids respectively, and Benson and Strickland (1960) detected [^{14}C]GPS among the hydrolysate products of total ^{14}C-labelled Chlorella lipids. Though this evidence is not conclusive, the presence of PS in plants is also suggested by the finding of PS labelled from [U-^{14}C]serine in tomato root (Willemot and Boll, 1967; see below). Other minor phospholipids are N-methyl and N-acyl derivatives of PE; the former has only been detected in diatoms (Volcani and Kates, 1966) and the latter in seeds (Aneja et al., 1969; Dawson et al., 1969). Also, Debuch and Rotsch (1966) have isolated a novel polyglycerophosphatide from spinach leaves, the precise structure of which is unknown.

A unique characteristic of all plant photosynthetic tissues, except photosynthetic bacteria, is the high content of glycolipids. These consist chiefly of monogalactosyl and digalactosyl diglycerides and sulfoquinovosyl diglyceride, of which monogalactosyl diglyceride is the major single component. In leaves, minor glycolipids include glucocerebrosides (Sastry and Kates, 1964; Carter and Koob, 1969), sterol and acylsterol glycosides (Eichenberger and Menke, 1966; Kiribuchi et al., 1966 and 1967) and a complex sphingolipid called phytoglycolipid (Carter and Koob, 1969).

In non-photosynthetic plant tissues, the lipid

composition is qualitatively similar to that of photosynthetic tissues, but the proportions of PG and glycolipids are much less (see Kates, 1970). Even more striking differences are apparent between the lipid composition of plant photosynthetic tissue, and that of animals and bacteria. Thus, in all animal tissues except brain, the glycolipid content is very low, and sulfoquinovosyl diglyceride is completely absent. A major phospholipid of photosynthetic tissue, PG, is only a trace component in animal tissues, while the latter tissue contains much higher amounts of cardiolipin, and also contains sphingomyelin and phosphatidyl inositol phosphates which are absent from photosynthetic tissue (Gray, 1964; Wells and Dittmer, 1966; and Fleischer and Rouser, 1965). In bacteria, the major phospholipids are PE and PG, but PC is completely absent except as a minor component in Thiobacillus novellus and in certain species of soil bacteria including the Agrobacteria, the nitrifying bacterium Nitrosocystis oceanus, the nitrogen fixing bacterium Azotobacter agilis and some of the photosynthetic bacteria (see Goldfine, 1972).

The fatty acid composition of the glycerolipids of all photosynthetic tissues, except photosynthetic bacteria and certain species of blue-green algae, is characterized by a high content of polyunsaturated species of which α-linolenic acid is the major component (see Kates, 1970). A unique fatty acid is trans-3-hexadenenoic acid, which is found in all

photosynthetic tissues except blue-green algae and photosynthetic bacteria, but is absent in etiolated and non-photosynthetic tissues (see reviews by Kates, 1970, and Hitchcock and Nichols, 1971). This acid is located specifically at the 2-position of the PG component in the chloroplast lamellae of leaves, green algae and Euglina gracillus (Haverkate and Van Deenen, 1965 a,b,). The major molecular species of the PG component of leaves is 1-linolenoyl-2-(trans-3'-hexadecenoyl)-sn-3-glycerophosphoryl-1"-sn-glycerol (Haverkate and Van Deenen, 1965 a).

2. Subcellular Localization of Phospholipids

In the leaves of spinach and tobacco, about 50% and 75% respectively of the total cell content of PG has been found localized in the chloroplasts, in which it is the major phospholipid component (see Table 1, Wintermans, 1960 and 1963; Ongun et al., 1968).

Concerning the subcellular localization of PC and PE, these have been found to be the major phospholipid components of purified mitochondria from non-photosynthetic plant tissue (Schwertner and Biale, 1973), and are also probably the major phospholipid components of mitochondria from photosynthetic tissue. The important question of whether PC and PE are also present in leaf chloroplasts can only be answered by lipid analysis of chloroplasts freed from mitochondrial contamination. This has only been attempted satisfactorily by Douce et al. (1968), who showed that in intact spinach chloroplasts

judged to be free of mitochondria by electron microscopic examination, PC accounted for 27% of phospholipid while only trace amounts of PE were detected. Douce's results are consistent with the finding by Allen et al. (1966) of 24% PC, but no PE, in the phospholipids of purified spinach chloroplast lamellae. Schwertner and Biale (1973) found a similar concentration of PC in purified cauliflower chloroplasts, but in contrast a much higher concentration of PE than that found by Douce et al and Allen et al for spinach chloroplasts. These studies strongly suggest that PC is a component of leaf chloroplasts and possibly localized in the lamellae membrane. However, since data on the amounts of PE in leaf chloroplasts is contradictory, the question of whether this lipid is present in chloroplasts cannot be answered at present.

Concerning the intracellular localization of PI and cardiolipin, the former has been found in both leaf chloroplasts (Table 1) and in the mitochondria of non-photosynthetic tissue (10% of phospholipid), while the latter has been found in mitochondria from non-photosynthetic tissue (10% of phospholipid) but appears to be only a trace component of leaf chloroplasts (see Table 1; Douce et al., 1968; Schwertner and Biale, 1973).

Much more detailed knowledge exists on the phospholipid distribution among rat liver subcellular membranes (McMurray and Dawson, 1969; Kleinig, 1970; Keenan and Morré, 1970; Ray et al., 1969). All subcellular membranes contain

PC, PE, PI and PS, of which PC and PE are the major components. PG is found only in mitochondria and the plasma membrane, while cardiolipin is exclusively localized in the mitochondria. Important differences also exist between the inner and outer mitochondrial membrane in that the inner membrane contains a higher proportion of cardiolipin but a lower proportion of PS and PI than the outer membrane.

III. Biosynthesis of Phospholipids in Plants, Animals and Bacteria

1. Pathways of Biosynthesis

a) General Principles. The currently accepted pathways for the biosynthesis of phospholipids which have been elucidated mainly by studies with animals and bacteria, are shown in Fig. 1. There are three main types of reaction:

i) de novo generation of a monophosphate ester linkage. The elucidation of this type of reaction was largely the work of Kennedy and co-workers who demonstrated that these reactions always involve cytidine coenzymes. This type of reaction can be further subdivided as follows:

i-a) donation of a phosphatidyl group from CDP-diglyceride to either serine, inositol, glycerol or phosphatidyl glycerol, to form phosphatidyl serine, phosphatidyl inositol, phosphatidyl glycerophosphate or diphosphatidyl glycerol (cardiolipin), respectively.

i-b) donation of a phosphorylbase group from CDP-

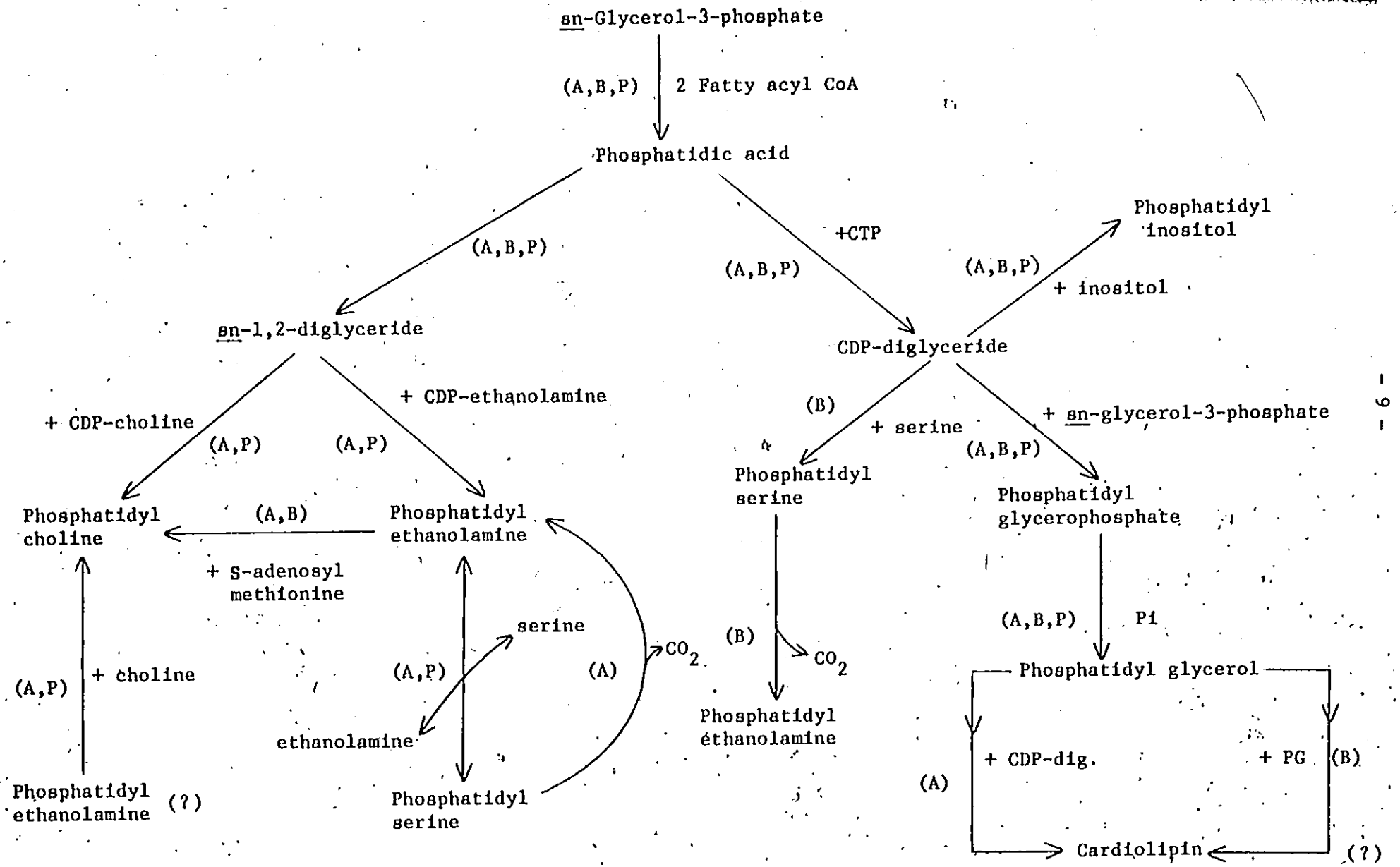


FIG. 1. PATHWAYS FOR THE BIOSYNTHESIS OF PHOSPHOLIPIDS
A, B or P denotes pathway known in animals, bacteria or plants, respectively.

base to sn-1,2-diglyceride. This type of reaction is involved in the biosynthesis of phosphatidyl choline and phosphatidyl ethanolamine from CDP-choline and CDP-ethanolamine respectively.

ii) interconversion of phospholipids without breakage of monophosphate ester linkages. The main examples of this type are the decarboxylation of phosphatidyl serine to phosphatidyl ethanolamine, and the methylation of phosphatidyl ethanolamine to phosphatidyl choline.

iii) interconversion of phospholipids by an exchange of polar head group. Examples are the exchange of the ethanolamine moiety of phosphatidyl ethanolamine for free serine to form phosphatidyl serine, and vice versa, and the exchange of the glycerol moiety of phosphatidyl glycerol for another molecule of phosphatidyl glycerol to form cardiolipin. It should be pointed out that though this type of reaction involves breaking and reforming a monophosphate ester linkage, there is no net de novo generation of such a linkage, and thus, as predicted by the principle elucidated by Kennedy (see i) above), no cytidine coenzymes are needed in such reactions.

b) Phosphatidic acid (PA). Four pathways are known for the biosynthesis of PA, as follows:

i) stepwise acylation of sn-glycerol-3-phosphate by acyl CoA:

$$\text{sn-Glycerol-3-phosphate} + \text{acyl CoA} \longrightarrow \text{lyso PA} + \text{CoA} \quad (1)$$

$$\text{Lyso PA} + \text{acyl CoA} \longrightarrow \text{PA} + \text{CoA} \quad (2)$$

These reactions have been shown to be catalyzed by separate enzymes in liver (Lands and Hart, 1964) and in E. coli (Ray et al., 1970; Hechemy and Goldfine, 1971). The biosynthesis of PA in spinach leaves has been studied by Cheniae (1965) and Sastry and Kates (1966). They demonstrated the in vitro incorporation of sn-glycerol-3-phosphate into PA and into small amounts of lyso PA, under conditions in which acyl CoA was being actively synthesized. Thus, PA in leaves is probably biosynthesized by the above pathway.

ii) acylation of dihydroxyacetonephosphate, followed by reduction and reacylation. These reactions have been demonstrated in rat liver microsomes (Hajra and Agranoff, 1968; Hajra, 1968; and Puleo et al., 1970), but have so far not been detected in bacteria or plants.

iii) phosphorylation of sn-1,2-diglyceride with ATP, catalyzed by diglyceridekinase. This enzyme is present in brain (Hokin and Hokin, 1959) and E. coli (Pieringer and Kunnes, 1965). It has been found in only one plant tissue so far, namely, peanut cotyledons (Bradbeer and Stumpf, 1960).

iv.) phosphorylation of monoglyceride with ATP to lyso PA, followed by acylation with acyl CoA to PA. These reactions have been demonstrated in brain (Pieringer and Hokin, 1962 a and b) and E. coli (Pieringer and Kunnes, 1965), but has so far not been demonstrated in plants.

c) CDP-diglyceride. The only known pathway for the biosynthesis of CDP-diglyceride is that catalyzed by

CTP:phosphatidic acid cytidyltransferase:

CTP+phosphatidic acid $\longrightarrow$ CDP-diglyceride+Pi-Pi (3)

The first clue to this pathway was the finding by Agranoff et al. (1958) of incorporation by liver extracts of CMP into a cytidine liponucleotide which, though not rigorously identified, was claimed to be CDP-diglyceride. These authors interpreted this finding as suggesting that CMP was first incorporated into CDP-choline which then reacted with PA in a cytidyltransferase reaction to give CDP-diglyceride. This interpretation was shown to be incorrect by Paulus and Kennedy (1960), who demonstrated that the cytidine moiety of CDP-diglyceride was derived from CTP by the reaction shown in eqn. (3), and not from CDP-choline. The rat liver enzyme system has been described in detail by Carter and Kennedy (1966). The same enzyme system can also synthesize dCDP-diglyceride from dCTP and PA (Ter Schegget et al., 1971). The synthesis of CDP-diglyceride by eqn. (3) also takes place in the brain (Petzold and Agranoff, 1967) and in bacteria (Carter, 1968; Patterson and Lennarz, 1971; McCaman and Finnerty, 1968; and Bell et al., 1971).

The central role of CDP-diglyceride in the biosynthesis of bacterial phospholipids is beyond doubt (see below). However, there had been no real proof of the natural occurrence of CDP-diglyceride in Nature until Raetz and Kennedy (1973) were able to isolate small amounts of ^{32}P-labelled CDP-diglyceride from E.coli cells grown in glycerol-

3-[^{32}P]phosphate. Interestingly, equivalent amounts of ^{32}P-labelled dCDP-diglyceride were also isolated. The very low level of total labelled cytosine liponucleotide relative to labelled PA lead Raetz and Kennedy to suggest that the conversion of PA to liponucleotide may be the rate determining step in the overall process of phospholipid biosynthesis in E.coli.

Recent evidence suggests that CDP-diglyceride is also biosynthesized by the above pathway in plants. Thus, Douce (1968), Bahl et al. (1970) and Sumida and Mudd (1970 a) have demonstrated the incorporation of ^{32}P from [α-^{32}P]CTP into CDP-diglyceride by cell free preparations from spinach leaves, maize and cauliflower inflorescence. However, these workers were unable to show a dependence of CDP-diglyceride synthesis on added PA, presumably because of high levels of endogenous PA in the cell free preparations.

d) Phosphatidyl inositol (PI). The currently accepted pathway for the de novo biosynthesis of PI in animal tissues is as follows:

CDP-diglyceride + myo-inositol $\rightleftharpoons$ phosphatidyl inositol+CMP (4)

This pathway was first postulated by Agranoff et al. (1958). Their evidence was based on the incorporation of inositol into PI by guinea pig liver and isolated liver mitochondria. Since CDP-choline and PA were found to stimulate inositol incorporation into PI by isolated mitochondria, these

authors suggested that CDP-diglyceride was formed from a cytidyltransferase reaction between CDP-choline and PA. However, direct proof that CDP-diglyceride was a substrate in PI biosynthesis was first provided by Paulus and Kennedy (1960) who showed that incorporation of inositol by chicken liver microsomes into PI in the presence of Mn^{2+} was accompanied by release of [^{32}P]CMP from [α-^{32}P]CDP-diglyceride. Ironically, though the postulate by Agranoff et al. for the involvement of CDP-diglyceride in PI biosynthesis was proved correct, the suggestion advanced by these workers for the biosynthesis of CDP-diglyceride itself was proved incorrect by Paulus and Kennedy (1960), as described above in Section c.

Though the de novo biosynthesis of PI in liver tissue proceeds as in eqn. (4), Paulus and Kennedy (1960) demonstrated that inositol, in the presence of Mn^{2+}, could be incorporated by liver microsomes into PI in the absence of added CDP-diglyceride. These authors suggested that this occurred by an exchange reaction with endogenous PI and that the exchange reaction and the CDP-diglyceride reaction were part of the same enzyme system, as follows:

$$\text{CDP-diglyceride} + \text{enzyme} \rightleftharpoons \text{phosphatidyl-enzyme} + \text{CMP} \qquad (5)$$

$$\text{Phosphatidyl-enzyme} + \text{inositol} \rightleftharpoons \text{phosphatidyl inositol} + \text{enzyme} \qquad (6)$$

It should be pointed out that the enzyme involved in the exchange reaction with inositol is different from the enzyme described below for exchange of serine, ethanolamine and choline, in that the inositol reaction requires Mn^{2+}

whereas the other exchange reactions exclusively require Ca^{2+}. It is not known whether the inositol exchange system functions in vivo.

CDP-diglyceride:inositol phosphatidyltransferase has since been found in a wide variety of animal tissues (Benjamins and Agranoff, 1969; Prottey and Hawthorne, 1967). However, little is known concerning the biosynthesis of PI in the few species of bacteria in which it is found.

The biosynthesis of PI in non-photosynthetic plant tissue has been studied by Sumida and Mudd (1970 b). A mitochondrial preparation from cauliflower inflorescence was found to catalyze the incorporation of inositol into PI in the presence of Mn^{2+}, and this incorporation could be greatly stimulated by the addition of CDP-diglyceride. CTP was also found to be as effective as CDP-diglyceride, presumably because of its conversion to the latter by reaction with endogenous PA. This evidence suggests that in non-photosynthetic plant tissue PI is biosynthesized by the same pathway as in animals (eqn. 4). The same pathway may also operate in spinach leaves since Sastry and Kates (1966) found that ^{32}P from sn-glycerol-3-[^{32}P]phosphate could be incorporated into PI, in the presence of CTP and Mn^{2+} and spinach cell fractions activily synthesizing [^{32}P]PA.

e) Phosphatidyl glycerol (PG). The only known pathway for the biosynthesis of PG involves the intermediate formation of phosphatidyl glycerophosphate (PGP) from CDP-

diglyceride and sn-glycerol-3-phosphate, followed by dephosphorylation to PG:

sn-Glycerol-3-phosphate+CDP-diglyceride ⟶ 3-sn-phosphatidyl-I'-sn-glycerol-3'-phosphate+CMP (7)

3-sn-Phosphatidyl-I'-sn-glycerol-3'-phosphate ⟶ 3-sn-phosphatidyl-I'-sn-glycerol+Pi (8)

This pathway was first demonstrated by Kiyasu et al. (1963) in rat and chicken liver, and by Kanfer and Kennedy (1964) in E.coli. Their evidence was based mainly on the CDP-diglyceride dependent incorporation of [^{14}C]GP into PG, and of [^{32}P]GP into PGP, by cell free extracts of these tissues. The pathway has since been demonstrated in sheep brain (Possmayer et al., 1968; Stanacev et al., 1968; Davidson and Stanacev, 1970), in beef and rat heart (Stanacev et al., 1969; Stuhne-Sekalec and Stanacev, 1970), in rat ventral prostate (Stanacev et al., 1970), in a Bacillus megaterium mutant (Patterson and Lennarz, 1971) and in Salmonella typhimurium (Bell et al., 1971).

Both of the enzymes catalyzing the above reactions have been isolated from the particulate fraction of E.coli and partially purified by Chang and Kennedy, (1967 a and b). The partially purified sn-glycerol-3-phosphate: CDP-diglyceride phosphatidyltransferase (PGP synthetase) requires Mn^{2+} or Mg^{2+} for activity, and is inactive with sn-glycerol-1-phosphate, glycerol, myo-inositol and serine. The partially purified PGP phosphatase is much more active than the phosphatidyl-

transferase, which explains why PGP has not been detected in E.coli lipids since it does not accumulate. The enzyme also requires Mg^{2+}, and does not act on GP or PA. Unlike the phosphatidyltransferase it is strongly inhibited by sulfydryl reagents. In contrast, the PGP phosphatase in a strain of Bacillus has been reported to be uninhibited by sulfydryl reagents (Patterson and Lennarz, 1971).

Surprisingly, it has only been very recently that the specificity of PGP synthetase towards different liponucleotides has been studied. Raetz and Kennedy (1973) found that the activity of non-cytosine nucleoside diphosphate diglycerides was measurable but much lower than that of either CDP-diglyceride or its deoxy analogue. Concerning the specificity between CDP-diglyceride and dCDP-diglyceride, the latter was almost twice as effective as CDP-diglyceride at high concentrations, though whether this was also true at low concentrations was not determined. At certain concentrations, dCDP-diglyceride was also found to be more effective than CDP-diglyceride in the synthesis of PS (see Section g). Since dCDP-diglyceride has been found to accumulate to the same extent as CDP-diglyceride in growing E.coli cells (Raetz and Kennedy, 1973), these findings raise the question, at present unresolved, as to which of the two cytosine containing liponucleotides is functioning in vivo in E.coli in the biosynthesis of PG and PS.

The same reactions as shown in eqns. 7 and 8 have been proposed for the formation of PG in photosynthetic tissues

since the plant PG also has the 3-sn-phosphatidyl-1'-sn-glycerol configuration (Benson and Miyano, 1961; Haverkate and Van Deenen, 1965 a and b). The first evidence for this proposal was presented by Sastry and Kates (1966) who showed that crude cell fractions from spinach leaves could incorporate ^{32}P from sn-glycerol-3-[^{32}P]phosphate into PG as well as PA, in a system containing CTP, ATP, Mg^{2+}, CoA and fatty acids. However, the direct involvement of CDP-diglyceride in the formation of PG was not demonstrated. The above pathway may also function in Chlorella vulgaris since PG is rapidly labelled with ^{32}P from DL-α-glycerol[^{32}P]phosphate during photosynthesis (Sastry and Kates, 1965). Concerning the biosynthesis of PG in non-photosynthetic tissue, Douce (1968) and Douce and Dupont (1969) presented good evidence that in cauliflower inflorescence PG is biosynthesized by the above reactions. Their evidence was as follows:

i) ^{14}C activity from sn-[^{14}C]glycerol-3-phosphate was incorporated into PG in the presence of Mg^{2+} by a crude mitochondrial fraction actively synthesizing CDP-diglyceride *in situ* from CTP and endogenous PA. Furthermore, $HgCl_2$ caused inhibition of PG formation and an accumulation of PGP (Douce and Dupont, 1969).

ii) ^{32}P activity from [α-^{32}P]CTP was incorporated into CDP-diglyceride by a crude mitochondrial fraction, and accumulation of labelled CDP-diglyceride was decreased and [^{32}P]CMP was released in the presence of sn-glycerol-3-phosphate (Douce, 1968).

Though the above evidence is consistent with the biosynthesis of PG by equations 7 and 8, no evidence was presented concerning kinetic properties of the PG synthesizing system nor of the ability of exogenous CDP-diglyceride to act as substrate. These studies also failed to tackle the question concerning the biosynthesis of PG in photosynthetic tissue.

An alternative pathway has been suggested (Yang et al., 1967; Dawson, 1967 a), namely, the transfer of a phosphatidyl unit from phosphatidyl choline to glycerol, catalyzed by phospholipase D:

$$\text{Phosphatidyl choline} + \text{glycerol} \xrightleftharpoons{\text{phospholipase D}} \text{phosphatidyl glycerol} + \text{choline} \qquad (9)$$

However, this pathway cannot possibly be operating in leaves since the PG formed by this transphosphatidylation has been shown to be phosphatidyl-rac-1'-glycerol (Yang et al., 1967), a mixture of diasterioisomers rather than the one sterioisomer known to be present in leaves. Also, Schantz et al. (1972) showed that in Euglina gracillus growing auxotrophically, no ^{32}P was incorporated into PG from PC and PE pulse labelled from [^{32}P]orthophosphate, even though PG was being rapidly synthesized. This clearly demonstrated that in this organism at least, the phosphatidyl moiety of PG is not derived from PC or PE by an exchange reaction catalyzed either by phospholipase D or by a separate exchange enzyme.

f) Diphosphatidyl glycerol (Cardiolipin). Two pathways are at present known for the biosynthesis of cardiolipin:

Phosphatidyl glycerol+CDP-diglyceride ⟶ cardiolipin+CMP (10)

2 Phosphatidyl glycerol ⟶ cardiolipin+glycerol (11)

Both pathways involve the transfer of a phosphatidyl group to PG, but in one pathway the phosphatidyl group is derived from CDP-diglyceride (eqn. 10), while in the other it is derived from another molecule of PG (eqn. 11).

In 1967, Stanacev, Chang and Kennedy proposed that cardiolipin in E.coli is biosynthesized by the reaction shown in eqn. 10. Their evidence was based on the stimulation of [^{14}C]cardiolipin synthesis from phosphatidyl-[^{14}C]glycerol in an E.coli particulate fraction by high concentrations of exogenous unlabelled CDP-diglyceride.

At the time of this proposal, cardiolipin biosynthesis by the alternate reaction (eqn. 11) was not known. However, in 1970, Stanacev and Stuhne-Sekalec showed the feasibility of such a reaction by demonstrating the CDP-diglyceride independent conversion of small amounts of PG into cardiolipin by a crude phospholipase D preparation from cabbage. The first indication that this alternate pathway might be functioning in bacteria was the finding by De Siervo and Salton (1971) of an almost quantitative conversion of PG to cardiolipin by a particulate fraction from M.lysodeikticus

in the absence of CDP-diglyceride. These studies prompted a re-examination of the biosynthesis of cardiolipin in E.coli. Thus, Hirschberg and Kennedy (1972) found that the $^{3}H/^{32}P$ ratio in cardiolipin formed by an E.coli particulate fraction was half that of the corresponding ratio of [^{32}P]phosphatidyl [^{3}H]glycerol substrate. These workers confirmed the original observation by Stanacev et al. (1967) that CDP-diglyceride stimulated cardiolipin biosynthesis, but they proved conclusively that this compound was not functioning as a phosphatidyl doner by the finding that ^{14}C from [^{14}C]PG, but no ^{32}P from [β-^{32}P]CDP-diglyceride, was incorporated into cardiolipin. Hostetler, Van den Bosch and Van Deenen (1972) have made similar observations in E.coli, and the work of Rampini et al. (1971) strongly suggests that the same CDP-diglyceride independent pathway operates in Staphylococcus aureus. The evidence so far thus favours the formation of cardiolipin in E.coli by the reaction shown in eqn. 11.

The pathway of cardiolipin biosynthesis in animal tissue has been the subject of much recent research, mainly contributed by the laboratories of Stanacev and of Van Deenen. Davidson and Stanacev in 1971 showed that sn-glycerol-3-phosphate could be incorporated into cardiolipin by guinea pig liver mitochondria actively synthesizing CDP-diglyceride in situ, and simultaneously, Hostetler et al. (1971) showed the dependence of glycerophosphate and PG incorporation into cardiolipin by rat liver mitochondria on the presence of

exogenous CDP-diglyceride. Stanacev et al. (1973) later demonstrated more directly the substrate role of CDP-diglyceride by showing the incorporation of ^{14}C into cardiolipin from CDP-[^{14}C]diglyceride. Another crucial piece of evidence was provided by Hostetler et al. (1972) who showed that [2-^{3}H] phosphatidyl-[1-^{14}C]glycerol was converted into cardiolipin with conservation of the $^{3}H/^{14}C$ ratio, a finding which was consistent with the CDP-diglyceride dependent pathway and at the same time ruled out the possibility that the bacterial CDP-diglyceride independent pathway was also operating.

Little is known about the properties of the enzyme catalyzing the CDP-diglyceride dependent pathway. However, Domazet et al. (1973) have confirmed earlier reports that Mg^{2+} or Mn^{2+} are required for activity. This is in contrast to the animal PG synthetase system which does not require metal ions (Kiyasu et al., 1964).

Very little is known concerning the biosynthesis of cardiolipin in plants. Douce (1968) and Douce and Dupont (1969) found that ^{14}C from [^{14}C]GP was incorporated into cardiolipin as well as into PG by cauliflower inflorescence mitochondria in the presence of CTP and Mg^{2+}. However, it is not possible to conclude from this result as to which of the pathways discussed above was operating in this system. In view of the finding by Stanacev and Stuhne-Sekalec (1970) of an in vitro phospholipase D catalyzed formation of cardiolipin by eqn. 11, it might be thought that this enzyme also catalyzes the

formation of cardiolipin *in vivo* in plants. However, as pointed out below in Section j) phospholipase D can carry out many non-specific exchange reactions in addition to its hydrolyzing activity. It is therefore possible that the observed *in vitro* phospholipase D catalyzed formation of cardiolipin from 2 moles of PG, which is essentially an exchange of the glycerol moiety of PG for another molecule of PG, may simply reflect the non-specificity of phospholipase D catalyzed *in vitro* exchange reactions, and is therefore unlikely to have any physiological role *in vivo*.

g) Phosphatidyl serine (PS). The importance of CDP-diglyceride as a phosphatidyl donor substrate in the biosynthesis of PG, PI and cardiolipin has already been described above. Kennedy and co-workers have demonstrated that in *E.coli* this compound also serves as a phosphatidyl donor in the biosynthesis of PS, a minor lipid in *E.coli* which serves as the intermediate in the biosynthesis of PE. The reaction for the formation of PS, catalyzed by CDP-diglyceride: L-serine phosphatidyl transferance (PS synthetase), is as follows:

CDP-diglyceride+L-serine $\longrightarrow$ phosphatidyl serine+CMP (12)

This reaction was first described by Kanfer and Kennedy (1964), who found that serine incorporation into PS by an *E.coli* cell free extract was completely dependent on exogenous CDP-diglyceride. A striking property of the enzyme

was its dependence for optimal activity on both an added surface active agent, (either octanol or cutscum) and a sufficient ionic strength (0.1 mM) in the incubation medium. The above pathway has also been demonstrated in a mutant of Bacillus megatarium by Patterson and Lennarz (1971) and in Salmonella typhimurium by Bell et al. (1971).

The specificity of PS synthetase in E.coli towards different liponucleotides has recently been studied by Raetz and Kennedy (1973). They found that over a wide range of concentrations the activity of non-cytosine nucleoside diphosphate diglycerides was measurable, but much lower than that of CDP-diglyceride. However, the difference in specificity between CDP-diglyceride and dCDP-diglyceride was more complex. The former was apparently more active at high concentrations, and the latter more active at low concentrations. Whether this dual specificity observed in vitro has any physiological significance in vivo is as yet unknown.

The biosynthesis of PS by eqn. (12) is characteristic of bacteria and no such reaction has been detected either in liver (Paulus and Kennedy, 1960) or in the protozoa Tetrahymena pyriformis (Dennis and Kennedy, 1970). Instead, in these tissues the evidence available so far suggests that PS is biosynthesized by an exchange reaction involving free serine and the ethanolamine moiety of phosphatidyl ethanolamine (see Section j).

Few studies on the biosynthesis of PS in plants

have been carried out, and most of these have utilized only non-photosynthetic tissue. The presence of an active synthesis of PS from serine in tomato roots is suggested by the work of Willemot and Boll (1967) who demonstrated the incorporation of ^{14}C from [U-^{14}C]serine into the serine moiety of PS by excised tomato root. The biosynthesis of PS in Chlorella vulgaris by eqn. (12) was suggested by Sastry and Kates (1965) from the finding that growing cells of this organism incorporated ^{32}P from [^{32}P]GP into PE. This incorporation most likely arose by the formation of CDP-diglyceride from GP followed by conversion of CDP-diglyceride to PS and then decarboxylation (eqns. 1-3, 12-13), since this is the only known route in which the phosphate moiety of PE is derived from that of GP. However, it is not possible from these in vivo studies to establish with certainty the pathway of PS biosynthesis. Vandor and Richardson (1968) showed that a microsomal fraction from etiolated pea seedlings catalyzed the incorporation of serine into PS by an exchange reaction (see Section j), but no results were reported on the presence of PS synthetase in this tissue.

h) Phosphatidyl ethanolamine (PE). Two pathways are at present known for the de novo bicsynthesis of PE. The first, (pathway 1, eqns. 12-13) which is present in bacteria, involves the formation and decarboxylation of PS, while the second (pathway 2, eqns. 14-16), which is present in animals,

involves the intermediate formation of CDP-ethanolamine.

Pathway 1.

CDP-diglyceride+L-serine ⟶ phosphatidyl serine+CMP (12)

Phosphatidyl serine ⟶ phosphatidyl ethanolamine+CO_2 (13)

Kanfer and Kennedy (1963) found that PE was heavily labelled but PS only slightly labelled with ^{32}P in E.coli grown in ^{32}P-labelled orthophosphate. These authors subsequently demonstrated the presence of a PS decarboxylase in cell free extracts from E.coli and showed it to be much more active than PS synthetase (Kanfer and Kennedy, 1964). The properties of PS synthetase have been described in detail in Section g above.

PS decarboxylase has recently been isolated from E.coli and purified to homogeneity by Wickner and Kennedy (1971). They found that the pure enzyme was inhibited by hydroxylamine and 4-bromo-3-hydroxylbenzyloxyamine, which they suggested indicated the involvement of a pyridoxal cofactor. The inhibition of PS decarboxylase by hydroxylamine has been used to demonstrate the operation of pathway 1 (eqns. 12-13) in vivo by Raetz and Kennedy (1972), who showed that when E.coli cells were grown in the presence of hydroxylamine the accumulation of ^{14}C from [1-^{14}C]serine into PS increased. PS decarboxylase, together with PS synthetase, has also been detected in other bacteria, namely a Bacillus megaterium mutant (Patterson and Lennarz, 1971) and in Salmonella typhimurium (Bell et al., 1971).

PS decarboxylase has also been detected in rat liver (Borkenhagen et al., 1961) and in the protozoum Tetrahymena pyriformis (Dennis and Kennedy, 1970). However, in these tissues, the enzyme is not involved in the de novo biosynthesis of PE, since the PS itself is apparently derived from PE by an exchange reaction with free serine (see Section j), and not by PS synthetase which is absent in these tissues. Thus the decarboxylation and exchange reactions in these tissues form a metabolic cycle in which, as pointed out by Kennedy (1962b), involves the net production of free ethanolamine from free serine.

Pathway 2.

Ethanolamine+ATP $\longrightarrow$ phosphorylethanolamine+ADP (14)

Phosphorylethanolamine+CTP $\longrightarrow$ CDP-ethanolamine+Pi-Pi (15)

CDP-ethanolamine+sn-1,2-diglyceride $\longrightarrow$ phosphatidyl ethanolamine+CMP (16)

The enzyme catalyzing the first step (eqn. 14), namely ethanolaminekinase, has been detected in a wide variety of animal tissues; it is inhibited by choline and localized specifically in the cytoplasm (Sung and Johnstone, 1967; Weinhold and Rethy, 1972).

The enzymes catalyzing the second and third steps (eqns. 15 and 16) were first described by Kennedy and Weiss (1956). Their evidence for CTP: phosphorylethanolamine cytidyl transferase (eqn. 15) was the incorporation by a rat liver

20,000xg supernatant fraction in the presence of CTP, of ^{32}P from [^{32}P]phosphorylethanolamine into a cytidine containing compound tentatively identified chromatographically as CDP-ethanolamine. CDP-ethanolamine was also shown to be naturally occurring in rat liver. Their evidence for CDP-ethanolamine: diglyceride phosphorylethanolamine transferase (eqn. 16) was the incorporation of ^{32}P from synthetic [β-^{32}P]CDP-ethanolamine into PE by a rat liver homogenate in the presence of endogenous diglyceride. The phosphorylethanolaminetransferase has also been found in brain (McMurray, 1964; Ansell and Metcalfe, 1966; Ansell and Chojnacki, 1966; Porcellati et al., 1970).

Another pathway observed for ethanolamine incorporation into PE in isolated liver and brain-particulate fractions is by an exchange reaction (see Section j), in which ethanolamine apparently exchanges with the serine moiety of PS (Borkenhagen et al., 1961; Kanfer, 1972; Porcellati et al., 1971). However, since PS is itself derived from PE in an exchange reaction this pathway cannot represent de novo synthesis of PE.

The biosynthesis of PE in plants has not been investigated in detail. However, Sastry and Kates (1965) showed that growing Chlorella vulgaris could incorporate ^{32}P from [^{32}P]GP into PE, a finding which as discussed above in Section g is consistent with the biosynthesis of PE in Chlorella vulgaris proceeding via the formation and decarboxy-

lation of PS by eqns. 1-3 and 12-13. The presence of PS decarboxylase in plants is further supported by the finding by Willemot and Boll (1967) that ^{14}C from [U-^{14}C]serine, but not from [1-^{14}C]serine, was incorporated by excised tomato roots into PE. Also, Vandor and Richardson (1968) provided indirect evidence for the presence of PS decarboxylase in pea microsomal suspensions by showing the formation of $^{14}CO_2$ from [U-^{14}C] serine under conditions in which the [U-^{14}C]serine was being incorporated into PS by the serine exchange system (see below, Section j).

If, as these studies suggest, PE can be biosynthesized in plants via the intermediate formation of PS (the "bacterial pathway") it is clearly of interest to know whether the CDP-ethanolamine "animal pathway" is also operating in plants. In this connection, Kennedy and Weiss (1956) reported but presented no evidence for the presence of the cytidyltransferase in carrot root, and Mudd and Macher (1972) have presented preliminary results which support the presence of the phosphorylethanolaminetransferase in spinach leaves. Also, Willemot and Boll (1967) demonstrated a very active incorporation of [^{14}C]ethanolamine into PE by excised tomato root, which suggests the operation of the CDP-ethanolamine pathway in this tissue; however, it cannot be ruled out that this incorporation may have arisen instead by an exchange reaction.

i) Phosphatidyl choline (PC). Two major pathways are known for the biosynthesis of PC, namely the nucleotide pathway utilizing CDP-choline (eqns. 17-19), which has been found only in animals and plants, and the methylation pathway from PE (eqns. 20-22), which has been found in animals and some bacteria, but not so far in plants.

The Nucleotide Pathway.

Choline+ATP $\longrightarrow$ phosphorylcholine+ADP (17)

Phosphorylcholine+CTP $\longrightarrow$ CDP-choline+Pi-Pi (18)

CDP-choline+sn-1,2-diglyceride $\longrightarrow$ phosphatidyl choline+CMP (19).

The first demonstration of cholinekinase was by Wittenberg and Kornberg (1953) who partially purified the enzyme from brewers' yeast. Cholinekinase activity has since been detected in a variety of animal tissues (McCaman, 1962) and in Ehrlich ascites cells (Sung and Johnstone, 1967).

The involvement of phosphorylcholine in PC biosynthesis in liver was first shown by Kornberg and Pricer (1952), who reported the incorporation of phosphorylcholine into phospholipid by a liver homogenate. However, the intermediate reactions were not known until the classical work of Kennedy and co-workers (Kennedy and Weiss, 1956; Borkenhagen and Kennedy, 1957; Weiss, Smith and Kennedy, 1958), who demonstrated that the synthesis of PC in rat liver cell free extracts involved the intermediate formation of CDP-choline from phosphorylcholine and CTP (eqn. 18) followed by transfer

of phosphorylcholine to sn-1,2-diglyceride (eqn. 19). This pathway was supported by many lines of evidence, among which were the incorporation of ^{32}P from [^{32}P]phosphorylcholine into CDP-choline in the presence of CTP, and the diglyceride stimulated incorporation of ^{14}C from chemically synthesized CDP-[^{14}C]choline into PC.

CDP-choline: diglyceride phosphorylcholinetransferase has also been demonstrated in brain (Strickland et al., 1963; Chojnacki, 1964; Ansell and Chojnacki, 1966; Miller and Dawson, 1972), lung (Vereyken et al., 1972) and other animal tissues. The enzyme is also present in the yeast Saccharomyces cervisiae (Waechter et al., 1969) and in the protozoum Tetrahymena pyriformis (Smith and Law, 1970).

Concerning the specificity of the individual enzymes in the nucleotide pathway in animal tissues, the cytidyl transferase catalyzing the second step is inactive with non cytosine nucleotides (Borkenhagen and Kennedy, 1957), and the phosphorylcholine transferase catalyzing the third step is equally inactive with non cytosine containing nucleoside diphosphate choline esters (Kennedy and Weiss, 1956). In the cytidyl transferase reaction, dCTP is as effective as CTP (Kennedy et al., 1959). However, while Kennedy et al. (1959) have claimed that in the phosphorylcholine transferase reaction dCDP-choline is as effective as CDP-choline, the more detailed results of Schneider and Behki (1963) strongly suggest that CDP-choline is the preferred substrate.

Chojnacki (1964) and Ansell and Chojnacki (1966) found that in brain dispersions, ethanolamine and its mono and dimethyl derivatives and choline were incorporated from their CDP esters into the corresponding phospholipid to the same extent. However, the formation of mono and dimethyl PE almost certainly has no significance *in vivo* since these phospholipids have not been detected in brain, neither can their methylation to PC take place in this tissue (see below). It therefore appears that the phosphorylbasetransferase activity in brain may be nonspecific towards the structure of the base. It is therefore possible that in brain the synthesis of PE and PC from CDP-ethanolamine and CDP-choline respectively may be catalyzed by a single enzyme. In the original studies of Kennedy and Weiss (1956), the two phosphorylbasetransferases in rat liver were both present in the 18,000xg pellet and no attempt was made to separate them. The only evidence in support of two separate enzymes was the retention of phosphorylcholinetransferase activity, but the loss of phosphorylethanolaminetransferase activity, upon lyophilization of the 18,000xg pellet. However, in a recent publication, Dennis and Kennedy (1972) have stated that inactivation of interfering enzymes may have been responsible for these observations. Clearly, the question of whether separate enzymes are involved in the two phosphorylbasetransferase reactions (eqns. 16 and 19) in brain and liver can only be answered by purification and separation of the two enzyme activities.

There is considerable evidence that the nucleotide pathway is present in photosynthetic and non-photosynthetic plant tissue. The presence of the phosphorylcholine transferase has been conclusively demonstrated in spinach leaves by Devor and Mudd (1971). Their evidence was based on the incorporation of ^{14}C into PC both from CDP-[^{14}C]choline and sn-1,2-[^{14}C]diglyceride by a microsomal fraction; the incorporation from CDP-[^{14}C]choline was stimulated by added diglyceride, and that from [^{14}C]diglyceride by added CDP-choline. Cholinekinase has been found in rapeseed (Ramasara and Wetter, 1957) and in leaves from a wide variety of species including spinach (Tanaka et al., 1966). Morré et al. (1970) have demonstrated the presence of all three enzymes in cell free fractions from onion stem. The cytidyltransferase was similar to the corresponding enzyme in animal tissue in its specificity for cytidine containing nucleotide.

The Methylation Pathway.

This pathway consists of the step-wise methylation of PE to PC by S-adenosylmethionine (SAM), with the intermediate formation of phosphatidyl monomethylethanolamine (PE-Me) and phosphatidyl dimethylethanolamine (PE-diMe)(eqns. 20-22). S-adenosylhomocysteine (SAHC) is the non-lipid product. Thus,

$$\text{PE} + \text{SAM} \longrightarrow \text{PE-Me} + \text{SAHC} \qquad (20)$$

$$\text{PE-Me} + \text{SAM} \longrightarrow \text{PE-diMe} + \text{SAHC} \qquad (21)$$

$$\text{PE-diMe} + \text{SAM} \longrightarrow \text{PC} + \text{SAHC} \qquad (22)$$

Very early work on choline metabolism in liver indicated that choline might be biosynthesized by methylation of ethanolamine by methionine (du Vigneud et al., 1941; Pilgeram et al., 1953). The involvement of phospholipid intermediates in the pathway was suggested by Crowder and Artom (1952) who showed the incorporation of dimethylethanolamine into phospholipid prior to its methylation to choline. Another valuable early contribution was by Stekol et al., (1955 and 1958) who were able to demonstrate the involvement of SAM in the third step (eqn. 22).

However, it was not until the early 1960s that more concrete evidence for the methylation pathway was forthcoming. The involvement of PE-Me and PE-diMe as intermediates in the pathway was strongly suggested by the work of Bremer and Greenberg (1961) and Gibson, Wilson and Udenfriend (1961), who showed that ^{14}C from [Me-^{14}C]SAM could be incorporated by rat liver microsomes into PE-Me and PE-diMe as well as into PC. Furthermore, Bremer and Greenberg (1961) and Cooksey and Greenberg (1961) found that the ^{14}C-incorporation from [Me-^{14}C]SAM into PC was stimulated by exogenous PE-Me and PE-diMe. However, a more direct demonstration of PE-Me and PE-diMe as intermediates was given by Artom and Lofland (1960) and Artom (1964), who demonstrated the direct methylation of ^{14}C-labelled PE-Me and PE-diMe to PC by unlabelled SAM.

The rat liver microsomal methylating enzyme system has been found to be incapable of methylating exogenous PE

(Bremer and Greenberg, 1961; Cooksey and Greenberg, 1961; Gibson et al., 1961). This finding could of course be explained by there being saturating levels of endogenous PE in the microsomal preparations used. However, a dispersed rat liver microsomal preparation freed of endogenous lipid was found to methylate exogenous PE-Me and PE-diMe, but not PE (Rehbinder and Greenberg, 1965). In fact, the only evidence in support of the first step was given by Gibson et al. (1961) who demonstrated that ^{14}C incorporation from microsomal bound phosphatidyl[^{14}C]ethanolamine into PC was stimulated five times by the addition of unlabelled SAM. However, it is not possible to determine from their data the extent of conversion of PE to PC. Thus, concrete evidence for the existence of the first step, namely the methylation of PE to PE-Me (eqn. 20), is lacking in rat liver.

The methylation pathway has since been studied in a wide variety of living organisms. There is good evidence, based mainly on the formation of ^{14}C-labelled PE-Me, PE-diMe and PC from [Me-^{14}C]SAM in cell free preparations containing endogenous PE, that the pathway functions in the bread mold Neurospora crassa (Scarborough and Nyc, 1965), the yeast Saccharomyces cervisiae (Waechter et al., 1969; Steiner and Lester, 1969), and the protozoa Ochromonas malhamenis and Tetrahymena pyriformis (Lust and Daniel, 1965; Smith and Law, 1970). The pathway is not present in bacteria with the exception of three species of Agrobacterium (Kaneshiro

and Law, 1964; Goldfine and Ellis, 1964), Hyphomicrobium vulgare (Goldfine and Hagen, 1968), and in the photosynthetic bacterium Rhodopseudomonas spheroides (Gorchein et al., 1968). The bacteria Clostridium butyricum and Proteus vulgaris, which do not contain PC, are of novel interest since PE-Me is a major phospholipid component and can be labelled with ^{14}C when the cells are grown in [Me-^{14}C]methionine (Goldfine, 1962; Goldfine and Ellis, 1964).

Several of the above studies have contributed significantly to our understanding of the methylation pathway. Thus, Kaneshiro and Law (1964) in their studies with Agrobacterium tumefacians, showed that ^{14}C incorporation into PE-Me, PE-diMe and PC from [^{14}C]SAM by microsomal and cytoplasmic fractions was almost totally dependent upon exogenous PE. They also isolated and partially purified an enzyme from the cytoplasm with PE:SAM methyltransferase activity. This was the first conclusive demonstration ever of the first step (eqn. 20) in the methylation pathway. In their studies with Neurosporra, Scarborough and Nyc (1965) found that microsomes from a mutant of this organism were deficient in PE:SAM methyltransferase activity, while microsomes from a second mutant, where the lesion was at a single genetic locus, could not catalyze the methylation of either PE-Me or PE-diMe. This finding was important in showing that in Neurosporra a single enzyme catalyzes the first step, while a second enzyme catalyzes both the second and third steps. The methylation pathway in other

organisms most likely also involves separate enzymes for the first and subsequent methylation. The finding of PE-Me, but no PC, in Cl.butyricum and P.vulgaris is consistent with this idea.

The evidence available at present, although indirect, suggests that the methylation pathway also exists in photosynthetic and non-photosynthetic plant tissues. Thus, Sastry and Kates (1965) showed that in Chlorella vulgaris grown for 12 h in [^{32}P]glycerophosphate, the ^{32}P-accumulation began to decrease in PE but increase in PC. Also, Willemot and Boll (1967) found that ^{14}C was incorporated by excised tomato roots from [^{14}C]ethanolamine and [U-^{14}C]serine not only into PE, but also into PC. More direct evidence is available for the occurrence of the methylation pathway in photosynthetic bacteria. Gorchein et al. (1968) demonstrated the incorporation of ^{14}C from [Me-^{14}C]methionine by growing cells of Rhodopseudomonas spheroides into the base moieties of PE-Me, PE-diMe and PC; they also demonstrated the incorporation of ^{14}C from [Me-^{14}C]SAM by a particulate fraction of this organism into the base moieties of PE-Me and PC. Tipton and Swords (1966) claimed to have provided evidence for the presence of the methylation pathway in Euglina gracillus, but the results were equivocable due to the lack of proper controls.

An interesting problem concerning the biosynthesis of PC is the relative importance of the methylation and nucleotide pathways. With some tissues, this problem does not exist

because only one pathway functions. Thus, in A.tumerfacians, only the methylation pathway is present (Kaneshiro and Law, 1964; Sherr and Law, 1965), and this is also likely true for other bacteria containing PC. In contrast, in animal brain, the nucleotide pathway exists but not the methylation pathway (Chojnacki et al., 1964; Marshall et al., 1965; Ansell and Spanner, 1965). In animal tissues having both pathways, e.g. kidney, heart, lung, liver and intestine, the methylation pathway is a very minor route in all tissues except liver. (Bremer and Greenberg, 196I; Bjørnstad and Bremer, 1966; Vereyken et al., 1972). The results of in vivo experiments by Bjørnstad and Bremer (1966) indicate that one-fifth of liver PC is synthesized by the methylation pathway, while Wise and Elwyn (1965) and Tinoco et al., (1970) have calculated that the amount of choline formed by this route in rat liver may equal the amount of choline in the diet. However, the relative importance of the methylation pathway appears to depend upon the amount of choline in the diet. Thus, in rats fed a choline deficient diet PC synthesis by the methylation pathway increased, apparently as a result of an increase in the amount of the methyltransferase enzyme(s) (Thompson et al., 1969; Fallon et al., 1969; Glen and Austin, 1971). Steiner and Lester (1969) have observed a similar effect of choline on the methylation pathway in the yeast S.cervisiae. It was found that the microsomal synthesis of PC by the methylation pathway was much less in cells grown in the presence of choline,

apparently as a result of a decrease in the amount of methyltransferase enzyme(s). It is thus possible that in rat liver and yeast externally supplied choline may repress synthesis of the methyltransferase enzyme(s).

j) Exchange reactions. The main principle of the Kennedy scheme of phospholipid biosynthesis is that the de novo generation of the monophosphate ester linkage in phospholipids involves a cytidine containing coenzyme as a "high energy" intermediate. Interest was therefore aroused by the finding by Hübscher et al. in 1959 that free L-serine could be incorporated into lipid by a rat liver cell free homogenate in a Ca^{2+} stimulated reaction which did not require nucleotide intermediates, but apparently involved exchange of free serine with the base moieties of endogenous phospholipids. This finding was confirmed by Borkenhagen et al., (1961) who identified the lipid formed from L-serine as PS. These workers also showed incorporation of ethanolamine into PE in the presence of Ca^{2+}, and presented evidence which suggested that the incorporation of serine and ethanolamine into lipid was catalyzed by a single enzyme. They also proposed, though presented no concrete evidence for, that these reactions involved an interconversion of PE and PS:

phosphatidyl ethanolamine+serine $\rightleftharpoons$ phosphatidyl serine + ethanolamine (23)

Borkenhagen et al. (1961) reported that an analogous "exchange" reaction involving free choline did not occur. However, Dils and Hübscher (1961) have conclusively demonstrated such a reaction in vitro and this has been confirmed by in vivo studies, though the relative importance of this pathway in PC synthesis in vivo is still a matter of controversy, (Treble et al., 1970; Sundler et al., 1972).

A Ca^{2+} stimulated incorporation of ethanolamine, serine and choline has also been detected in particulate fractions from brain tissue (Porcellati et al., 1971; Kanfer, 1972) and in pea seedlings (Vandor and Richardson, 1968).

The mechanism of these exchange reactions is still a matter of speculation. Since cytidine coenzymes are not required, the Kennedy principle predicts that these reactions do not involve a net de novo formation of monophosphate diester linkage. Hübscher et al (1959) and Dils and Hübscher (1961) have suggested that the reactions simply reflect the reversibility of phospholipase D hydrolysis:

$$\text{Phosphatidyl-O-R}+H_2O \xrightleftharpoons{Ca^{2+}} \text{phosphatidic acid} + \text{R-OH} \qquad (24)$$

where ROH can be serine, ethanolamine or choline. However, proof of this mechanism must await the conclusive demonstration of phospholipase D in animal tissues. So far, the only evidence in favour of phospholipase D in animal tissues is the finding (Dils and Hübscher, 1961) of a small release of PA when rat liver microsomes were incubated with Ca^{2+}, and

the observed Ca^{2+} dependent release of ^{14}C-labelled serine, ethanolamine and choline on incubation of rat brain microsomes containing ^{14}C-labelled phospholipids (Kanfer, 1972). However, in contrast, Bjørnstad (1966) found no phospholipase D activity in liver microsomes towards PE, though phospholipase D activity towards PC was not ruled out. Thus, the question of the presence of phospholipase D in animal microsomes requires further investigation.

A better case for the phospholipase D hypothesis might appear to be made for the exchange enzyme system in pea seed microsomes, since phospholipase D is known to be widely distributed in plant tissues including leaves and seeds (Kates, 1954; Davidson and Long, 1958; Quarles and Dawson, 1969). Indeed, Yang et al. (1967) have discovered that a partially purified phospholipase D from cabbage can catalyze in the presence of Ca^{2+} not only the hydrolysis of PC but also the exchange of the choline moiety with other alcohol moieties such as ethanol, ethanolamine, glycerol and L-serine. This exchange activity has been detected in phospholipase D preparations from leaves of cabbage, spinach, radish and other species by several independent investigators (Bartels and Van Deenen, 1966; Douce et al., 1966; Dawson, 1967a).

To account for both the hydrolyzing and exchange activity of phospholipase D, Yang et al. (1967) have postulated the following mechanism involving the formation of a common phosphatidyl-enzyme complex:

Phosphatidyl choline+enzyme $\rightleftharpoons$ phosphatidyl-enzyme+choline (25)

Phosphatidyl-enzyme + H_2O $\longrightarrow$ phosphatidic acid+enzyme (26)

Phosphatidyl-enzyme + ROH $\rightleftharpoons$ phosphatidyl-O-R+enzyme (27)

where ROH is a free alcohol.

However, it is very unlikely that phospholipase D catalyzes the above exchange reactions *in vivo*. Concerning the phospholipase D catalyzed exchange reaction between the choline of PC and free glycerol, this reaction cannot possibly function *in vivo* for the reasons discussed above (see Section e). This conclusion is further supported by the striking differences in the properties of the exchange system in pea microsomes (Vandor and Richardson, 1968) and that catalyzed by isolated leaf phospholipase D (Yang *et al*., 1967). Thus, the pH optimum of the former is 8.5, while the pH optimum of the latter is equal to that of its hydrolyzing activity, i.e. 5.5-6.0. Also the former system was inactive towards ethanol and glycerol, while the latter was very active with these substrates.

2. Subcellular Localization of Biosynthesizing Enzymes

There is general agreement that in *E.coli* the enzymes involved in the biosynthesis of PA (eqns. 1-2), CDP-diglyceride (eqn. 3), PG (eqns. 7-8), and cardiolipin (eqn. 11) are localized in the cell envelope (Kanfer and Kennedy, 1964; Chang and Kennedy, 1967a and b; Carter, 1968; Kito and Pizer, 1969; Ailhaud and Vagelos, 1967; Van den Bosch and Vagelos, 1970; Hirschberg and Kennedy, 1972). Furthermore, White *et al*

(1971) and Bell et al. (1971) have demonstrated that these enzymes are localized in the "inner membrane of the cell envelope" (plasma membrane).

Concerning the subcellular localization of the enzymes involved in PE synthesis, namely PS synthetase and PS decarboxylase (see eqns. 12-13), there clearly must be an efficient coupling of the activity of these enzymes in vivo since PS does not accumulate in E.coli. One might expect therefore that the two enzymes would be located at the same subcellular site. While there is strong evidence suggesting the localization of PS decarboxylase in the plasma membrane (White et al., 1971; Bell et al., 1971; Raetz and Kennedy, 1972), there are conflicting reports concerning the subcellular localization of PS synthetase. Thus, Raetz and Kennedy, (1972) have reported that the enzyme was localized in the membrane free supernatant fraction after disruption of E.coli cells either by vigorous sonication or by more gentle enzymatic lysis methods. Furthermore, the enzyme was found to be predominantly associated with the ribosomes. These findings are consistent with the report by Bell et al. (1971) that in the E.coli cell envelope the specific activity of PS synthetase was very much lower than that of the other phospholipid biosynthesizing enzymes. However, in contrast, Cronan and Vagelos (1972) have claimed that a considerable portion of the total PS decarboxylase activity in E.coli is present in the washed envelope fraction when the cells are gently lysed,

and White et al. (1971) have reported that in the cell envelope the specific activity of the enzyme is comparable to that of the other phospholipid biosynthesizing enzymes. Also, Patterson and Lennarz (1971) found that all of the activity in a strain of Bacillus megaterium was localized in the cell envelope. Thus, the question of the subcellular localization of PS synthetase in bacteria requires further clarification.

Present knowledge concerning the subcellular localization of phospholipid biosynthesizing enzymes in animals and plants is summarized in Table 2. Definite conclusions concerning the subcellular localization of the sn-glycerol-3-phosphate:acyl CoA acyltransferase(s) in animal liver cannot be made at present because of the contradictory data available. Thus, Eibl et al. (1969) and Davidson and Stanacev (1972) have reported that the enzyme is localized in the microsomal fraction only, while Zborowski and Wojtczak (1969), Stoffel and Schiefer (1968), Shepard and Hübscher (1969) and Sarzala et al. (1970) have claimed the localization of this enzyme in mitochondria. In spinach leaves the enzyme has been reported localized in the microsomal fraction (Cheniae, 1965). The reason for the contradictory data may be the difference in the methods of assay used, and this has been critically discussed by Davidson and Stanacev (1972). As discussed above (Section b), PA can also be biosynthesized in rat liver by the dihydroxyacetone pathway, but the subcellular localization of this enzyme system is at present unknown.

TABLE 2. SUB-CELLULAR LOCALIZATION OF PHOSPHOLIPID BIOSYNTHETIC ENZYMES IN ANIMALS AND PLANTS

Enzyme and Reaction Catalyzed	Tissue	Localization	Reference
Acyl CoA:glycerophosphate acyltransferase (eqns. 1-2)	rat and guinea pig liver	mitochondria? microsomal?	1,2,3,4 5,6
	spinach leaves	microsomal?	7
CTP:phosphatidic acid cytidyltransferase (eqn. 3)	rat brain.	mitochondria	8
	rat liver and cauliflower inflorescence	microsomal; also in mitochondria (inner membrane)	9, 10, 11
	spinach leaves	microsomal (mitochondria?)	10
CDP-dig:inositol phosphatidyltransferase (eqn. 4)	guinea pig pancreas and brain, rat brain	microsomal	8,12,13
	cauliflower infloresence	mitochondria?	14
CDP-dig:glycerophosphate phosphatidyltransferase and	chicken liver, rat brain, beef and rat heart	mitochondria	15,16,17,18
PGP phosphatase (eqns. 7-8)	rat liver	mitochondria (inner membrane)	9
CDP-dig:PG phosphatidyltransferase ("Cardiolipin synthetase") (eqn. 10)	guinea pig liver	mitochondria	19
	rat liver	mitochondria (inner membrane)	9,20
CTP:phosphoryl-ethanolamine cytidyltransferase (eqn. 15)	rat liver	?	21

Table 2 continued

Enzyme and Reaction Catalyzed	Tissue	Localization	Reference
CTP:phosphorylcholine cytidyltransferase (eqn. 18)	rat liver and onion stem	cytoplasm? microsomal? golgi apparatus	22,23,24, 25
CDP-ethanolamine: diglyceride phosphoryl-ethanolaminetrans-ferase (eqn. 16)	rat liver and brain	microsomal	26,27,28, 29
CDP-choline:diglyceride phosphoryl-cholinetransferase (eqn. 19)	rat liver and brain	microsomal	4,22,23,26, 27,30
	spinach leaves	microsomal	31
PS decarboxylase (eqn. 13)	rat liver	mitochondria	32
SAM methyltransferases (eqns. 20-22)	rat liver	microsomal (mitochondria?)	33
"PE:serine phosphatidyl transferase"	rat liver	microsomal	32
	rat and chicken brain	microsomal	34,35
	pea seedlings	microsomal (mitochondria?)	36

1. Stoffel and Schiefer (1968)
2. Zborowski and Wojtczak (1969)
3. Shepard and Hübscher (1969)
4. Sarzala et al., (1970)
5. Eibl et al., (1969)
6. Davidson and Stanacev (1972)
7. Cheniae (1965)
8. Cotman et al., (1971)
9. Hostetler and Van den Bosch (1972)
10. Bahl et al., (1970)
11. Douce et al., (1972)
12. Prottery and Hawthorne (1967)
13. Benjamins and Agranoff (1969)
14. Sumida and Mudd (1970b)
15. Kiyasu et al., (1963)
16. Stanacev et al., (1969)
17. Davidson and Stanacev (1970)
18. Stuhne-Sekalec and Stanacev (1970)
19. Davidson and Stanacev (1971)
20. Hostetler et al., (1971)
21. Kennedy and Weiss (1956)
22. Wilgram and Kennedy (1963)
23. Schneider (1963)
24. Morré et al., (1968)
25. Morré et al., (1970)
26. McMurray and Dawson (1969)
27. Jungalwala and Dawson (1970)
28. Porcellati et al., (1970)
29. Ansell and Metcalfe (1968)
30. Miller and Dawson (1972)
31. Devor and Mudd (1971)
32. Dennis and Kennedy (1972)
33. Bremer and Greenberg (1960)
34. Porcellati et al., (1971)
35. Kanfer (1972)
36. Vandor and Richardson (1968)

The CTP:PA cytidyltransferase in animal liver is localized primarily in the microsomal fraction, but in rat liver the enzyme has also been found to a lesser extent in mitochondria, in which it is specifically localized in the inner membrane (Table 2). However, in brain the enzyme is localized exclusively in mitochondria. Concerning the localization of this enzyme in plant tissue, Bahl et al. (1970) showed that the microsomal fraction had the highest specific activity of subcellular fractions isolated from spinach and maize leaves and cauliflower inflorescence. No activity was found in spinach and maize chloroplasts. However, they also presented evidence strongly suggesting that a separate but relatively much less active mitochondrial enzyme system existed in cauliflower inflorescence. This latter finding is supported by the later work of Douce et al. (1972) who demonstrated the presence of the enzyme in the inner, but not the outer, mitochondrial membrane from etiolated bean hypocotyles. It therefore appears that non-photosynthetic plant tissues resemble animal tissues in possessing both a microsomal and a mitochondrial enzyme system.

The CDP-diglyceride: inositol phosphatidyltransferase of animal tissue is localized in the microsomal fraction (Table 2). The enzyme was found to be absent from purified rat brain mitochondria (Cotman, 1971). The localization of the enzyme in non-photosynthetic tissue is not known for certain. However, Sumida and Mudd (1970b) found that 70% of

the total enzyme activity from cell free homogenates of cauliflower inflorescence was localized in an 18,000xg crude mitochondrial pellet, which indicated a mitochonridal location in this tissue.

The CDP-diglyceride: GP phosphatidyltransferase of animal tissues is localized in the mitochondria (Table 2). However, in rat liver the enzyme is present to a lesser extent also in the plasma membrane (Victoria et al., 1971). Hostetler and Van den Bosch (1972) have shown using purified subcellular fractions from rat liver that in this tissue the mitochondrial enzyme is localized in the inner membrane. The results of Stuhne-Sekalec and Stanacev (1970) indicate that this is also true in beef heart. The subcellular localization of CDP-diglyceride:GP phosphatidyl transferase in non-photosynthetic tissue is not known. Douce (1968) and Douce and Dupont (1969) detected the enzyme in a crude mitochondrial fraction from cauliflower inflorescence, but the presence of the enzyme in other cell fractions was not investigated.

The CDP-diglyceride: phosphatidyl glycerol phosphatidyltransferase ("cardiolipin synthetase") in liver tissue is localized in the mitochondria, and in rat liver the enzyme has been shown to be located specifically in the inner membrane (Table 2).

Concerning the subcellular localization of the enzymes in the nucleotide pathways for PE and PC biosynthesis in animal tissue, little is known concerning the localization

of the respective cytidyltransferase activities. The association of CTP: phosphorylcholine cytidyltransferase with certain cell fractions is apparently dependent on the osmotic strength of the medium used in homogenizing the liver tissue. Thus, Schneider (1963) has reported that the enzyme can be found associated with either the cytoplasm or a total particulate mitochondrial plus microsomal fraction depending on whether the medium is hypertonic or hypotonic, respectively. Also, Wilgram and Kennedy (1963) reported an equal distribution of the enzyme between the cytoplasm and the microsomal fraction when isotonic medium was used. It is therefore not clear whether the enzyme is microsomal or cytoplasmic. Furthermore, our understanding of the localization of this enzyme in animal tissue is further complicated by the finding by Morré et al. (1968) that the enzyme is also present in the Golgi apparatus. Morré et al. (1970) have also studied the subcellular distribution of the phosphorylcholine cytidyltransferase in onion stem. The enzyme was apparently present in all cell fractions but with the highest specific activity in purified Golgi apparatus membranes.

Nothing definite is known about the localization of CTP: phosphorylethanolamine cytidyltransferase in animal tissue. However, in the original studies of Kennedy and Weiss (1956) the enzyme was detected along with CTP: phosphorylcholine cytidyltransferase activity in a 20,000xg supernatant fraction from rat liver, which suggests that the enzyme may

have the same localization as the phosphorylcholine cytidyltransferase.

The phosphorylethanolamine and phosphorylcholine transferases for the biosynthesis of PE and PC respectively in animal tissues are localized in the microsomal fraction (Table 2). However, whether a separate but minor phosphorylcholinetransferase also exists in animal mitochondria has been the subject of recent controversy. The enzyme has been reported to be present in isolated mitochondria from rat brain and bovine heart muscle (McCaman and Cook, 1966) and rat liver (Stoffel and Schiefer, 1968). Also, choline has been reported to be incorporated into the phospholipids of isolated rat liver mitochondria in a CTP stimulated reaction (Bygrave and Kaiser, 1968). However, more recent work strongly suggests that these observations arose from microsomal contamination of the isolated mitochondria, and that purified mitochondria from liver and brain do not contain the phosphorylcholinetransferase (McMurray and Dawson, 1969; Sarzala et al., 1970; Miller and Dawson, 1972). Concerning the subcellular localization of the phosphorylcholinetransferase in non-animal tissues, the enzyme in spinach leaves appears to be localized in the microsomal fraction (Table 2), while that in the protozoum Tetrehymena pyriformis is unique in being localized in the mitochondria (Smith and Law, 1970).

The methyltransferases involved in the methylation of PE to PC in rat liver have been reported by Bremer and

Greenberg (1960) to be localized in the microsomal fraction (Table 2). However, these workers also observed considerable methyltransferase activity in the crude mitochondrial fraction, and it was not determined whether this was due to a separate mitochondrial enzyme system or to microsomal contamination. In the studies by McMurray and Dawson (1969) and Sarzala et al. (1970) described above on the question of whether purified rat liver mitochondria can synthesize PC by the nucleotide pathway, these workers did not unfortunately test their pure mitochondrial preparations for the presence of the methylation pathway. Thus whether rat liver mitochondria can synthesize PC by the methylation pathway is at present unknown.

The serine-ethanolamine exchange enzyme ("PE:serine phosphatidyltransferase") of animal liver, brain and pea seedlings is localized in the microsomal fraction (Table 2). The choline exchange enzyme system in liver (see Section j) is also microsomal (Dils and Hübscher, 1961). Vandor and Richardson (1968) have reported exchange enzyme activity in a pea seedling crude mitochondrial fraction and which was half that of the microsomal fraction on a specific activity basis. However, whether this activity arose from a separate mitochondrial system or from microsomal contamination was not determined.

IV. Aims of the Research

The prime aim of the research was to elucidate the pathways in the biosynthesis of phospholipids in spinach leaves. PG is a major phospholipid in this tissue and it was of interest to determine whether its pathway of biosynthesis is the same as that operating in animals and bacteria involving the intermediate formation of PGP from CDP-diglyceride and sn-glycerol-3-phosphate. This pathway was investigated by following the incorporation of ^{14}C and ^{32}P from [^{14}C+^{32}P]GP, and of ^{14}C from [^{14}C]GP, into PGP and PG by cell free preparations in the presence of CDP-diglyceride. The biosynthesis of PI from CDP-diglyceride and [^{14}C]inositol was also investigated.

It was also of interest to study the biosynthetic pathways for nitrogenous phospholipids. As discussed above, there are two known pathways for the de novo biosynthesis of PE in Nature. One pathway, which is present in animals, involves the intermediate formation of CDP-ethanolamine from ethanolamine, while the second, which is present in bacteria, involves the intermediate formation of PS from CDP-diglyceride and serine followed by decarboxylation of PS to PE. It was of interest to determine which of these pathways functions in the de novo biosynthesis of PE in spinach leaves. This question was investigated in vivo by determining the incorporation of ^{14}C from ^{14}C-labelled serine and ethanolamine into PS and PE by whole leaves and leaf slices. The CDP-ethanolamine

"animal" pathway was tested *in vitro* using cell free preparations and ^{14}C-labelled ethanolamine, phosphorylethanolamine and CDP-ethanolamine as substrates, and the PS "bacterial" pathway by the formation of [^{14}C]PS from [^{14}C]serine and CDP-diglyceride and its subsequent decarboxylation. *In vitro* studies were also carried out concerning the possibility of exchange reactions for PS and PE synthesis.

With regard to PC biosynthesis, it has already been established that the nucleotide "animal" pathway operates (Tonaka *et al.*, 1966; Devor and Mudd, 1971). It was of interest to confirm this pathway, and to determine whether the alternative "bacterial" pathway for PC biosynthesis, involving the stepwise methylation of PE by SAM, also operates. The methylation pathway was studied *in vivo* by following the incorporation of ^{14}C from ^{14}C-labelled ethanolamine and methionine into PC and intermediates of the pathway, and *in vitro* by testing PE, PE-Me and PE-diMe as methyl-acceptors for SAM. Also studied were the exchange reaction for PC synthesis, and the involvement of SAM in the synthesis of non-phospholipids.

In spinach leaves, PG is the major phospholipid of the chloroplasts and PC and PE are the major phospholipids of the mitochondria. It was of interest therefore to investigate whether these lipids can be synthesized in situ by the organelle in which they occur. This problem was approached by determining the subcellular distribution of phospholipid

biosynthesizing enzymes among chloroplasts, mitochondrial, microsomal and cytoplasmic cell fractions.

B. MATERIALS AND METHODS

I. Materials

1. Enzymes and Co-factors were obtained from: Phospholipase D (Cabbage, B grade, Calbiochem); Phospholipase C (C.Welchii, B grade, Calbiochem); Glycerokinase (C.F. Boehringer & Sohns, Germany); Cytochrome C (A grade, Sigma Chemical Co.); NAD (Calbiochem), CTP and ATP disodium salts (P-L Biochemicals, Inc.).

2. Unlabelled substrates

a) Commercially available. The following compounds were obtained from: dipalmitoyl PE-Me and PE-diMe (Synthetic A grade, Calbiochem); phosphatidyl serine and phosphatidyl inositol (Pierce Chemical Co.); CMP-morpholidate (B grade, Calbiochem); myo-inositol and glycerol (Anachemia Chemicals Ltd.); CDP-diglyceride (Koch-Light Laboratories Ltd.). CDP-diglyceride was freed from breakdown products (phosphatidic acid and CMP) by preparative TLC as described in Methods II.1.b., to give a purified material containing 5.40 % P (calcd., 5.90% P; based on average mol. wt. of 262 for fatty acids). Its fatty acid composition, determined as described in Methods III.3, was 56% palmitate, 1% palmitoleate, 4% stearate and 37% oleate.

b) Preparation

i) CDP-diglyceride

Spinach leaf phosphatidyl choline (PC) (isolated

by procedure of Sastry and Kates, 1964) was hydrolyzed to phosphatidic acid (PA) by Phospholipase D, essentially according to the method of Kates and Sastry (1969), in a reaction mixture (total volume 15 ml) containing PC (49 mg), 80 mM Na acetate buffer (pH 5.6), 16 mM $CaCl_2$, diethylether (3 ml) and phospholipase D (60 mg). After 3 h incubation at 30°C, the ether was removed in a stream of N_2 and lipids were extracted as described in Methods VI.1. The final chloroform solution was concentrated down under N_2 to approximately 0.4 ml and diluted with 2 ml of methanol. After 3 h at 4°C, the precipitate of PA calcium salt was isolated by centrifugation and washed with 2 ml of cold methanol. The PA was converted to the free acid form as follows: To a solution of the calcium salt in chloroform (4 ml) was added methanol (4 ml) and 0.5 N HCl (3.6 ml). The chloroform phase was diluted with benzene and concentrated to dryness under N_2. The residual PA was dried in a desiccator over KOH pellets; yield 30.4 mg, 60%. It was judged pure by TLC in solvents (A) and (C) (Methods II.1.a.) (R_f, 0.9 and 0.05 respectively).

PA was converted to CDP-diglyceride essentially according to the procedure of Kates et al. (1971): a reaction mixture containing PA (30.4 mg) and CMP-morpholydate (50 mg) in dry pyridine (15 ml) was stirred at room temperature for 65 h; the pyridine was evaporated under reduced pressure and the residue dried in vacuo in a desiccator over concentrated sulphuric acid. The residue was dissolved in 20 ml of

chloroform-methanol (1:1) and diluted with 9 ml of 0.5N HCl; the mixture was centrifuged, the chloroform phase was withdrawn, and the aqueous phase washed with 3 ml of chloroform. The combined chloroform phases, containing CDP-diglyceride and unreacted PA, were concentrated to a small volume under N_2 and the CDP-diglyceride isolated by preparative TLC in solvent (B) (Methods II.1.a). The band of CDP-diglyceride (R_f, 0.2; R_f of PA, 0.9) was visualized by spraying the plate with water, and the CDP-diglyceride was extracted from the band by the Bligh and Dyer "acidic" procedure (see Methods VI.2.b). The final solution of CDP-diglyceride in chloroform was concentrated to dryness under N_2 and the residue was dried over KOH pellets in vacuo and finally dissolved to a known concentration in chloroform-methanol (2:1, by vol.) and stored at -15°C. The product, CDP-diglyceride diammonium salt (yield, 15 mg; 33%) migrated as one spot on TLC in solvents (B) and (E) (Methods II.1.a) with R_f, 0.20 and 0.48, respectively, and contained 5.70% P (calcd, 5.90%; based on average mol. wt. of 262 for fatty acids). Its fatty acid composition, determined as described in Methods III.3, was 31% palmitate, 13% oleate, 25% linoleate and 30% linolenate.

ii) sn-1,2-Diglyceride

A modification of the method described by MacFarlane and Knight (1941) and Zamecnick et al. (1947) was employed: PC (150 mg, isolated from eggs as described in Methods I.2.b.iv) was dispersed in 4 ml of 0.1M potassium

borate (pH 7.2) by sonication as described in Experimental II.3.b.ii, and 0.04 ml of 1M $CaCl_2$ and 3 mg of phospholipase C added. After incubation for 2-1/2 h at 37°C, the reaction was stopped and lipids extracted as described in Methods VI.1. The diglyceride was freed from unreacted PC by preparative TLC in solvent H (Methods II.1.a) (R_f of diglyceride and PC, 0.25 and 0.0, respectively). Yield of diglyceride, 74 mg (61%).

iii) Phosphatidyl ethanolamine

From Spinach Leaves

40 g of fresh leaves were torn into small pieces and added to 120 ml of hot isopropanol. The mixture was homogenized in a Waring blender for 1-2 min and filtered. The filter residue was washed with 80 ml of hot isopropanol, and rehomogenized with 80 ml of chloroform-isopropanol (1:1 by vol.); the mixture was filtered and the residue washed with 40 ml of chloroform-isopropanol (1:1 by vol.). The combined filtrates were concentrated to dryness in vacuo and the residual lipids (650 mg) in chloroform solution were applied to a column of silicic acid (50 mg, 1.8 x 38 cm). Elution was carried out with the following solvents: 1) chloroform, 750 ml (pigments and neutral lipids), 2) chloroform-methanol (5:1, by vol.), 25 ml (MGD) followed by 3) chloroform-methanol (5:1), 500 ml (PE, PG and DGD). Following removal of PG by preparative TLC in solvent system A (Methods II.1.a and b) (R_f PG, 0.6; R_f PE+DGD, 0.72), the PE and DGD were separated

on a column of silicic acid (3 g, 1.0 x 7.5 cm) as follows: DGD was eluted with 20 ml of acetone and PE eluted with 200 ml of chloroform-methanol (5:1 by vol.). The eluate containing PE was concentrated to dryness in vacuo and the residue (8.7 mg) dissolved in chloroform. The PE migrated as a single spot with standard egg yolk PE on TLC in solvents A (R_f, 0.80) and D (R_f, 0.60); it gave a positive reaction with the ninhydrin and phosphate stains (Methods II.1.a).

From Egg Yolk

This phospholipid was isolated according to the procedure of Lea, Rhodes and Stoll (1955).

iv) Phosphatidyl choline

This phospholipid was isolated by the procedure of Lea, Rhodes and Stoll (1955) from egg yolk. Its fatty acid composition was 36% palmitate, 15% stearate, 37% linoleate and 12% linolenate.

v) sn-Glycerol-3-phosphate

This compound (as barium salt) was prepared by the method of Baer and Fischer (1939).

II. Chromatography

1. Thin Layer Chromatography (TLC)

a) Analytical TLC was carried out on 20 x 20 cm plates coated with silica gel H (E. Merck AG) (0.25 mm). Samples were spotted under nitrogen on plates previously

activated for 2 h at 120°C. The solvent systems used for the development of chromatograms were as follows:

A Chloroform-methanol-acetic acid-water (65:25:8:4 by vol.).

B Chloroform-90% acetic acid-methanol (30:20:4 by vol.).

C Chloroform-methanol-28% ammonia (65:25:5 by vol.).

D Chloroform-methanol-water (65:25:4 by vol.).

E Diisobutyl ketone-acetic acid-water (40:25:5 by vol.).

F Diisopropyl ether-acetic acid (96:4 by vol.).

G Petroleum ether-diethylether-acetic acid (90:10:1 by vol.).

H Heptane-isopropylether-acetic acid (60:40:4 by vol.).

Following development of the chromatogram, spots were located by spraying the plate with the following general reagents: a) iodine, b) 40% sulphuric acid in ethanol followed by charring of plate, or by the following specific reagents, c) acidified ammonium molybdate which specifically stains phosphate containing lipids (Vaskovsky and Kostetsky, 1968 and Dittmer and Lester, 1964) and d) 0.25% ninhydrin in acetone-lutidine (9:1 by vol.), which specifically stains amino containing lipids (Marinetti, 1962 and 1964). Radioactive spots were located by autoradiography as described in Methods V.3.

b) Preparative TLC Lipid samples (40 mg/plate) were streaked on activated 20 x 20 cm silica gel H plates (1 mm thickness) using a "Pelick Streaker" (Applied Science Laboratories, State College, Pa.). Solvent systems used for development of the chromatogram were as described above.

Radioactive bands were located by autoradiography, and non-radioactive bands by spraying the plate with Rhodamine 6G solution (0.01%) followed by viewing the plate under U.V. light (366 nm). Lipids were eluted from the bands as described in Methods VI.2.

2. Paper Chromatography

a) Lipids

Analytical and preparative chromatography was carried out on silicic acid impregnated Whatman 3 MM paper, prepared as described by Kates (1967), with ascending development in diisobutyl ketone-acetic acid-water (8:5:1 by vol.). Lipids were detected by staining the chromatogram with the following reagents:

Rhodamine 6G general stain (see Marinetti 1962, 1964). The chromatogram was immersed in a 0.0012% aqueous solution of Rhodamine 6G for 5 min and immediately viewed under ultraviolet light (366 nm). Acidic lipids fluoresced blue or purple, and neutral lipids as orange or yellow.

Periodate-Schiff reagent, specific for lipids containing vicinal diol groups; the procedure used was an adaptation to silicic acid impregnated paper (Sastry and Kates, 1964) of the method devised by Baddiley et al.(1956).

Ninhydrin reagent, specific for lipids containing amino groups; chromatograms were sprayed with 0.25% ninhydrin in acetone-lutidine (9:1 by vol.) as described by Marinetti (1962 and 1964).

Radioactive lipids were detected by autoradiography as described in Methods V.3. Lipids were eluted from preparative paper chromatograms as described in Methods VI.3.

b) Water soluble compounds. Ascending chromatography was carried out on Whatman No. 1 paper in the following solvent systems:

I phenol-water (100:40 W/W).

J n-butanol-acetic acid-water (5:3:1 by vol.).

K n-butanol-acetic acid-water (5:2:3 by vol.).

L isopropanol-water-conc. ammonia (7:2:1 by vol.).

M methanol-98% formic acid-water (80:13:7 by vol.).

N methanol-98% formic acid-water (80:30:7 by vol.).

O 1M ammonium acetate-absolute ethanol (35:65 by vol.).

Compounds containing phosphate were detected by the salicylsulfonic acid-ferric chloride procedure (Vorbeck and Marinetti, 1965): the chromatogram was dipped in a solution made by dissolving 1.5 g $FeCl_3 . 6\ H_2O$ in 30 ml of 0.3N HCl and diluting with 970 ml of acetone; the chromatogram was dried and dipped in a 1.5% solution of salicylsulfonic acid in acetone; the phosphates appeared as white spots on a mauve background. Labelled compounds were detected by autoradiography as described in Methods V.3.

III. Methods for the Chemical Degradation of Lipids

1. Deacylation by mild alkaline hydrolysis. Lipids were subjected to mild alkaline deacylation by a modification of the procedure of Dawson (1967b): A solution of the lipids in 0.5 ml of chloroform-methanol (2:3, by vol.) was treated with 0.5 ml of 0.2N methanolic NaOH at room temperature for 15 min. The mixture was immediately diluted with 1 ml of chloroform-methanol (4:1, by vol.) and 0.9 ml of water, and then centrifuged for 1 min at 400g. The upper methanol-water phase was removed, the chloroform phase washed with 0.5 ml methanol-water (10:9, by vol.) and the combined methanol-water extract and washing immediately neutralized or slightly acidified with 0.3-0.5 ml of cation exchange resin (Rexyn RG 50 or Dowex 50). The resin mixture was centrifuged, the supernatant removed and the resin washed twice with 0.5 ml methanol-water (10:9, by vol.). The chloroform and methanol-water extracts were counted for ^{14}C. The methanol-water extract was made slightly alkaline with 1.5M methanolic NH_4OH and concentrated almost to dryness under a stream of N_2 at 40°C. The residue was dissolved in 0.1 ml of methanol-water (10:9, by vol.) and analyzed by paper chromatography on Whatman No. 1 paper in the solvent systems described in Section II.2.b.

2. Saponification. A modification of the procedure described by Kates (1972) was used, as follows: to a sample

of ^{14}C-labelled lipid in a glass stoppered 15 ml centrifuge tube was added 1 ml of 0.2N sodium hydroxide in 90% methanol, and the mixture was heated at 55°C for 1 h. After extracting the unsaponifiable material twice with 1 ml of petroleum ether (b.p. 30-60°C), aliquots of the aqueous-methanol and ether phases were counted for ^{14}C. For ^{14}C-methyl fatty acid esters, the remainder of the aqueous-methanol phase was transferred to a microdistillation apparatus and heated in a hot water bath (60-65°C) to distil over methanol, which was then counted for ^{14}C.

3. Methanolysis Procedure for the determination of Fatty acid composition. The following procedure described by Kates (1972) was employed: an aliquot of the chloroform solution of lipids (containing 15-30 mg of lipids) was pipetted into a side arm flask and the solvent evaporated under a stream of N_2; 4.5 ml of 2.5% HCl in methanol (made by dissolving 2.5 g of HCl gas in 100 ml of methanol) was added and the mixture was heated under reflux ($CaCl_2$ tube on condenser) for 1-2 h; 0.5 ml of water was added and the volume was adjusted with methanol-water (9:1, by vol.) to fill the side tube. Fatty acid methyl esters were then extracted with 3 to 4 portions (5 ml) of petroleum ether (b.p. 30-60°C); the ether extract was concentrated to dryness under N_2 and the residue dissolved in a known volume of chloroform. An aliquot (containing 100 µg of fatty ester) was analyzed by gas liquid chromatography on a column of butanediol succinate at 175°C

(N_2 carrier gas, 0.5 kg/cm^2), using a Carlo Erba instrument, and the fatty acid methyl ester composition calculated as described by Kates (1972).

IV. Analytical Procedures

1. Protein was measured essentially by the procedure of Lowry et al. (1951) as follows: Prior to analysis, cell fractions were diluted with water (100,000xg fraction by 10-fold; all other fractions 25-fold). To 0.1 ml of diluted fraction was added 6 ml of a solution prepared by adding 50 ml of 2% sodium carbonate in 0.1N sodium hydroxide to 1 ml of a fresh solution of 0.5% copper sulphate in 1% sodium potassium tartrate. After 10 min., 0.6 ml of Folin-Ciocalteau reagent diluted with water (1:1, by vol.) was added with Vortexing during the addition, and the mixture left at room temperature for 30 min. The absorption was measured at 730 nm in cuvettes 1.6 cm in diameter using a Coleman Junior II Spectrophotometer. Bovine serum albumin (Fraction V, Nutritional Biochemical Corporation) was used as a protein standard. Since cell fractions contained different amounts of chlorophyll the absorption at 730 nm from chlorophyll was determined by preparation of a suspension of 0.05 ml of cell free homogenate in 6 ml of 2% Na_2CO_3 in 0.1N NaOH. The spectrum of this suspension showed a maximum due to chlorophyll at 640 nm and minima of zero absorbance at 600 and 730 nm. The wavelength of 730 nm was therefore chosen for protein analysis.

2. Chlorophyll was measured by the procedure of Arnon (1949): to 2 ml of cell fraction suspension was added 8 ml of acetone, the mixture shaken and centrifuged, and the absorbance of the supernatant read at 652 nm.

3. Phosphorus was measured by the modified procedure of Allen (1940).

V. Radioisotopic Procedures

1. Labelled compounds

a) Commercially available. [U-^{14}C]L-serine (128 mCi/mmole), [1,2-^{14}C]ethanolamine (2.7 mCi/mmole), [1,2-^{14}C] choline chloride (5.2 mCi/mmole) and [U-^{14}C]myo-inositol (215 mCi/mmole) were purchased from the New England Nuclear Corporation. Phosphoryl[1,2-^{14}C]ethanolamine (1.7 mCi/mmole), CDP-[1,2-^{14}C]ethanolamine (60 mCi/mmole), CDP-[1,2-^{14}C]choline (9.3 mCi/mmole), [Me-^{14}C]methionine (25 mCi/mmole) and S-adenosyl[Me-^{14}C]methionine (50 mCi/mmole) were purchased from the International Chemical and Nuclear Corporation (ICN). [1-^{14}C]Glycerol (15.3 mCi/mmole) and 5-[Me-^{14}C]tetrahydrofolic acid (45 mCi/mmole) were purchased from Amersham/Searle. All compounds were stored at -15°C and checked periodically for purity by paper chromatography.

b) Preparation

i) sn-[1,3-^{14}C]Glycerol 3-phosphate. This substrate was synthesized by the enzymatic phosphorylation of [1-^{14}C]glycerol essentially according to the procedure

of Kennedy (1962a) as modified by Chang and Kennedy (1967a): a reaction mixture of total volume 5 ml containing 1.54 mM [1-^{14}C]glycerol (2.61 x 10^8 dpm), 2.7 mM unlabelled glycerol, 20 mM ATP, 20 mM $MgCl_2$, 50 mM Tris HCl (pH 8.0), 20 mM β-mercaptoethanol, 5 mg of bovine serum albumin and 50 µg of glycerolkinase (sp.act. 1 x 10^4 units/mg protein) was incubated at 37°C for 4 h. The mixture was heated to 100°C for 10 min. to stop the reaction, and then applied to a column of Dowex-1 formate prepared by washing a column of Dowex-1 chloride (200-400 mesh; 16 x 1.3 cm) free of chloride ion with 4N formic acid and finally overnight with distilled water. Gradient elution was then carried out with an arrangement of apparatus similar to that described by Hurlbert et al. (1954) with 250 ml of water in the lower mixing chamber and 1ℓ of 4N formic acid in the upper reservoir. With a flow rate of 1.5 ml/min., fractions of 10 ml each were collected and assayed for radioactivity. The fractions (tubes 1-8) containing [^{14}C]glycerol were discarded. The fractions (tubes 30-41) containing [^{14}C]GP were combined, and taken to dryness under reduced pressure at 30°C in a rotary evaporator. The product was dissolved in 10 ml of water, neutralized with ammonia, and shaken for 1 h with 150 mg of acid washed animal charcoal (Norite) to remove any adenine nucleotides (Pande et al., 1967). The charcoal was filtered off and the [^{14}C]GP filtrate made up to a known volume with water and stored at 4°C. The product migrated with standard

sn-3-glycerophosphate as a single radioactive spot on Whatman No. 1 paper in solvent systems (J) and (O) (R_f GP, 0.14 and 0.42 respectively; R_f glycerol, 0.6 and 1.0 respectively). Yield of sn-3-glycerophosphate as determined by the enzymatic procedure described by Kennedy (1962) was 15.2 µmoles (72%); specific activity 1.48 x 10^7 dpm/µmole. The product was diluted with unlabelled sn-3-glycerophosphate prepared as described in Methods I.2.b.v. Final specific activity is specified in the appropriate section of Results.

ii) sn-[1,3-^{14}C]Glycerol-3-[^{32}P]Phosphate. This substrate was synthesized from [1-^{14}C]glycerol by the same procedure as described above, using [γ^{32}P]ATP prepared as described by Glynn and Chapell (1964). The sn-[1,3-^{14}C] glycerol-3-[^{32}P]phosphate was counted for total radioactivity using a Nuclear Chicago gasflow Geiger counter (model 1042). The ^{32}P activity was approximately determined by measuring the radioactivity (^{32}P only) transmitted through two layers of parafilm placed over the planchet containing the sample, and the ^{14}C activity by difference.

iii) Phosphatidyl[1,3-^{14}C]glycerol from E.coli enzyme system. This compound was prepared from sn-[1,3-^{14}C] glycerol-3-phosphate and CDP-diglyceride by using the PG-synthesizing enzyme system of E.coli (Kanfer and Kennedy, 1964): a reaction mixture of total volume 1.0 ml, containing 0.82 mM sn-[1,3-^{14}C]glycerol-3-phosphate (1 x 10^6 dpm), 5 mM CDP-

diglyceride (Koch-Light Co.), 20 mM $MgCl_2$, Triton X-100 (1.5 mg/ml), 0.16M Tris HCl (pH 8.0), 1 mM β-mercaptoethanol and 3 mg of E.coli 40,000xg pellet suspension, was incubated for 3 h at 37°C. The reaction was stopped by the addition of methanol (2.5 ml) and chloroform (1.25 ml) and total lipids extracted as described in Methods VI.1. [^{14}C]PG was isolated by preparative paper chromatography (Methods II.2.a) in diisobutyl ketone-acetic acid-water (8:5:1, by vol.) (R_f PG, 0.58). ^{14}C activity of product, 8.0×10^5 dpm (8% yield).

iv) Phosphatidyl[1,3-^{14}C]glycerol from Spinach enzyme system. This compound was synthesized in an assay system (total vol. 5 ml) containing 1 mM CDP-diglyceride (Koch-Light Co.), 1.06 mM sn-[1,3-^{14}C]glycero-3-phosphate (4×10^6 dpm), 30 mM $MgCl_2$, Triton X-100 (1.5 mg/ml), 0.27M Tris HCl (pH 7.4), 1 mM β-mercaptoethanol and 1.25 ml of Spinach 40,000xg fraction prepared as described in Experimental II.1 except that the homogenizing medium contained potassium phosphate (pH 8.0) instead of Tris HCl. Incubation, 3 h at 38°C. Lipids were extracted as described in Methods VI.1.a.ii, and [^{14}C]PG purified by preparative paper chromatography, (see Methods II.2.a), in diisobutylketone-acetic acid-water (8:5:1, by vol.) (Rf of PG, 0.58).

v) Phosphatidyl[1,3-^{14}C]glycerophosphate. This compound was synthesized in the assay system under the same conditions as described above in section iii, except that

β-mercaptoethanol was replaced by 20 mM $HgCl_2$ in order to inhibit PGP phosphatase (Chang and Kennedy, 1967b). Paper chromatography of the total lipid extract in diisobutylketone-acetic acid-water (8:5:1, by vol.) showed a single ^{14}C spot with R_f, 0.41; no ^{14}C PG (R_f 0.60) was present.

vi) [^{14}C]Phosphatidyl serine. This substrate was isolated from cells of E.coli grown in hydroxylamine and [U-^{14}C]serine, essentially as described by Raetz and Kennedy (1972): An inoculum of E.coli stain B cells was grown for 16 h at 37°C in 5 ml of medium containing 0.11M glycerol, 3 mM $MgSO_4$, 2 mM KCl, 0.5 mM K_3PO_4, 5 mM $(NH_4)_2SO_4$, 0.01 mM $FeSO_4$ and 0.1M Tris HCl (pH 7.0); 2 ml of inoculum were transferred to 25 ml of fresh medium in a 500 ml side arm flask. Cells were grown for 6 h on a rotary shaker at 120 rpm until the late exponential phase, when 0.5 ml of 1.25M hydroxylamine hydrochloride was added. Incubation was continued for 20 min, and then 1 ml of 0.42 mM [U-^{14}C]L-serine (1.11×10^8 dpm) was added; after 20 min further incubation, cells were killed by the addition of methanol (66 ml) and chloroform (33 ml). ^{14}C-Labelled lipids were extracted as described in Methods VI.1. TLC of total lipids in solvent C, showed [^{14}C]PS (R_f, 0.16) to be the major ^{14}C product; small amounts of ^{14}C were also associated with PE (R_f, 0.44) and with unknown components migrating with Rf 0.22, 0.31 and 1.0. [^{14}C]PS was purified by repeated preparative TLC in solvent C; it was extracted from the TLC plate by the Bligh

and Dyer acidic procedure described in Methods VI.2.b. TLC analysis in solvents (A) and (D) showed a ^{14}C-labelled spot migrating with standard PS (R_f 0.53 and 0.15 respectively) and accounting for 95% of total ^{14}C activity; a minor (5%) unknown component remaining at the origin in solvent C was also present. The ratio of ^{14}C in GPS to ^{14}C in the fatty acid moiety was 1.67 (see Table 15); GPS was identified by paper chromatography on Whatman No. 1 in solvent system (L) (see Fig. 19).

2. Measurement of Radioactivity

Solutions of ^{14}C lipid in chloroform were counted in a scintillation mixture consisting of 0.03% POPOP and 0.5% PPO in toluene-methanol (87:13 by vol.). The instrument used was a Beckman LS-150 scintillation counter. Each sample was counted to 1% statistical error and corrected for quenching by the external standard ratio (ESR) method (Baillie, 1960) using a series of Amersham-Searle standards containing equal amounts of ^{14}C toluene (2.03×10^5 dpm) but different amounts of the quenching agent chloroform. Since chlorophyll was present in many of the lipid samples counted, the quenching due to this compound was determined by preparing standards containing equal amounts of ^{14}C toluene (1.92×10^5 dpm) but different amounts of total spinach lipids (equivalent to 5-50 µg chlorophyll). It was found that the plot of % counting efficiency vs. ESR for the chlorophyll standards was

very similar to the corresponding plot for the chloroform standards (see Fig. 2). Since chloroform mimiced the quenching action of chlorophyll, the chloroform standards used routinely were adequate in determining the degree of quench in lipid samples containing chlorophyll (ESR values were generally in the range 0.25-0.70).

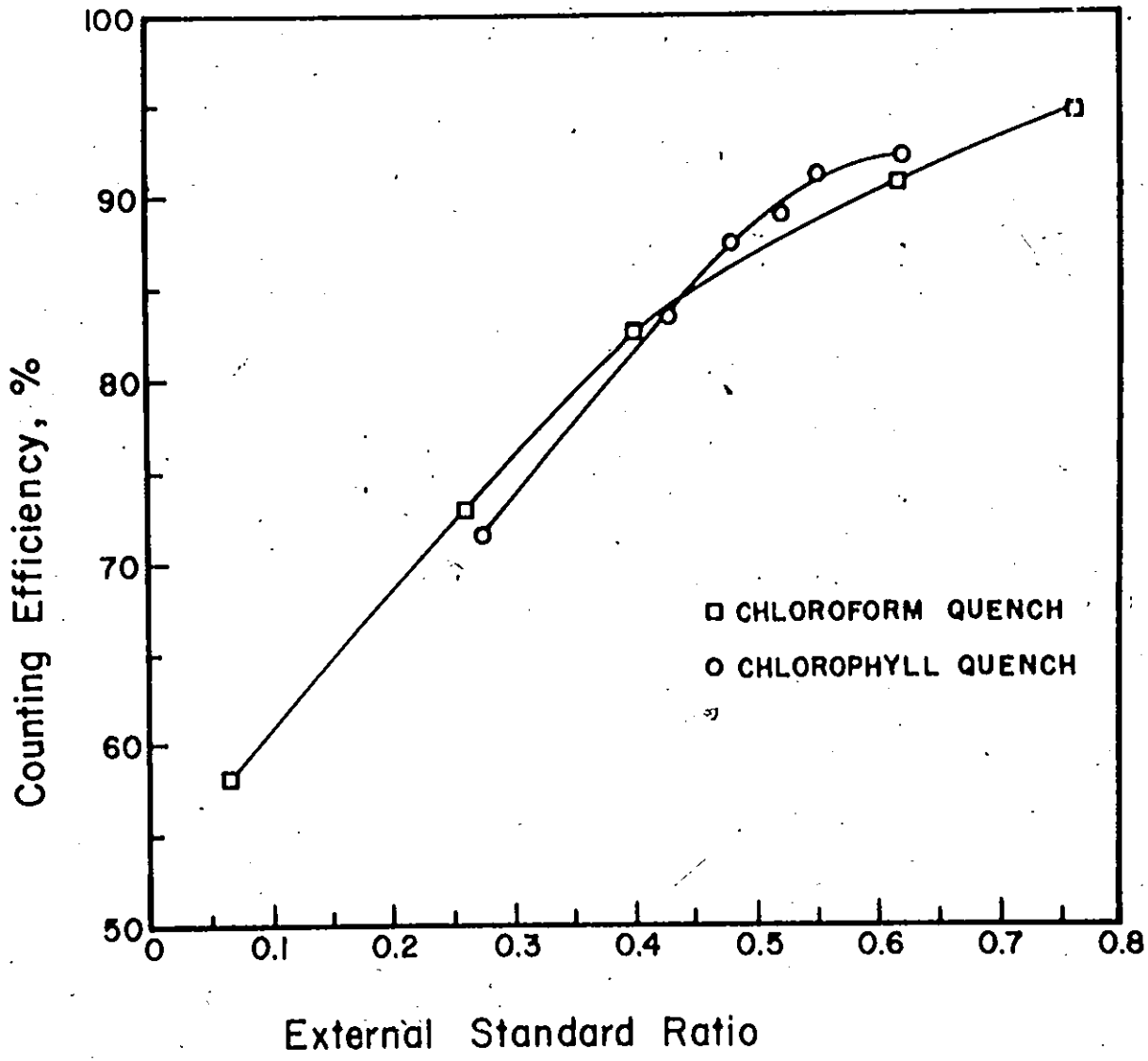
Aliquots (<0.1 ml) of aqueous solutions containing ^{14}C were counted in the above scintillation mixture supplemented with Beckman Bio-solv solubilizer (10% by vol.); quench corrections were made using the chloroform standards described above. ^{14}C spots on TLC plates were scraped directly into the same scintillation mixture and counted; ^{14}C spots on paper chromatograms were cut out and added directly to the scintillation mixture.

3. Autoradiography

^{14}C-Labelled lipid spots on TLC and paper chromatograms were detected by exposing the chromatograms to a sheet of Kodak Blue Brand X-ray film for a suitable period of time depending on the ^{14}C activity of labelled spot (on TLC, 2 days for 1000 dpm; on paper chromatograms, 20 days for 1000 dpm). The film was developed and fixed by standard photographic procedures. The ^{32}P and ^{14}C of doubly labelled lipid spots on paper chromatograms were detected independently by exposing the chromatogram to two layers of X-ray film, the lower layer detecting both ^{32}P and ^{14}C activity, and the upper layer detecting only ^{32}P activity.

FIGURE 2

Percentage counting efficiency as a function of external standard ratio for quenching from chloroform and chlorophyll.


100
90
80
70
60
50
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
Counting Efficiency, %
External Standard Ratio
CHLOROFORM QUENCH
CHLOROPHYLL QUENCH

VI. Extraction of Lipids by the Bligh and Dyer Procedure (Bligh and Dyer, 1959)

1. From enzyme assay mixtures

Enzyme assay mixtures (1.0 ml) were diluted with 2.5 ml of methanol and 1.25 ml of chloroform to form a one phase mixture containing methanol-chloroform-water in the ratio 2:1:0.8, by vol. The mixture was left at room temperature for 15 min, and diluted with 1.25 ml each of chloroform and water to produce a biphasic mixture containing methanol-chloroform-water in ratio 1:1:0.9, by vol. The mixture was gently Vortexed and briefly centrifuged; the lower chloroform phase was removed, and the upper methanol-water (10:9, by vol.) phase was washed twice with 1 ml of chloroform. The combined chloroform phases were washed twice with 1 ml of methanol-water (10:9, by vol.), then diluted with benzene and concentrated to dryness under an N_2 stream; the residue was made to a known volume with chloroform.

2. From preparative TLC chromatograms

a) "Neutral" procedure. Each band of silica containing lipid was scraped from the plate into a centrifuge tube and covered with 5 ml of methanol-chloroform-water (2:1:0.8, by vol.); the mixture was Vortexed and centrifuged, and the supernatant was removed. The process was repeated twice, the supernatants were combined (total vol. 15 ml) and chloroform and water were added (3.95 ml each). The resultant

biphasic mixture was centrifuged, and the chloroform phase was removed and washed twice with 2 ml of methanol-water (10:9, by vol.). When bands from plates developed in solvent (A) were extracted, any acetic acid remaining in the chloroform phase at this stage was neutralized with 0.2N methanolic ammonia. The lipid solution in chloroform was then diluted with benzene and concentrated to dryness under a stream of N_2; the residue was made up to a known volume with chloroform.

b) "Acidic" procedure. In the purification of CDP-diglyceride and [^{14}C]PS by preparative TLC (see Methods I.2.b.i and V.1.b.vi respectively) it was found that these compounds could not be extracted from the silica by methanol-chloroform-water (2:1:0.8, by vol.). Complete extraction could however be achieved by extracting the silica three times as rapidly as possible with methanol-chloroform-0.2 NHCl (2:1:0.8, by vol.). The chloroform phase, isolated as described in section a) above, was immediatly neutralized with 0.2N methanolic ammonia, and concentrated to dryness under an N_2 stream in the presence of benzene; the residue was made up to a known volume with chloroform.

3. From preparative paper chromatograms

Lipid material on Whatman 3 MM silicic acid impregnated chromatograms was eluted with chloroform-methanol-water (1:2:0.8) as described by Kates (1972).

C. EXPERIMENTAL PROCEDURES

I. In vivo studies

1. Incorporation of ^{14}C-labelled substrates into lipid

a) By whole leaves. This study was carried out essentially according to the procedures described by Eberhardt and Kates (1957) and Kates (1960, 1972). Spinach leaves, each approximately 6 x 4 cm and weighing 2-3 g, were selected from a batch of leaves bought from the local supermarket. ^{14}C Labelled substrate was supplied to each leaf by transpiration through the petiole, as follows: the petiole of each leaf was first placed in water, the tip of the petiole cut off and the leaf illuminated by a 500W light bulb at a distance of 1.5 ft for 15 min. The petiole was then immersed in 0.5 ml of an aqueous solution of labelled substrate ([U-^{14}C] serine, [1,2-^{14}C]ethanolamine or [Me-^{14}C]methionine, 2-3 μCi) contained in a glass tube (0.5 x 5 cm), and the leaf was illuminated for a further 3.5 h during which time 80-100% of the substrate was taken up. The leaf was then transferred to a 40 ml centrifuge tube immersed in acetone-solid CO_2, and the leaf crushed with a glass rod. Lipids were extracted as follows: 10 ml of hot isopropanol were added and the mixture was immersed in boiling water for 1 min, and then centrifuged; the supernatant was removed, and the pellet was washed twice with 10 ml of hot isopropanol. The combined isopropanol extract and washings were concentrated to dryness

under an N_2 stream. To free the residue of non-lipid material, 8 ml of chloroform-methanol (1:1) were added, the mixture was clarified by centrifugation and 3.6 ml of water were added. The biphasic mixture was centrifuged and the chloroform phase removed, washed three times with 2 ml of methanol-water (10:9), and finally concentrated to dryness under an N_2 stream. The lipid residue was dissolved in chloroform to a known volume and the ^{14}C incorporation into individual lipids determined by the same TLC method as described for analysis of ^{14}C products from in vitro assays (see Experimental II.3.a). Individual lipids were also isolated by preparative TLC and deacylated by mild alkaline hydrolysis as described in Methods III.1, to determine distribution of ^{14}C between water-soluble and fatty acid moieties.

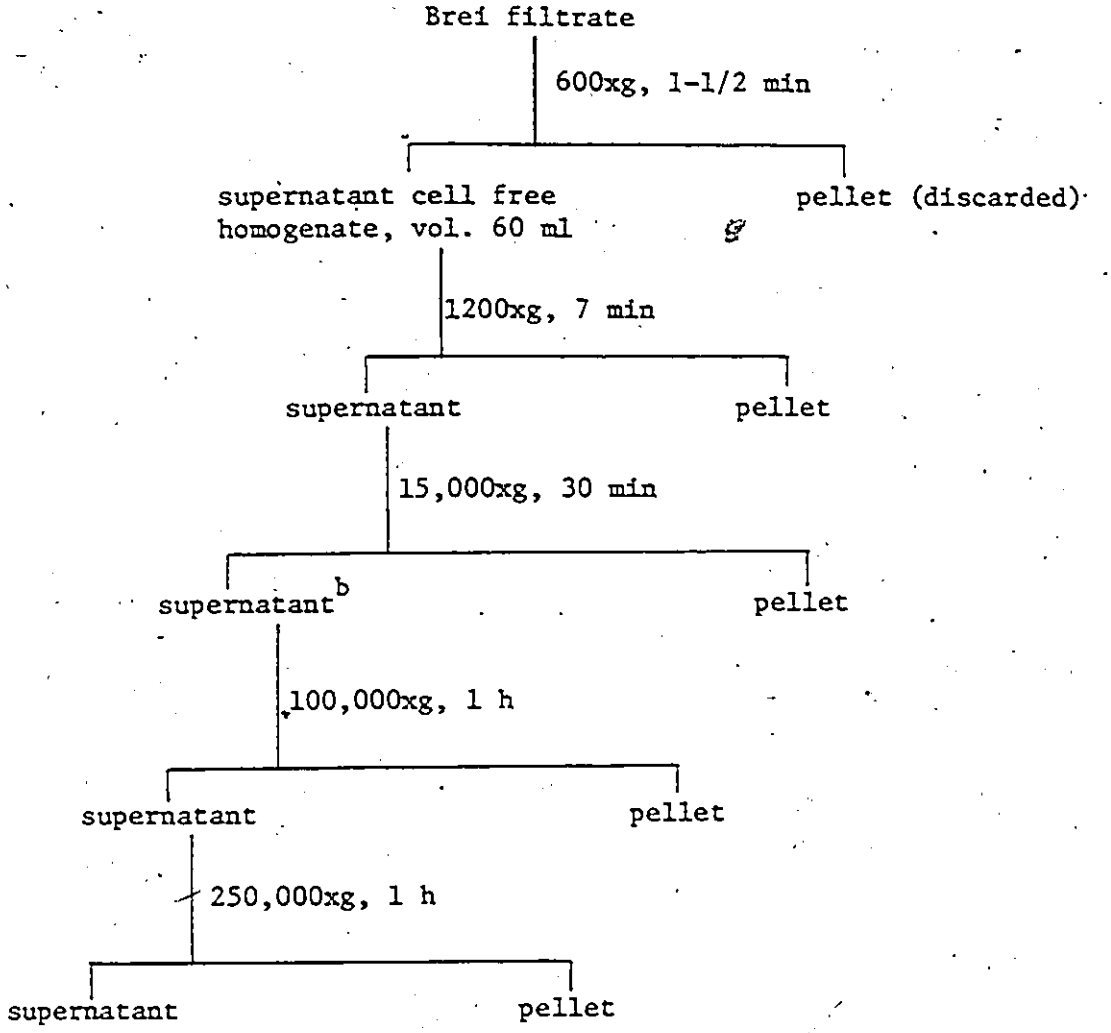
b) By leaf slices. ^{14}C-Labelled substrate was supplied to leaf slices as follows: Spinach leaves (approximately 2 x 4 cm) were first immersed in 0.1% chloramphenicol for 10 min to kill surface bacteria, then rinsed with water, dried, and cut with a scalpel into slices approximately 3 mm wide; 1 g of leaf slices was then immersed in 7 ml of a solution of labelled substrate ([U-^{14}C]serine, [1,2-^{14}C] ethanolamine, [1,2-^{14}C]choline or [Me-^{14}C]methionine, 10 µCi) in 0.15M potassium phosphate buffer (pH 7.5). After illumination by a bank of seven 35W fluorescent tubes (cool white, high output) at a distance of 1.5 ft. for 5 h in a shaking incubator at 37°C, the slices were transferred to a 40 ml

centrifuge tube immersed in acetone-solid CO_2, and lipids were extracted and analyzed as described in section a) above.

II. In vitro Studies

1. Subcellular Fractionation. Spinach leaves, bought from the supermarket, were washed with distilled H_2O and the main rib removed; 50 g of leaves (approximately 11 x 8 cm) were ground with sea sand (16 g) in a mortar cooled on ice, in 50 ml of ice-cold homogenizing medium of composition similar to that used by Appelquist et al. (1968), containing 0.01 M sodium chloride, 0.5 M sucrose, 1 mM EDTA disodium salt and 0.1 M Tris HCl (pH 8.0). After the leaves were crushed, grinding was continued a further 3 min; the brei was filtered through eight layers of cheese cloth and the residue washed with 10 ml of cold homogenizing medium. The combined filtrate and washing was fractionated by differential centrifugation at 4°C, as described in Fig. 3. In most studies, pellets were suspended as follows: to each pellet was added 6 ml of 2 mM Tris HCl (pH 7.5) and the pellet was loosened by scraping the centrifuge tube with a glass rod; the mixture was transferred to a Potter homogenizer of bore 1.5 cm, and the pellet was suspended by five slow strokes at 0°C. The suspension was made up to 10 ml with the same buffer. In studies on subcellular enzyme distribution, pellets were suspended as above except that the Tris HCl buffer was

FIGURE 3 CELL FRACTIONATION PROCEDURE[a]



a) Centrifugal forces are maximum values. A Sorvall RC-2B centrifuge was used for centrifugation up to 40,000xg, and a Beckman Model L2-65B for centrifugation at 100,000xg and 250,000xg. All pellets, other than the 600xg pellet, were washed lightly with homogenizing medium (5 ml) and the washings combined with the respective supernatant.

Pellet fractions prepared differently from those above will be described in the text by the supernatant fraction from which they were derived, e.g. a 1200-100,000xg fraction denotes the pellet obtained by centrifuging the 1200xg supernatant at 100,000xg.

b) For studies on PG biosynthesis this fraction was centrifuged at 40,000xg.

replaced by homogenizing medium. Minor modifications to the above procedure were occasionally used, and are described in the appropriate legend.

2. Marker enzyme assays. All spectrophotometric measurements were made on a Carey Model 15M.

a) Cytochrome c oxidase. This enzyme is one of the chain of enzymes involved in oxidative phosphorylation. In animals (Appelmans et al., 1955) and plants (Goddard and Bonner, 1960; Tolbert et al., 1968) the enzyme is localized in mitochondria. Within animal mitochondria, it is known to be situated only in the inner membrane (Parsons et al., 1966; Levy et al., 1967; Schnaitman and Greenwalt, 1968), and though the intramitochondrial localization of the plant enzyme has not been determined it is also most likely exclusively localized in the inner membrane.

In the present study, cytochrome c oxidase was measured spectrophotometrically essentially according to the procedure of Smith (1955), as follows: Prior to analysis, cytochrome c was reduced with sodium ascorbate, the excess ascorbate removed by dialysis (Wharton and Tzagoloff, 1967), and the concentration of reduced cytochrome c determined spectrophotometrically (Yonetani, 1965). Spinach cell fractions were diluted to 60 ml with homogenizing medium and then "solubilized" by gentle shaking for 1 min at 0°C in the presence of 0.2% lubrol. The decrease in absorbance (Ared) of reduced cytochrome c at 550 nm was then followed as a

function of time in a mixture (vol. 1 ml) containing 90 mM potassium phosphate (pH 7.0), 23 mM cytochrome c (88% reduced) and 0.03 ml of diluted cell fraction. After a reaction time of 1 min a few crystals of potassium ferricyanide were added and the absorbance (Aox) of fully oxidized cytochrome c was measured. The first order rate constant was determined from the log (Ared-Aox) vs. time plot, and the initial rate of oxidation of cytochrome c was calculated as described by Smith (1955).

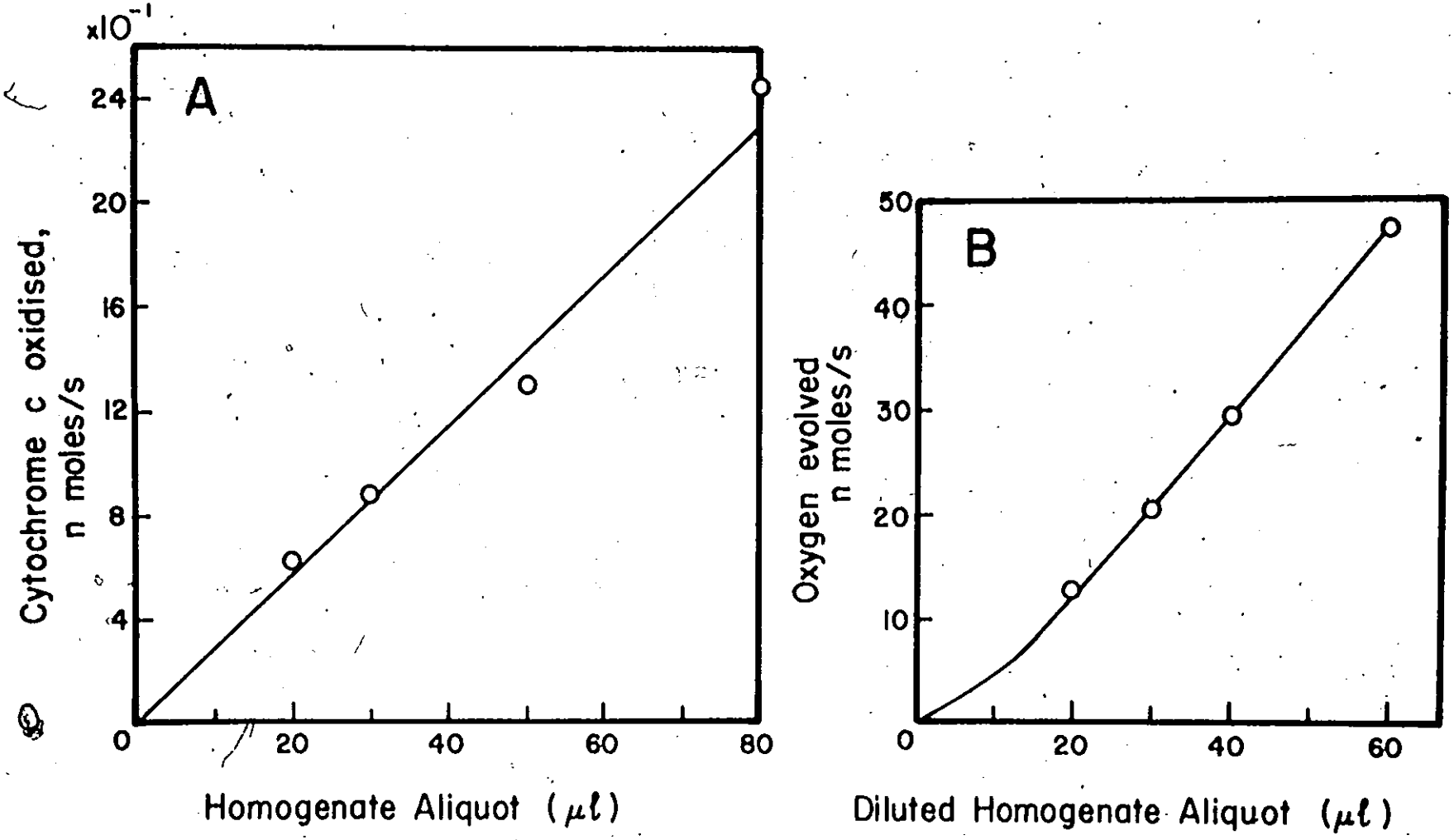
b) Catalase. This enzyme, which catalyzes the breakdown of hydrogen peroxide, is found in peroxisomes of leaves (Tolbert et al.; 1968) and liver and kidney (de Duve and Baudhuin, 1966). However, the ease of lysis of peroxisomes can give rise to activity in the supernatant fraction (Tolbert et al., 1968). Thus distribution studies with this marker enzyme should be interpreted with caution. In the present study, enzyme activity was measured spectrophotometrically essentially according to the procedure of Lück (1966), as follows: Prior to analysis, each cell fraction was diluted with homogenizing medium (cell free homogenate and 40,000xg supernatant by x5, and pellet fractions by x25), and the diluted fraction was "solubilized" by gentle shaking for 1 min at 0°C in the presence of 0.2% lubrol. The decrease in absorbance of hydrogen peroxide at 240 nm was then followed as a function of time in a mixture (vol. 1 ml) containing 67 mM potassium phosphate (pH 7.0), 14 mM hydrogen peroxide

FIGURE 4

Cytochrome c oxidase and catalase activities as a function of protein concentration.

A Cytochrome c

B Catalase


A
x10⁻¹
24
20
16
12
8
4
0
20
40
60
80
Cytochrome c oxidised,
n moles/s
Homogenate Aliquot (μl)
B
50
40
30
20
10
0
20
40
60
Oxygen evolved
n moles/s
Diluted Homogenate Aliquot (μl)

and 0.03 ml of diluted cell fraction. The first order rate constant was determined from the log (absorbance) vs. time plot, and the initial rate of reaction calculated as described by Lück (1966).

The activity of both cytochrome c oxidase and catalase was linear with enzyme concentration over the range assayed (Fig. 4). "Solubilization" of cell fractions with lubrol was necessary for proper dispersion of both enzymes; treatment of cell fractions with higher lubrol concentrations or for a longer time period than described above did not affect the activity of either enzyme.

3. Biosynthesis of Phospholipids and Neutral Lipids from ^{14}C-Labelled Substrates

Details of individual experiments are given in legends to appropriate tables and figures.

a) General method of assay. Reactions were carried out in 15 ml glass stoppered centrifuge tubes in a volume of 1.0 ml (unless otherwise stated) with shaking in a constant temperature bath at either 30° or 37°C. Reactions were stopped by the addition of methanol-chloroform (2:1; vol. 3.75 ml), and lipids were extracted by the Bligh and Dyer procedure described in Methods VI.1. ^{14}C-Labelled lipid products were analyzed as follows: a portion of the chloroform solution of lipids was counted for ^{14}C as described in Methods V.2. A second portion was then chromatographed either by TLC or on silicic acid impregnated paper (Methods II.1.a and II.2.a).

Radioactive spots were either scraped from the TLC chromatogram, or cut out of the paper chromatogram, and counted in the scintillation mixture supplemented with solubilizer (see Methods V.2). Total incorporation of labelled substrate into each lipid component was calculated from the relative distribution of radioactivity in the corresponding spot multiplied by the radioactivity incorporated into the total lipids. All results reported are from single experiments, unless stated otherwise. However, results from duplicate experiments on the incorporation of ^{14}C from [^{14}C]SAM into PC fatty acids (see legend to Fig. 29C) showed the general method of assay as applied to this enzyme system to be reproducible to within ±5%.

b) Dispersion of lipid substrates.

i) Vortexing. In studies utilizing CDP-diglyceride and [^{14}C]PS as substrates, these compounds were dispersed in the appropriate reaction mixture as follows: a suitable aliquot of substrate in chloroform-methanol (2:1) or chloroform was pipetted into an empty reaction tube and the solvent evaporated under an N_2 stream. The remaining non-enzymatic components were added and the mixture Vortexed vigorously for 15 sec. The above procedure resulted in the formation of clear dispersions of CDP-diglyceride (ca. 0.1 mM); and of [^{14}C]PS.

Unless stated otherwise, all other lipid substrates were dispersed prior to their addition to the assay systems

either by sonication or dialysis, as follows:

ii) Sonication. A mixture of 3-5 mg of lipid (PE, PE-Me or PE-diMe) in 1 ml of 20 mM Tris HCl (pH 8.0) was sonicated at room temperature for 15 sec. intervals over a 5-10 min period, with cooling in cold water. A Quigly-Rochester instrument fitted with a microtip oscillating at maximum output was used. The clarity of the lipid suspension produced was dependent on the lipid used: both spinach and egg PE produced almost clear suspensions, while both dipalmitoyl PE-Me and PE-diMe gave rise to opaque gel-like suspensions. There was no breakdown of lipids during sonication as shown by TLC analysis of the final suspensions.

iii) Dialysis. The procedure followed was that described by Fleischer and Klouwen (1961): A mixture of spinach PE (5 mg) in 1 ml of n-butanol-20% K-deoxycholate (pH 7.5) (87:13 by vol.) was dialyzed at 4°C for 7 days on a rocker dialyzer, against 300 ml of a solution (renewed every 24 h) containing 20 mM Tris acetate (pH 8.0) and 1 mM Na_2 EDTA. The resultant, almost clear, PE suspension (vol. 2.2 ml) was free of butanol but most likely contained some undialyzed deoxycholate. Concerning this, Fleishcer and Klouwen (1961) reported that in the dialysis of beef heart mitochrondrial total lipids the amount of undialyzed deoxycholate could not be decreased below 53 µg/mg lipid. In the experiment described in Table 23 the PE suspension was tested immediately following its preparation.

D. RESULTS

I. Biosynthesis of Phosphatidyl Glycerol

1. The Incorporation of sn-[1,3-^{14}C]Glycerol-3[^{32}P] phosphate and sn-[1,3-^{14}C]Glycerol-3-phosphate into Phosphatidyl Glycerol by a 40,000xg Spinach Cell Fraction

Preliminary studies were carried out to test sn-[1,3-^{14}C]glycerol-3-[^{32}P]phosphate ([^{14}C+^{32}P]GP) as a substrate in the biosynthesis of phosphatidyl glycerol (PG). It was found that a 40,000xg fraction, in an assay system at pH 8.0 containing CDP-diglyceride, Mg^{2+} and Triton X-100, incorporated 2% of total radioactivity (^{14}C+^{32}P) from [^{14}C+^{32}P]GP into lipid; under the same conditions a 90,000xg fraction incorporated 0.7% of total radioactivity (^{14}C+^{32}P) into lipid. With both fractions, one major radioactive product with mobility corresponding to that of PG (R_f, 0.58) and labelled only with ^{14}C was formed; a minor radioactive product migrating with R_f 0.38 was also detected on both "^{32}P only" and "^{14}C+^{32}P" films, showing that the minor product contained both ^{14}C and ^{32}P (Fig. 5).

Identification of these products was carried out subsequently using sn-[1,3-^{14}C]glycerol-3-phosphate ([^{14}C]GP) as substrate, since the doubly labelled GP was difficult to prepare and the ^{32}P decayed rapidly. The major product

FIGURE 5

Autoradiogram of a chromatogram of lipids labelled with ^{14}C and ^{32}P from [^{14}C+^{32}P]glycerophosphate.

1. 40,000xg fraction as enzyme source
2. 90,000xg fraction as enzyme source

Assay mixture (vol. 1 ml): 0.8 mM sn-[1,3-^{14}C] glycerol-3-[^{32}P]phosphate (6.7 x 10^4 cpm; ^{32}P:^{14}C, 1:1), 0.1 mM CDP-diglyceride (Koch-Light Co.), 10 mM $MgCl_2$, 0.25 M Tris HCl (pH 8.0), Triton X-100 (1.5 mg/ml), 13 mM β-mercaptoethanol, and 0.6 ml of cell fraction suspended in homogenizing medium. Cell fractions were prepared as described in Experimental II.1 except that homogenizing medium contained 0.1 M potassium phosphate (pH 8.0) instead of Tris HCl. Incubation was for 3 h at 37°C. Incorporation of total radioactivity into lipid by the 40,000xg and 90,000xg fractions was 1345 cpm (2%) and 470 cpm (0.7%) respectively.

Chromatography was on silicic acid impregnated paper in diisobutylketone-acetic acid-water (8:5:1 by vol.). The separate detection of ^{32}P and ^{14}C+^{32}P was carried out as described in Methods V.3.

Individual components in the mixture of spinach lipids were identified by their R_f values (Kates, 1972) and by the staining procedures described in Methods II.2.a.

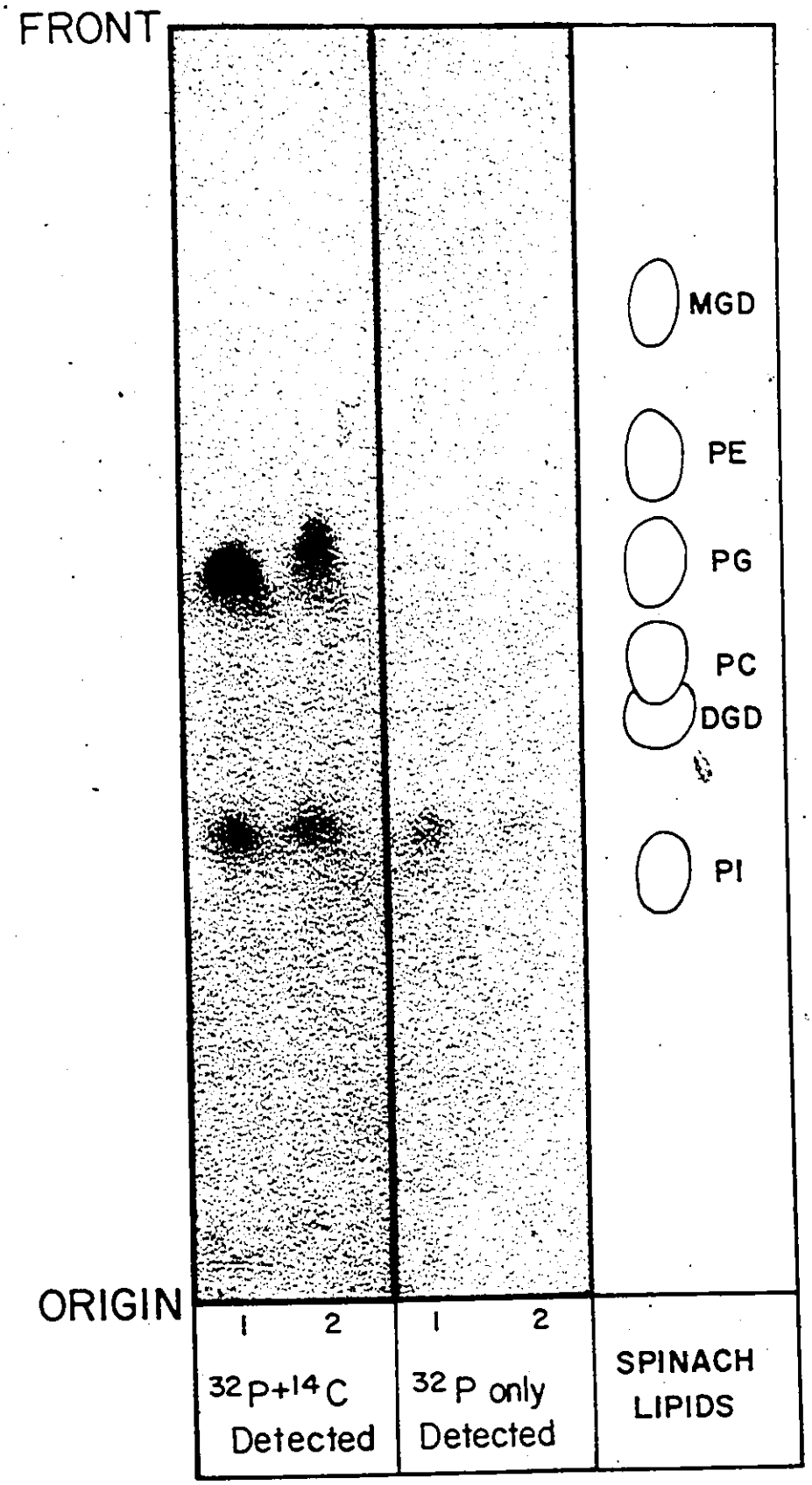

FRONT
MGD
PE
PG
PC
DGD
PI
ORIGIN
1
2
1
2
32P+14C Detected
32P only Detected
SPINACH LIPIDS

FIGURE 6

Autoradiogram of a chromatogram of phosphatidyl [1,3-^{14}C]glycerol synthesized by the 40,000xg cell fraction from spinach and E.coli.

1. Spinach [^{14}C]PG, synthesized as described in Methods V.1.b.iv.
2. E.coli [^{14}C]PG, synthesized as described in Methods V.1.b.iii.

Chromatography was on silica gel H plates in chloroform-methanol-acetic acid-water (65:25:8:4 by vol.).

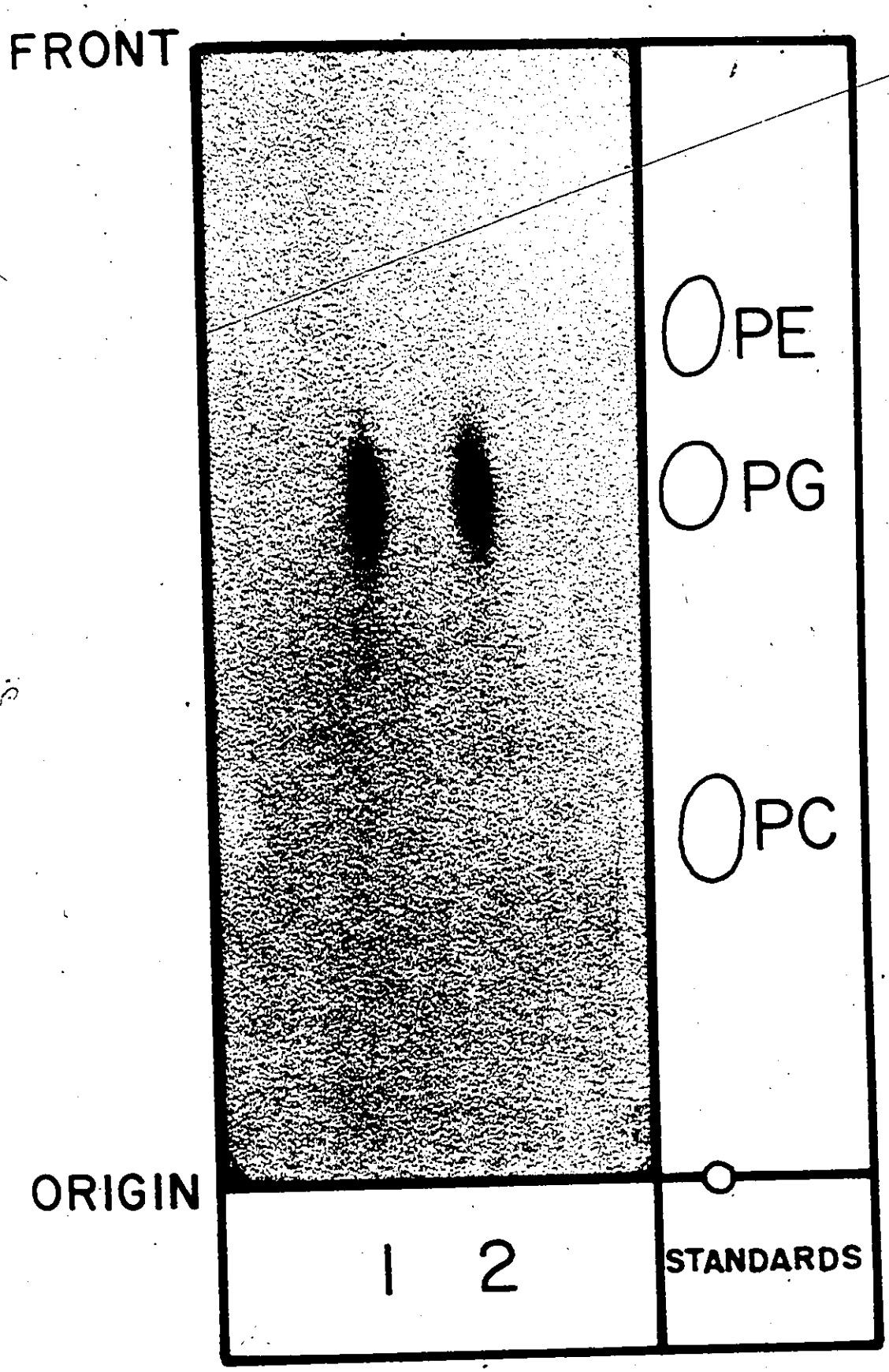
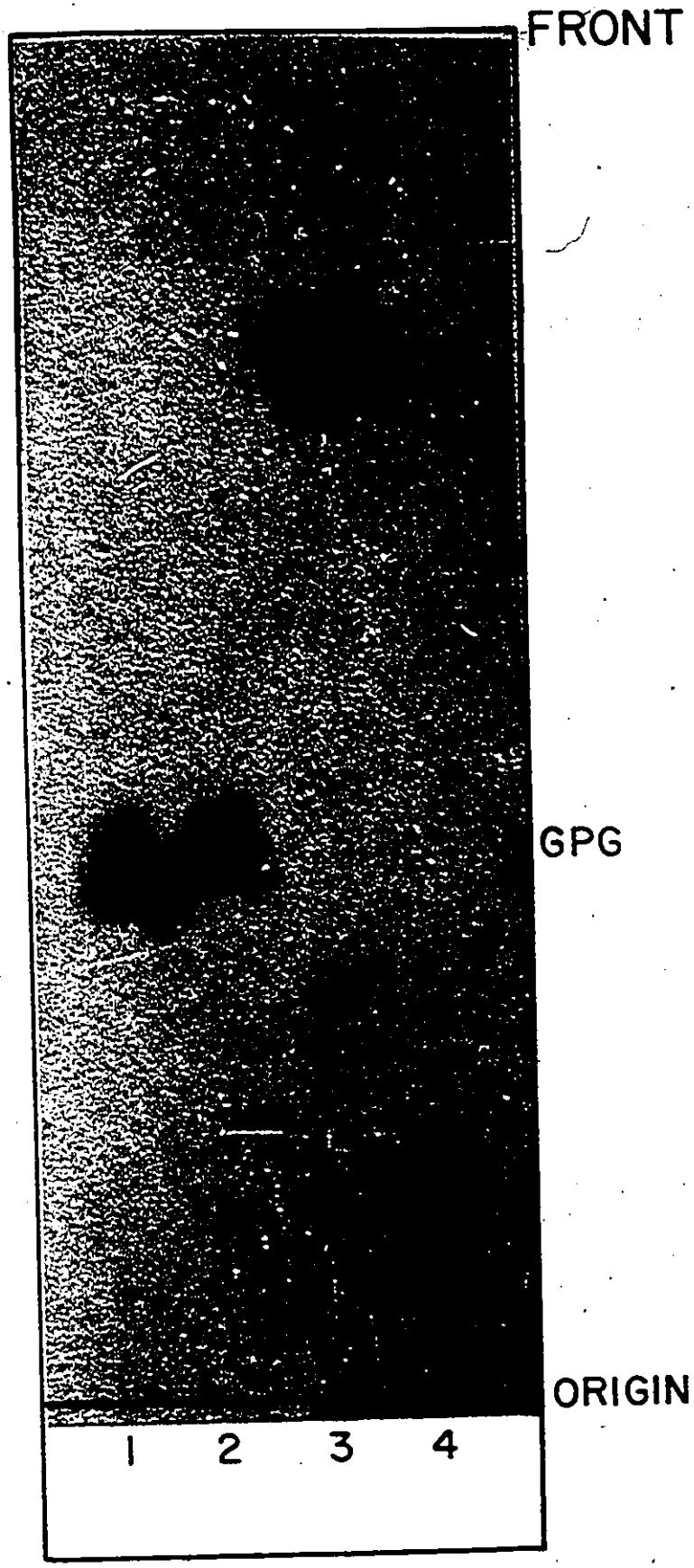

FRONT
PE
PG
PC
ORIGIN
1 2
STANDARDS

FIGURE 7

Autoradiogram of a chromatogram of glycerol phosphoryl [^{14}C]glycerol formed by deacylation of phosphatidyl [1,3-^{14}C]glycerol synthesized by the 40,000xg cell fraction from spinach and *E.coli*.

1. Spinach GP[^{14}C]G
2. *E.coli* GP[^{14}C]G
3. [1-^{14}C]Glycerol
4. *sn*-[1,3-^{14}C]Glycerophosphate

Chromatography was on Whatman No. 1 paper in phenol-water (100:40 w/w).


FRONT
GPG
ORIGIN
1
2
3
4

(Fig. 5) was identified as PG as follows: After incubation of [^{14}C]GP with the 40,000xg fraction, the major ^{14}C lipid product was isolated as described in Methods V.1.b.iv. The product migrated on TLC (see Fig. 6), with R_f value identical to that of authentic [^{14}C]PG (Methods V.1.b.iii). Deacylation gave a single radioactive water soluble product having an R_f value on paper chromatography in phenol-water (100:40, W/W) identical with that of [^{14}C]glycerophosphorylglycerol formed from authentic [^{14}C]PG (see Fig. 7).

The minor lipid product labelled with ^{14}C and ^{32}P from sn-[1,3-^{14}C]glycerol-3-[^{32}P]phosphate (see Fig. 5) was not rigorously identified, but it is likely to be phosphatidyl glycerophosphate since it had a similar mobility (R_f, 0.38, Fig. 5) to that of authentic phosphatidyl [1,3-^{14}C]glycerophosphate (R_f, 0.41; measured on a separate chromatogram) prepared as described in Methods V.1.b.v.

The question arose as to the pathway by which radioactivity from labelled GP was incorporated into PG and PGP. The possibility that GP was first dephosphorylated to glycerol which was then incorporated into PG by an exchange reaction was ruled out by the results of an experiment comparing the relative incorporation of [^{14}C]GP and [^{14}C]glycerol into PG under the same assay conditions. The results in Table 3 show that ^{14}C was incorporated into PG from [^{14}C]glycerophosphate but not from [^{14}C]glycerol. Furthermore, [^{14}C]glycerol could not be detected in the methanol-water soluble products from

TABLE 3. THE RELATIVE INCORPORATION OF [^{14}C] GLYCEROPHOSPHATE AND [^{14}C] GLYCEROL INTO PHOSPHATIDYL GLYCEROL

Substrate	^{14}C-Incorporation into PG[a] dpm
sn[1,3-^{14}C]glycerol-3-phosphate	1200
[1-^{14}C]glycerol	0

[a]Assay system (vol. 1 ml): 0.1 mM CDP-diglyceride (Koch-Light Co.), 10 mM $MgCl_2$, 1.5 mg Triton X-100, 0.25M Tris HCl (pH 7.3), 0.6 ml of 40,000xg fraction suspended in homogenizing medium, and either 800 µM sn-[1,3-^{14}C]glycerol-3-phosphate (1.6×10^5 dpm), or 3 µM [1-^{14}C]glycerol (8×10^4 dpm). Incubation for 3h at 37°C. The 40,000xg fraction was prepared as described in Fig. 5.

the [^{14}C]GP incubation when these were chromatographed on Whatman No. 1 paper in butanol-acetic acid-water (5:3:1 by vol.); unreacted [^{14}C]GP (R_f, 0.09) was the only product (R_f of standard glycerol, 0.48).

The formation of [^{14}C]PG was absolutely dependent on the presence of exogenous CDP-diglyceride (Table 4), strongly indicating that CDP-diglyceride was an obligatory substrate. Maximum synthesis of PG occurred at a CDP-diglyceride concentration of 0.04 mM, above which there was a marked inhibition (Table 4 and Fig. 8).

Thus, the above evidence shows that the 40,000xg cell fraction from spinach leaves contains an enzyme system catalyzing the biosynthesis of phosphatidyl glycerol from CDP-diglyceride and sn-glycerol-3-phosphate. Furthermore, the finding that PG formed from sn-[1,3-^{14}C]glycerol-3-[^{32}P] phosphate as substrate was labelled only with ^{14}C, whereas the ^{32}P was incorporated only into the "PGP" spot (Fig. 5), shows that the biosynthetic pathway most likely involves the intermediate formation of PGP, which is then dephosphorylated to PG (see eqns. 7-8).

The formation of PG from CDP-diglyceride and [^{14}C]GP by the 40,000xg fraction was linear with time of incubation up to 1 h, after a brief lag phase of about 2 min (Fig. 9), and with protein concentration up to 0.35 mg/ml (Fig. 10B). It should be pointed out that in these two studies the ^{14}C-incorporation into the total lipids was taken as a measure of

formation of PG, and no correction was made for any PGP that might have been present in the final products.

The pH optimum of PG formation in Tris HCl was 7.3 (Fig. 11A); over the range of pH covered only small amounts of PGP were detected (2-10% of total ^{14}C products). The temperature optimum of PG formation measured by the incorporation of ^{14}C into the total lipid extract was found to be 37°C (Fig. 11B). The thermal stability of the PG synthesizing system at the optimum temperature was determined by pre-incubating the 40,000xg fraction at 37°C for varying lengths of time up to 3 h, and then assaying for its activity. The rate of PG formation was constant for all pre-incubation times up to 1 h, but decreased thereafter to 70% of the initial activity after 3 h pre-incubation (Fig. 12). At no time was PGP formation observed, indicating that the PGP phosphatase is not more heat labile than the PGP synthetase. In some experiments (see Tables 7 and 8 and Fig. 11A), the 40,000xg fraction was stored at -10°C prior to assay. The loss of activity during a 3-day storage period was approximately 10%.

PG formation was dependent upon Mg^{2+}, half maximal activity being observed at 2 mM Mg^{2+} (Fig. 13); at all concentrations of Mg^{2+}, PG was the only product detected. Mn^{2+} was also found to be effective as a co-factor, but at a concentration of 10 mM it was only half as effective as Mg^{2+}. The effect of varying Triton X-100 concentration was not

TABLE 4. THE EFFECT OF CDP-DIGLYCERIDE CONCENTRATION ON PHOSPHATIDYL GLYCEROL SYNTHESIS

CDP-diglyceride concentration, mM	Phosphatidyl glycerol formed[a] n moles/h
0	0.0
0.02	14.3
0.04	14.8
0.08	12.9
0.16	10.2
0.25	9.5

[a]Assay system (vol. 1 ml): 1 mM [^{14}C]GP (8.5 x 10^5 dpm), 30 mM $MgCl_2$, 0.25M Tris HCl (pH 7.4), 1.5 mg Triton X-100, 0.2 ml (0.33 mg protein) of 40,000xg fraction suspended in 0.1M potassium phosphate (pH 8.0) containing 1 mM β-mercaptoethanol, and varying amounts of CDP-diglyceride (Koch Light Co.) as indicated. The 40,000xg fraction was prepared as described in Experimental II.1 except that homogenizing medium contained 0.1M potassium phosphate instead of Tris HCl. Incubation, 1h at 37°C. ^{14}C-Labelled lipid products were chromatographed on silicic acid impregnated paper in diisobutyl ketone-acetic acid-water (8:5:1 by vol.). [^{14}C]PG was the only product detected.

FIGURE 8

The effect of CDP-diglyceride concentration on phosphatidyl glycerol synthesis by the 40,000xg fraction.

Data obtained from Table 4.

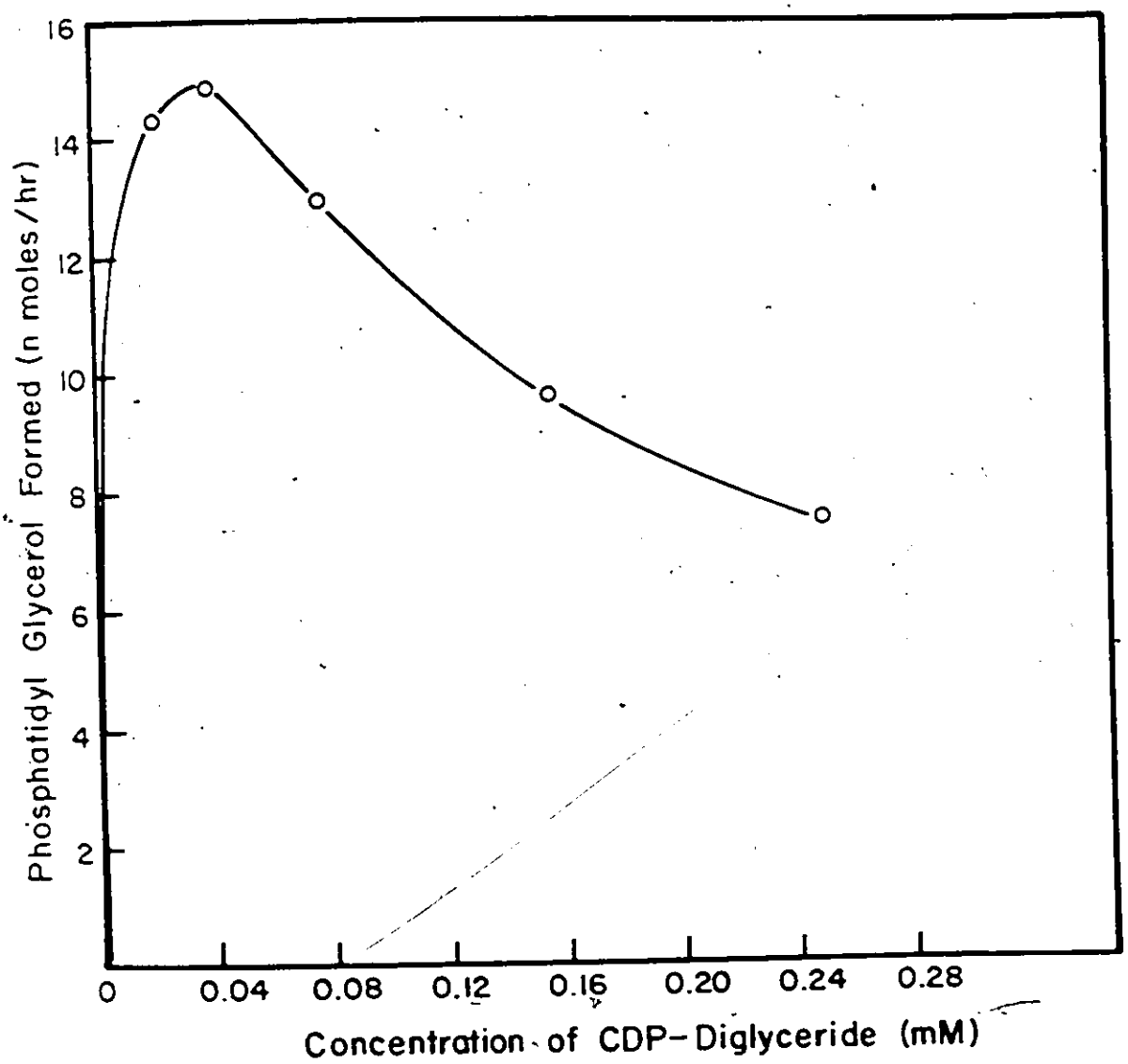

16
14
12
10
8
6
4
2
0
0.04
0.08
0.12
0.16
0.20
0.24
0.28
Phosphatidyl Glycerol Formed (n moles/hr)
Concentration of CDP–Diglyceride (mM)

FIGURE 9

Time course of phosphatidyl glycerol synthesis by the 40,000xg fraction.

Assay system (vol. 1 ml): 1 mM [^{14}C]GP (1×10^6 dpm), 0.1 mM CDP-diglyceride (Koch-Light Co.), 30 mM $MgCl_2$, 0.25 M Tris HCl (pH 7.3), 1.5 mg Triton X-100, and 0.2 ml of 40,000xg fraction suspended in 0.1 M potassium phosphate (pH 8.0), and prepared as described in Table 4.

Reactions were started at the same time and stopped at the times indicated. The incorporation of ^{14}C into total lipids was taken as a measure of PG formation.

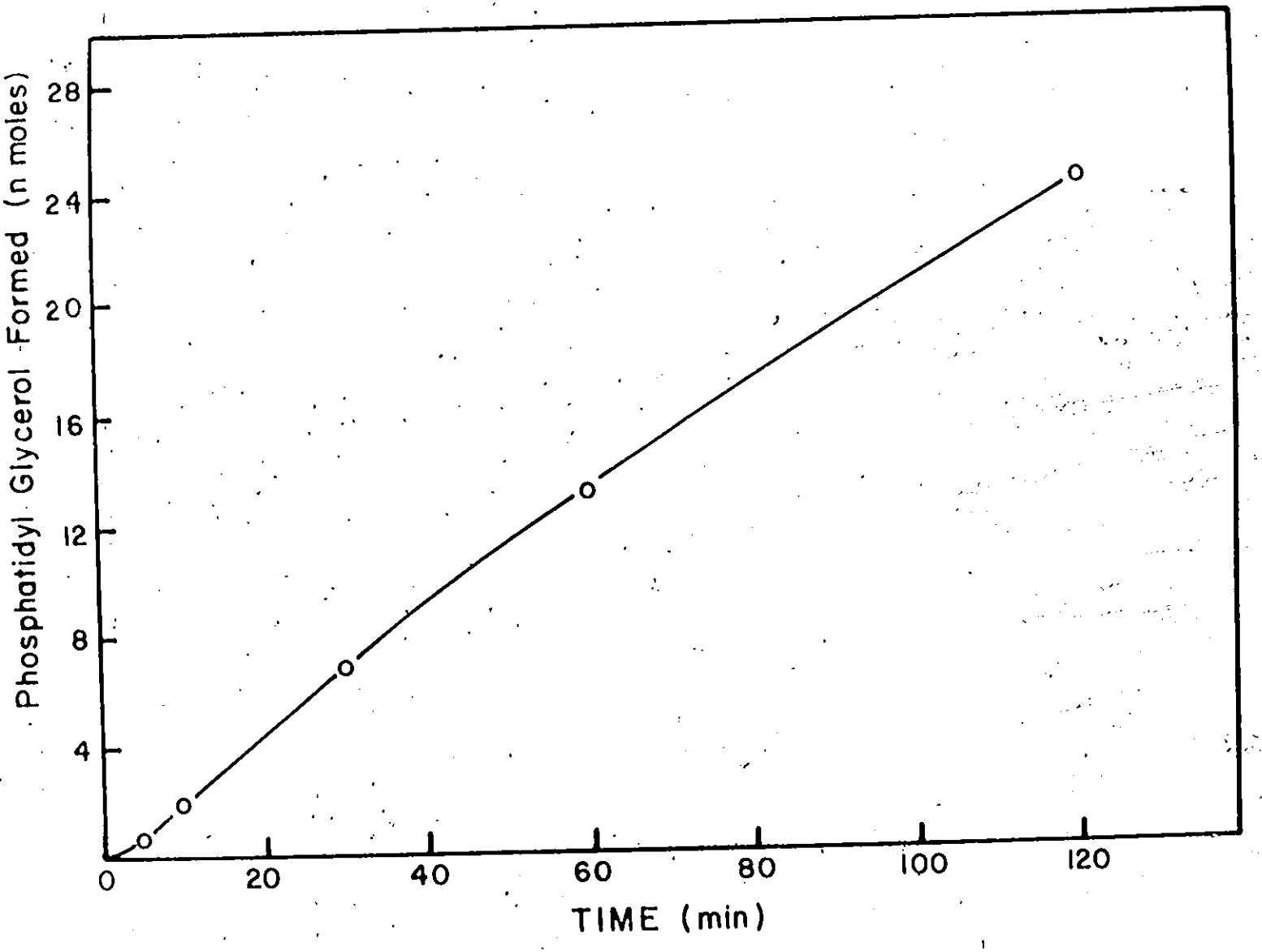

Phosphatidyl Glycerol Formed (n moles)
28
24
20
16
12
8
4
0
20
40
60
80
100
120
TIME (min)

FIGURE 10

The effect of protein concentration on phosphatidyl glycerol synthesis by the cell free homogenate and 40,000xg fractions.

A. Cell-free homogenate

B. 40,000xg fraction

Assay system as described in Table 7, but containing varying amounts of either cell free homogenate or 40,000xg fraction, as indicated. Cell fractions were those assayed in the subcellular distribution study described in Table 7. The incorporation of ^{14}C into the total lipids was taken as a measure of PG formation.

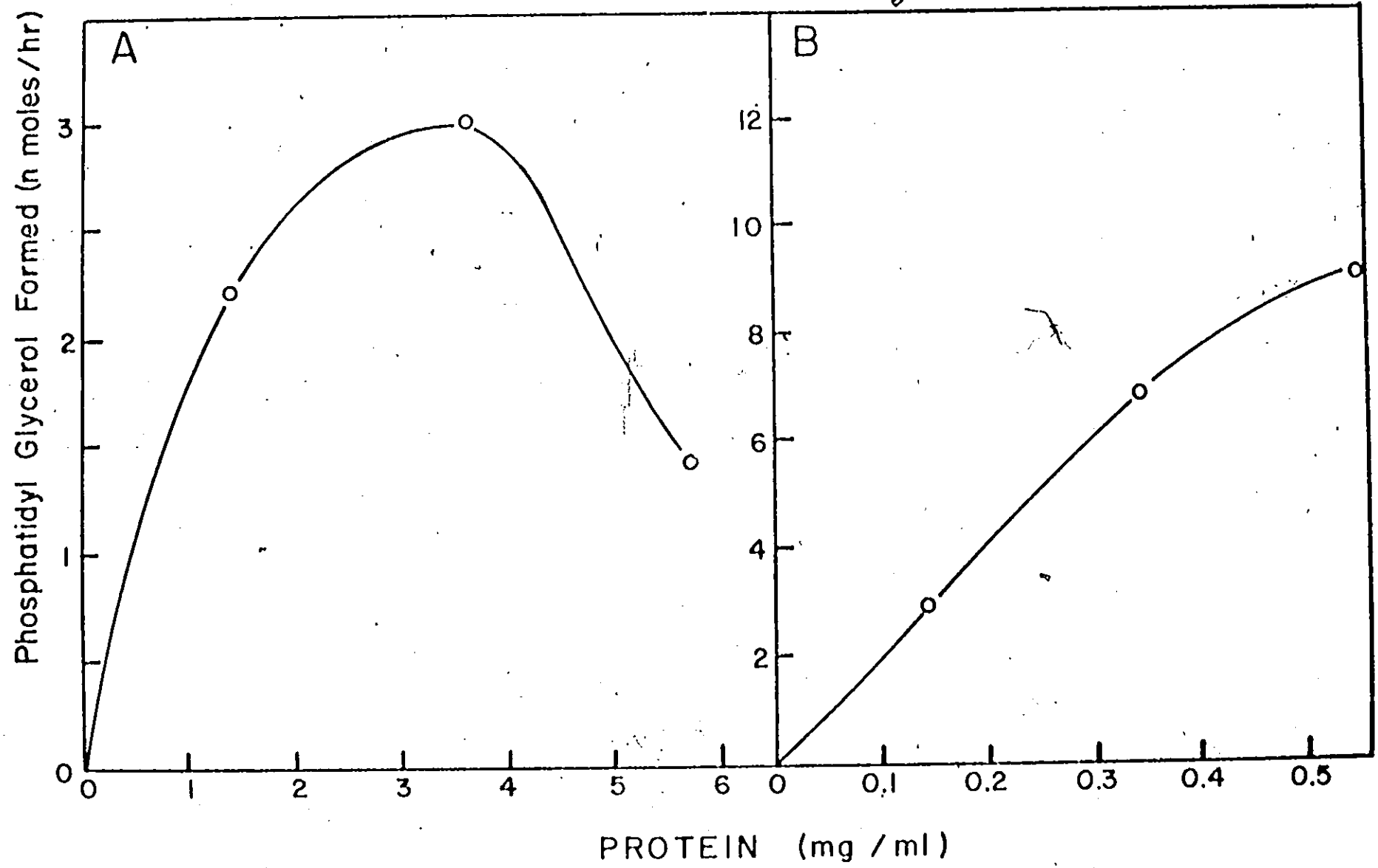

A
B
Phosphatidyl Glycerol Formed (n moles/hr)
0
1
2
3
0
1
2
3
4
5
6
2
4
6
8
10
12
0
0.1
0.2
0.3
0.4
0.5
PROTEIN (mg / ml)

FIGURE 11

The effect of pH and temperature on phosphatidyl glycerol synthesis by the 40,000xg fraction.

A. pH

Assay system as described in Figure 5, except that 0.25 M Tris HCl at pH 7.0, 7.5, 8.0, 8.5 or 9.0 was used, and enzyme source was 0.3 ml of 40,000xg fraction suspended in homogenizing medium and stored at -15°C for 3 days before assay. The preparation of 40,000xg fraction and chromatography of lipid as described in Figure 5. The pH values shown in the figure are the measured pH of the final reaction mixture.

B. Temperature

Assay system (vol. 1 ml): 1 mM [^{14}C]GP (8.5×10^5 dpm), 0.08 mM CDP-diglyceride (Koch-Light Co.), 30 mM $MgCl_2$, 0.25 M Tris HCl (pH 7.3), 1.5 mg Triton X-100 and 0.2 ml of 40,000xg fraction, prepared as described in Figure 5, suspended in 0.1 M pot. phosphate (pH 8.0) containing 1 mM β-mercaptoethanol. Incubation for 1 h at temperatures indicated. Incorporation of ^{14}C into total lipids was taken as a measure of PG formation.

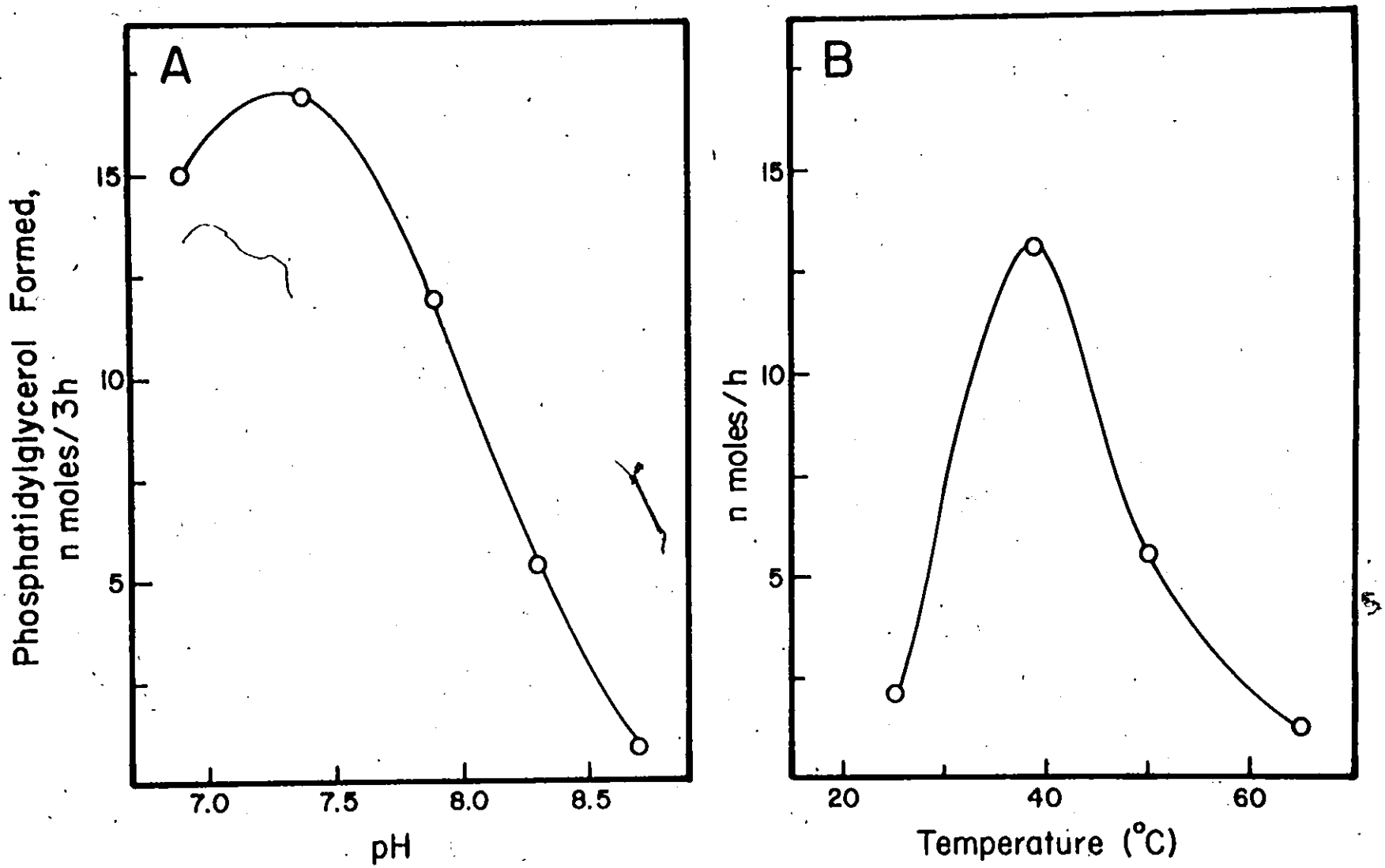

A
Phosphatidylglycerol Formed,
n moles/3h
15
10
5
7.0
7.5
8.0
8.5
pH
B
n moles/h
15
10
5
20
40
60
Temperature (°C)

FIGURE 12

Stability of the 40,000xg phosphatidyl glycerol synthesizing enzyme system at 37°C.

Assay system (vol. 1 ml): 1 mM [^{14}C]GP (8.1×10^5 dpm), 0.1 mM CDP-diglyceride (Koch Light Co.), 30 mM $MgCl_2$, 0.25 M Tris HCl (pH 7.4), 1.5 mg Triton X-100 and 0.25 ml of 40,000xg pellet suspended in 0.1 M potassium phosphate (pH 7.6) containing 5 mM β-mercapto-ethanol. Incubation, 1 h at 37°C. Prior to assay, the 40,000xg suspension was pre-incubated at 37°C for different times as indicated. Preparation of 40,000xg fraction and chromatography of lipid products as described in Figure 5.

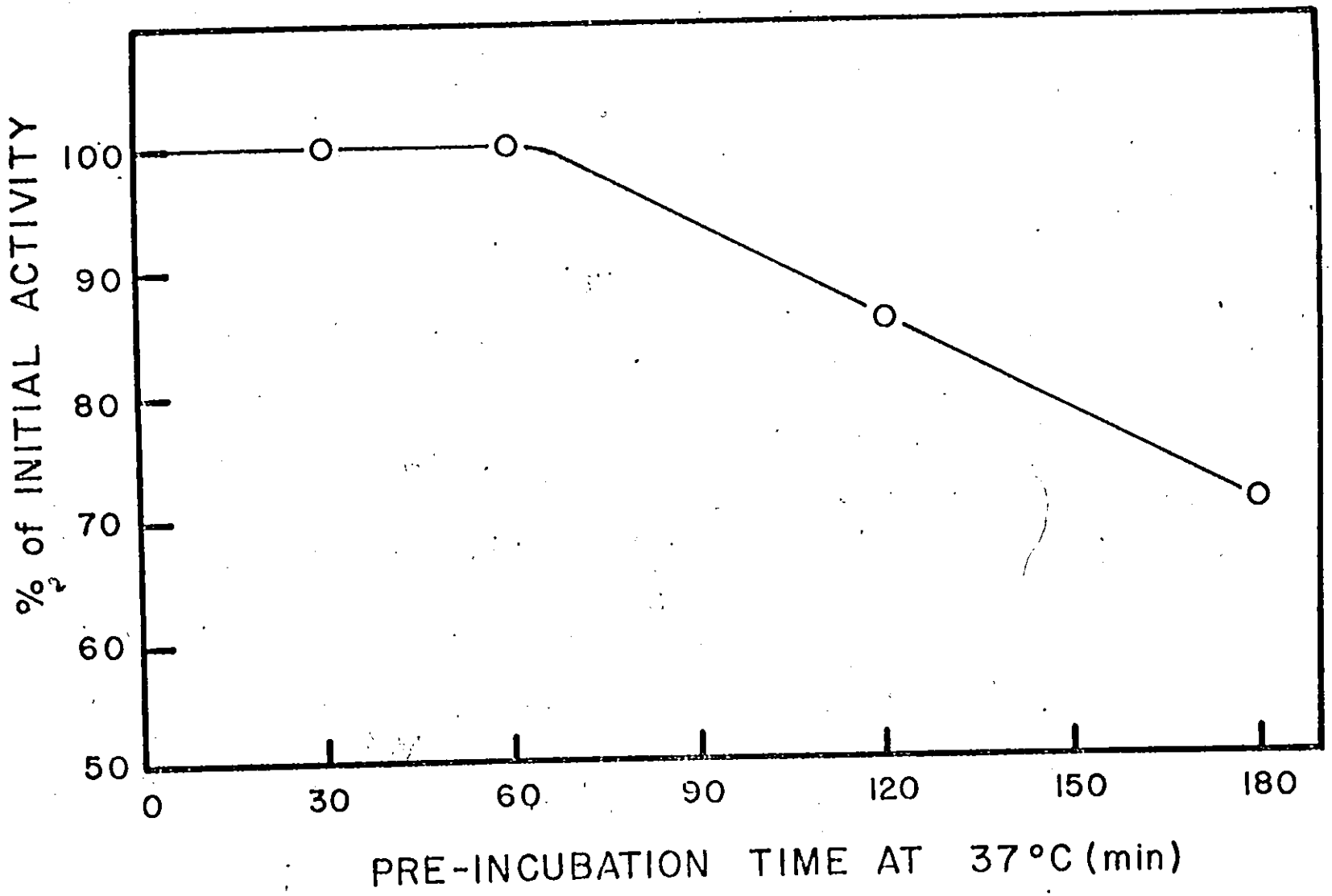
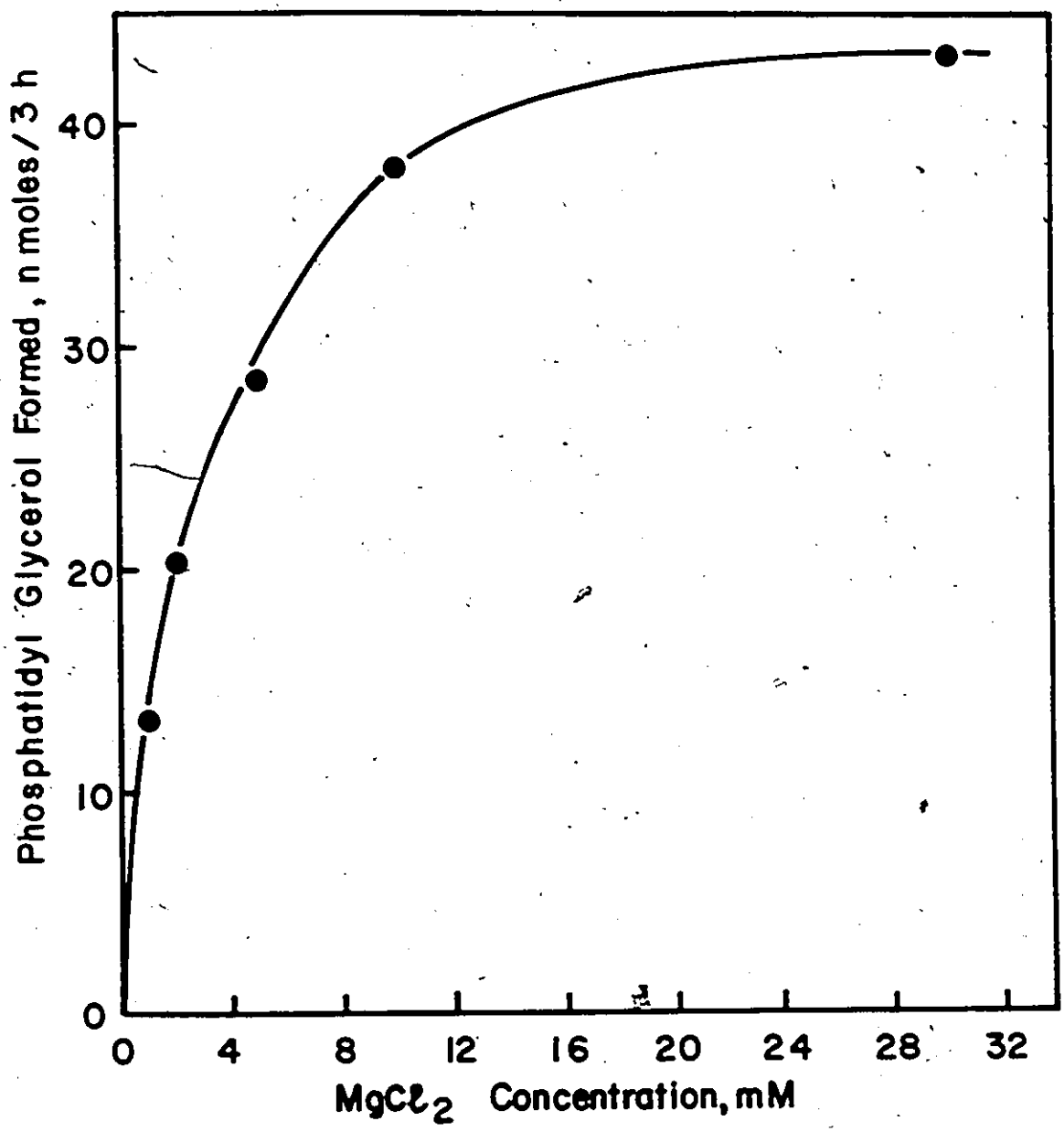

% of INITIAL ACTIVITY
100
90
80
70
60
50
0
30
60
90
120
150
180
PRE-INCUBATION TIME AT 37°C (min)

FIGURE 13

The effect of $MgCl_2$ concentration on phosphatidyl glycerol synthesis by the 40,000xg fraction.

Assay system (vol. 1 ml): 0.4 mM [^{14}C]GP (1.2×10^5 dpm), 0.1 mM CDP-diglyceride (Koch Light Co.), 0.25 M Tris HCl (pH 7.3), 0.2 ml of 40,000xg fraction suspended in homogenizing medium containing phosphate buffer (0.33 mg protein), and varying amounts of $MgCl_2$ as indicated. Incubation, 1 h at 37°C. Preparation of 40,000xg fraction and chromatography of lipid products as described in Figure 5.


Phosphatidyl Glycerol Formed, n moles / 3 h
0
10
20
30
40
0
4
8
12
16
20
24
28
32
MgCl2 Concentration, mM

studied, though it was observed that in the absence of Triton little or no PG was formed.

Data on the effect of sn-glycerol-3-phosphate concentration on the formation of PG and PGP is shown in Table 5 and Fig. 14. Under assay conditions of optimum pH and Mg^{2+} concentration, and a saturating concentration of CDP-diglyceride, the initial rate of formation of PG showed typical Michaelis-Menten kinetics and reached a maximum at a GP concentration of 1.3 mM (Fig. 14A). For a GP concentration of 1 mM the initial rate of formation of PG could be validly expressed as the amount of PG formed in 1 h, since the formation of PG was reasonably linear up to 1 h (see Fig. 9). At other GP concentrations, the formation of PG as a function of time was not determined, but was assumed to be reasonably linear up to 1 h.

In this particular experiment, an amount of PGP intermediate accumulated and reached a steady state above a GP concentration of 0.53 mM (Fig. 14A). However, at all concentrations of GP, PGP accounted for only a small proportion of total phosphatide formed (8-14%, Table 5), showing that the rate determining step in PG synthesis from GP is the first step catalyzed by PGP synthetase. Thus,

$$\text{CDP-dig} + \text{GP} \xrightarrow[\text{slow}]{} \text{PGP} + \text{CMP} \qquad \text{step 1}$$

$$\text{PGP} \xrightarrow[\text{fast}]{} \text{PG} + \text{Pi} \qquad \text{step 2}$$

The Michaelis-Menten constant for GP in the first step was

calculated as follows: Using the nomenclature of Dixon and Webb (1964), the rate equation for the first step, assuming there is no effect of one substrate on the affinity by the enzyme for the other, is

$$\frac{-d[GP]}{dt} = \frac{V_{max}[GP][CDP\text{-}dig]}{(K_a+[GP])(K_b+[CDP\text{-}dig])} \qquad (28)$$

where K_a and K_b are the Michaelis-Menten constants for GP and CDP-diglyceride, respectively.

Under conditions of saturation by CDP-dig, [CDP-dig] >> K_b, and the equation reduces to

$$\frac{-d[GP]}{dt} = \frac{V_{max}[GP]}{K_a+[GP]} \qquad (29)$$

Since GP forms the products PG and PGP, it follows that

$$\frac{-d[GP]}{dt} = \frac{d(PG + PGP)}{dt} \qquad (30)$$

Thus,

$$\frac{d(PG + PGP)}{dt} = \frac{V_{max}[GP]}{K_a + [GP]} \qquad (31)$$

The plot of rate of (PG+PGP) formation vs. GP concentration showed typical Michaelis-Menten kinetics (Fig. 14A), and from the Lineweaver-Burke plot, shown in Fig. 14B, the Michaelis-Menten constant for GP was calculated to be 0.25 mM. For comparison, the corresponding K_a obtained from the Lineweaver-Burke plot of PG formation alone, was 0.20 mM.

TABLE 5. THE EFFECT OF sn-GLYCEROL-3-PHOSPHATE CONCENTRATION ON PHOSPHATIDYL GLYCEROL AND PHOSPHATIDYL GLYCEROL PHOSPHATE SYNTHESIS

Glycerol phosphate concentration mM	Formation[a] of PG+PGP	PG	PGP
		n moles/h	
0.13	7.9	7.3(92)	0.6(8)
0.26	11.6	10.3(89)	1.3(11)
0.53	15.1	13.0(86)	2.1(14)
0.79	16.6	14.4(87)	2.2(13)
1.32	17.2	15.1(88)	2.1(12)

[a]Assay system (vol. 1 ml): 0.1mM CDP-diglyceride (Koch Light Co.), 30mM $MgCl_2$, 0.25M Tris HCl (pH 7.3), 1.5 mg Triton X-100, 0.2 ml of 40,000xg fraction prepared as described in Table 4, suspended in homogenizing medium, and varying amounts of [^{14}C]GP (sp.act. 7.7 x 10^5 dpm/µmole) as indicated. Incubation, 1h at 37°C. PG and PGP were separated on silicic acid impregnated paper in diisobutyl ketone-acetic acid-water (8:5:1 by vol.) and counted separately as described in Experimental II.3.a.

Figures in parenthesis denote amount of each lipid as % of PG+PGP.

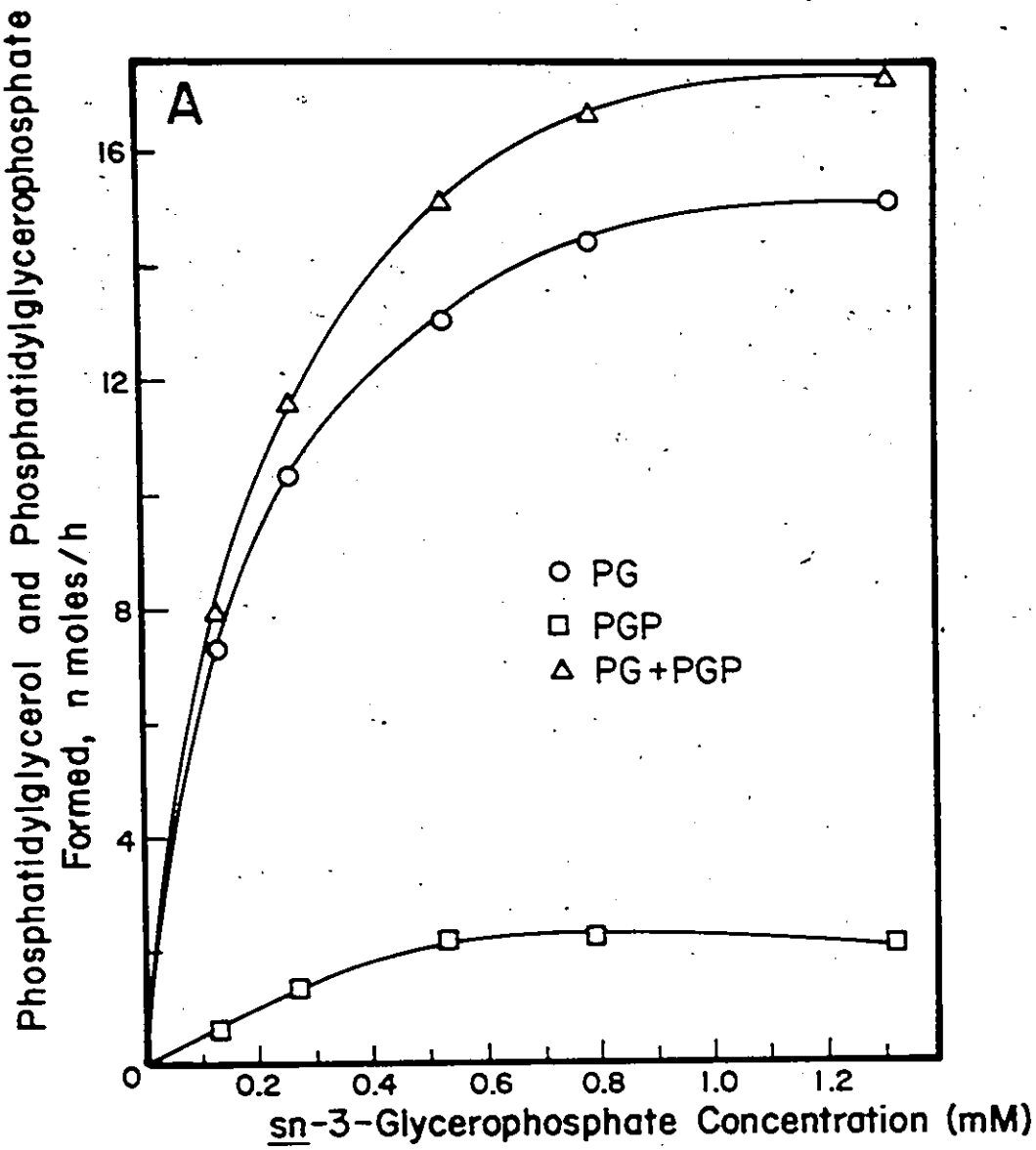
FIGURE 14

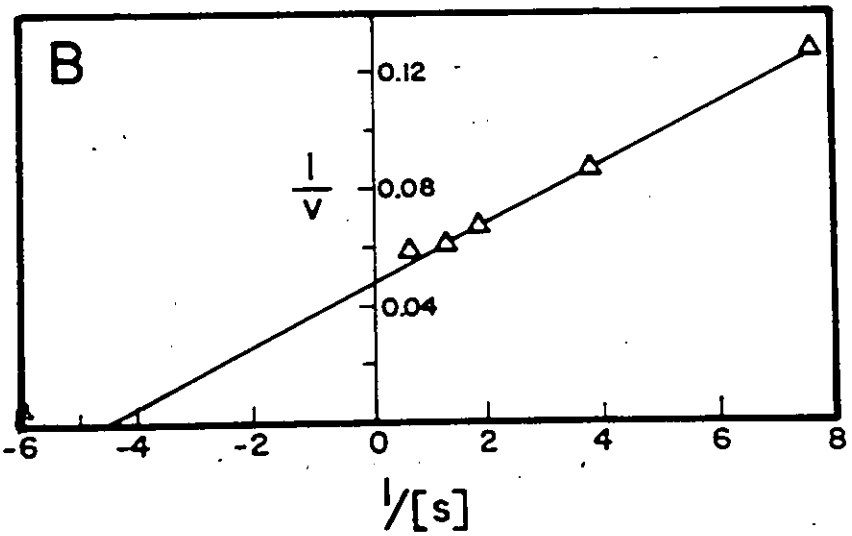
The effect of sn-glycerol-3-phosphate concentration on the synthesis of phosphatidyl glycerol and phosphatidyl glycerophosphate by the 40,000xg fraction.

Data obtained from Table 5.

A. Rate of lipid formation as a function of GP concentration.

B. Plot of 1/v vs 1/s, where v is the rate of (PG + PGP) formation and s is GP concentration.


A
Phosphatidylglycerol and Phosphatidylglycerophosphate Formed, n moles/h
16
12
8
4
0
PG
PGP
PG+PGP
0.2
0.4
0.6
0.8
1.0
1.2
sn-3-Glycerophosphate Concentration (mM)


B
0.12
1/v
0.08
0.04
-6
-4
-2
0
2
4
6
8
1/[s]

The effect of sulfhydryl reacting reagents on PG synthesis by the 40,000xg fraction is shown in Table 6. PG synthesis was not significantly inhibited (8%) by $HgCl_2$ at a concentration (10 mM) reported to be completely inhibitory for PG synthesis in cell free preparations from cauliflower inflorescence (Douce and Dupont, 1969), chicken liver (Kiyasu et.al., 1963) and E.coli (Chang and Kennedy, 1967). Furthermore, only 17% inhibition was observed on increasing the $HgCl_2$ concentration to 40 mM. The most pronounced inhibition was observed with p-chloromercuribenzoate, but still amounted to only 21%. No PGP was detected in any of the inhibition assay systems. It should be pointed out that a small amount of β-mercaptoethanol derived from the 40,000xg suspension was present in the reaction mixtures, but at a concentration (0.25 mM) too low to react with significant amounts of sulfhydryl reagent. Furthermore, it was found that prior dialysis of the 40,000xg fraction against phosphate buffer did not affect the results. Since none of the sulfhydryl reacting reagents inhibited PG synthesis to any great extent, nor resulted in accumulation of PGP, it was concluded that neither PGP synthetase nor PGP phosphatase contained sulfydryl groups actively taking part in their catalytic action.

TABLE 6. THE EFFECT OF SULFYDRYL GROUP REACTING REAGENTS ON PHOSPHATIDYL GLYCEROL SYNTHESIS BY THE 40,000xg FRACTION

Assay system[a]	PG formed n moles/h	% Inhibition
Complete	16.8	-
" + $HgCl_2$ (10mM)	15.3	8
" + $HgCl_2$ (40 mM)	14.0	17
" + PCMB (5mM)	13.3	21
" + Iodoacetate (5mM)	17.0	0

[a]Complete assay system as described in Table 4; concentration of CDP-diglyceride, 0.1mM. Enzyme source: 0.25 ml of a fresh suspension of the 40,000xg fraction (1.6 mg protein/ml) in 0.1M pot. phosphate buffer (pH 8.0) containing 1mM β-mercaptoethanol. Preparation of 40,000xg fraction and chromatography of assay products as described in Table 4.

Abbreviation: PCMB, p-chloromercuribenzoate.

2. Subcellular distribution of the phosphatidyl glycerol synthesizing system

The results of this study are shown in Table 7. The recovery of protein and chlorophyll, and of PG synthetase and cytochrome c oxidase activities after cell fractionation was virtually quantitative. Quantitative recovery of enzyme activity is usually obtained when the enzyme concentration for each cell fraction assayed lies in the linear range of the activity-enzyme concentration plot. For cytochrome c oxidase and catalase this condition was met (see Experimental II.2., Fig. 4). The low recovery of catalase activity may perhaps be connected with the long period of storage at -10°C of cell fractions prior to assay. In the PG synthetase assay, 0.04 ml (0.57 mg protein) of the cell-free homogenate and 0.2 ml (0.27 mg protein) of the 40,000xg pellet fraction were assayed, and at these protein levels, PG synthesis in the two fractions was linear with protein concentration (Fig. 9A and B). Therefore, for these two fractions the condition discussed above was met. For the 1200xg and 15,000xg pellets and the 40,000xg supernatant, 0.2 ml was assayed, containing 3.3, 1.7 and 0.9 mg protein respectively. The effect of protein concentration on PG synthesis in these fractions was not investigated, but since the recovery of enzyme activity was almost quantitative (108%), enzyme activity in these fractions at the levels of protein assayed was presumably linear with protein concentration.

TABLE 7. SUB-CELLULAR DISTRIBUTION OF PHOSPHATIDYL GLYCEROL SYNTHESIZING ACTIVITY[a]

Cell Fraction	Protein		Chloro-phyll		PG Synthesis[b]			Cytochrome C Oxidase[c]			Catalase[d]		
	mg	%	mg	%	Activity nmoles/hr	%	Specific Activity	Activity nmoles/s	%	Specific Activity	Activity nmoles/s	%	Specific Activity
Cell free homogenate	427	100	11.8	100	306	100	0.7	515	100	1.2	330	100	0.8
1200xg	98	23	7.4	63	13	4	0.1	97	19	1.0	16	5	0.2
15,000xg	50	12	4.8	41	46	15	0.9	363	71	7.3	66	20	1.3
40,000xg	8.5	2	0.3	3	139	45	16.0	13	3	1.5	0	0	0.0
40,000xg-supernatant[e]	304	71	0.2	2	133	44	0.4	32	6	0.1	113	34	0.4
% recovery		108		109		108			99			59	

[a]Sub-cellular fractionation was carried out as described in Experimental II.1 except that homogenizing medium contained 0.1M potassium phosphate (pH 8.0) instead of Tris HCl. Volume of cell fractions: cell-free homogenate, 38 ml; 1200xg and 15,000xg, 6.0 ml; 40,000xg, 6.2 ml; 40,000xg supernatant, 6.7 ml.

[b]Assay mixture (vol. 1 ml): 0.36 mM [^{14}C]GP (1.3 x 10^6 dpm), 0.04 mM CDP-diglyceride (Koch Light Co.), 30 mM $MgCl_2$, 0.25M Tris HCl (pH 7.3), 1.5 mg Triton X-100, and either 0.04 ml of cell free homogenate or 0.2 ml of remaining fractions suspended in homogenizing medium. Incubation, 1h at 37°C. Cell fractions were stored for one day at -10°C prior to assay. Lipid products chromatographed as described in Table 4. [^{14}C]PG was the only product formed by all fractions.

[c]Cell fractions stored for one day at -10°C, and then assayed as described in Experimental II.2.a; values are average of duplicate assays.

[d]Cell fractions stored for six days at -10°C, and then assayed as described in Experimental II.2.b; values are average of duplicate assays.

[e]In separate experiments, further centrifugation of this fraction at 90,000xg or 100,000xg for 1h sedimented about 25-30% of its PG synthesizing activity.

The study reported in Table 7 showed that the 40,000xg fraction contained a high proportion (45%) of phosphatidyl glycerol-synthesizing activity, and the highest specific activity. The 40,000xg supernatant also contained a high proportion of activity (44%), but with a low specific activity, and about 25% of this activity could be sedimented by further centrifugation at 90,000xg. A small (15%) but significant proportion of activity was associated with the 15,000xg fraction, but little activity (4%) was found in the 1200xg fraction. Essentially the same distribution pattern was obtained when the cell fractions were ultrasonicated prior to assaying for phosphatidyl glycerol-synthesizing activity.

Chlorophyll was concentrated in the chloroplast (1200xg) fraction; some was also present in the 15,000xg fraction, but was virtually absent from the 40,000xg fraction. Cytochrome c oxidase, the specific marker for mitochondria (Experimental II.2.a) was concentrated in the 15,000xg mitochondrial fraction; a small proportion (19%) was also found in the 1200xg fraction, but little activity (3%) was present in the 40,000xg fraction. Catalase, the specific marker for peroxisomes (Experiment II.2.b), was concentrated in the 15,000xg pellet and the 40,000xg supernatant fractions, but was completely absent from the 40,000xg fraction. The high proportion of activity in the supernatant is in agreement with that reported by Tolbert *et al.* (1968) for spinach.

The phosphatidyl glycerol-synthesizing system thus appears to be localized primarily in the 40,000xg fraction which, being essentially free of chloroplasts, peroxisomes and mitochondria, is most probably a microsomal fraction. The question whether the PG synthetase activity detected in the 15,000xg fraction was due to microsomal contamination or to a separate mitochondrial system cannot be answered unambiguously since the 15,000xg fraction was not assayed specifically for microsomal marker enzymes. In a separate experiment attempts were made to assay the cell fractions for glucose-6-phosphatase, which in animal tissues is localized in the microsome fraction (Ginsburg and Hers, 1960). However, using the assay procedure of Harper (1965), it was found that 70% of the original total cell-free homogenate activity was localized in the 90,000xg supernatant, while only 30% was present in the 600-90,000xg total particulate fraction. Since the 90,000xg supernatant would not be expected to contain a high proportion of microsomes it was concluded that under the conditions of sub-cellular fractionation employed, glucose-6-phosphatase was not a valid microsomal marker. Indeed, this marker enzyme is now known to be localized in microsomal preparations from animal tissues (Ginsburg and Hers, 1960), but in plant tissues this enzyme is distributed in the cytoplasm as well as in the microsomes (Thompson, 1969; Harwood and Stumpf, 1972). Therefore, glucose-6-phosphatase was not used in the present study to determine microsomal distribution among cell fractions.

TABLE 8. THE EFFECT OF SULFYDRYL GROUP REACTING REAGENTS ON PHOSPHATIDYL GLYCEROL SYNTHESIS BY THE 15,000xg AND 40,000xg FRACTIONS

Assay System[a]	Final Protein Concentration mg/ml	PG formed n moles/h	% Inhibition
Complete (15,000xg fraction)	1.2	1.5	-
" $+HgCl_2$ (10 mM)	1.2	1.4	7
Complete (40,000xg fraction)	0.2	2.1	-
" $+HgCl_2$ (10 mM)	0.2	1.6	24

[a]Complete assay system and preparation of cell fractions as described in Table 7. Enzyme source: 0.2 ml of each cell fraction suspended in 0.1M phosphate buffer (pH 8.0) containing no β-mercaptoethanol, following 3 weeks storage at -10°C.

The possibility that the PG synthesizing activity in the 15,000xg fraction represented a separate mitochrondrial system was further studied. Since mitochondrial PGP phosphatase is known to be inhibited by sulfhydryl reagents (Chang and Kennedy, 1967b) an experiment was carried out to determine whether PG synthesis in the 15,000xg fraction could be affected by sulfhydryl reacting reagents. It was found that PG synthesis in this fraction was only slightly inhibited by 10 mM $HgCl_2$ which also did not significantly inhibit PG synthesis in the 40,000xg fraction (Table 8). Furthermore, no PGP was detected among the products from the assays containing $HgCl_2$. It was therefore concluded that the PG synthetase activity in the 15,000xg fraction most likely arose from microsomal contamination, but the possibility that it was associated with a separate mitochondrial system resistant to sulfhydryl reagent must also be considered.

II. The Biosynthesis of Phosphatidyl inositol

Assays were carried out using a particulate fraction (1200-100,000xg pellet) incubated with [U-^{14}C]myo-inositol, Mn^{2+} and Triton X-100, either in the presence or the absence of CDP-diglyceride. The rate of [^{14}C]PI synthesized in the absence of CDP-diglyceride was 0.8 n moles/h, and was stimulated three fold by the addition of CDP-diglyceride (see Table 9). When Triton X-100 was omitted from the assay systems PI synthesis in the presence of CDP-diglyceride was

decreased, whereas PI in the absence of CDP-diglyceride was increased (Table 9). However, both activities required Mn^{2+} (5 mM) which could not be replaced by Mg^{2+} or Ca^{2+} at the same concentration. The synthesis of PI in the presence of CDP-diglyceride was investigated as a function of reaction time (Table 10), but the data are uncorrected for synthesis of PI by the CDP-diglyceride independent pathway. However, the data include a value (0.71 n moles) for CDP-diglyceride independent synthesis of PI at 60 min.

The [^{14}C]PI structure of the labelled product from reactions carried out in the presence and absence of CDP-diglyceride was confirmed as follows: the product from each reaction migrated on TLC with an R_f identical to that of standard PI (see Fig. 15A). The products were isolated by preparative TLC (see Fig. 15A for solvent system) combined, and deacylated (see Methods III.1). The ^{14}C-labelled deacylated lipid migrated as a single ^{14}C-labelled spot with R_f identical to that of standard glycerophosphorylinositol on Whatman No. 1 paper in methanol-98% formic acid-water (80:13:7, by vol.), as shown in Fig. 15B. Since the combined product yielded only [^{14}C]glycerophosphorylinositol after deacylation it was concluded that the product formed from [U-^{14}C]*myo*-inositol either in the presence or absence of CDP-diglyceride was [^{14}C]phosphatidyl inositol.

With regard to the pathways of PI biosynthesis, the observed CDP-diglyceride independent synthesis of PI

TABLE 9. COFACTOR REQUIREMENTS FOR PHOSPHATIDYL INOSITOL SYNTHESIS

System	Phosphatidyl inositol synthesized, n moles/h
A. + CDP-diglyceride	
Complete[a]	8.5
" minus Triton	0.6
" minus Triton and Mn^{2+}	0.0
" minus Triton and Mn^{2+} plus Mg^{2+} (5 mM)	0.0
" minus Triton and Mn^{2+} plus Ca^{2+} (5 mM)	0.0
" boiled enzyme	0.0
B. - CDP-diglyceride	
Complete[b]	0.8
" minus Triton	1.5
" minus Triton and Mn^{2+}	0.0
" minus Triton and Mn^{2+} plus Mg^{2+} (5 mM)	0.0
" minus Triton and Mn^{2+} plus Ca^{2+} (5 mM)	0.0

[a] Complete assay system (vol. 1 ml): 0.1 mM [U-^{14}C]-*myo*-inositol (2.4×10^5 dpm), 0.064 mM CDP-diglyceride (prepared as described in Methods I.2.b.i), 0.1M Tris HCl (pH 8.0), 5 mM $MnCl_2$, and 0.15 ml of 1200-80,000xg pellet suspension (1.2 mg protein). Incubation, 1h at 30°C. ^{14}C incorporation into total lipids was taken as measure of PI synthesized.

[b] As in a), minus CDP-diglyceride.

TABLE 10. PHOSPHATIDYL INOSITOL SYNTHESIS IN THE PRESENCE OF CDP-DIGLYCERIDE AS A FUNCTION OF REACTION TIME

Reaction Time min	PI formed[a], n moles	
	plus CDP-dig.	minus CDP-dig.
0	0.0	-
20	0.82	-
40	1.00	-
60	1.19	0.71

[a]Assay system (vol. 1 ml): 0.05 mM [U-^{14}C] myo-inositol (2.4×10^5 dpm), 0.05 mM CDP-diglyceride (prepared as described in Methods L2.b.i), 20 mM $MnCl_2$, 20 mM $MgCl_2$, 0.2M Tris HCl (pH 8.5), 1.5 mg Triton X-100 and 0.3 ml of 600-100,000xg pellet fraction suspended in 50 mM Tris HCl (pH 8.0) containing 3 mM β-mercaptoethanol. Incubation was at 30°C for varying times indicated above. ^{14}C-Incorporation into total lipids was taken as a measure of PI synthesized. Results are corrected for a zero time blank (0.11 n moles). Dash (-) denotes data unavailable.

FIGURE 15

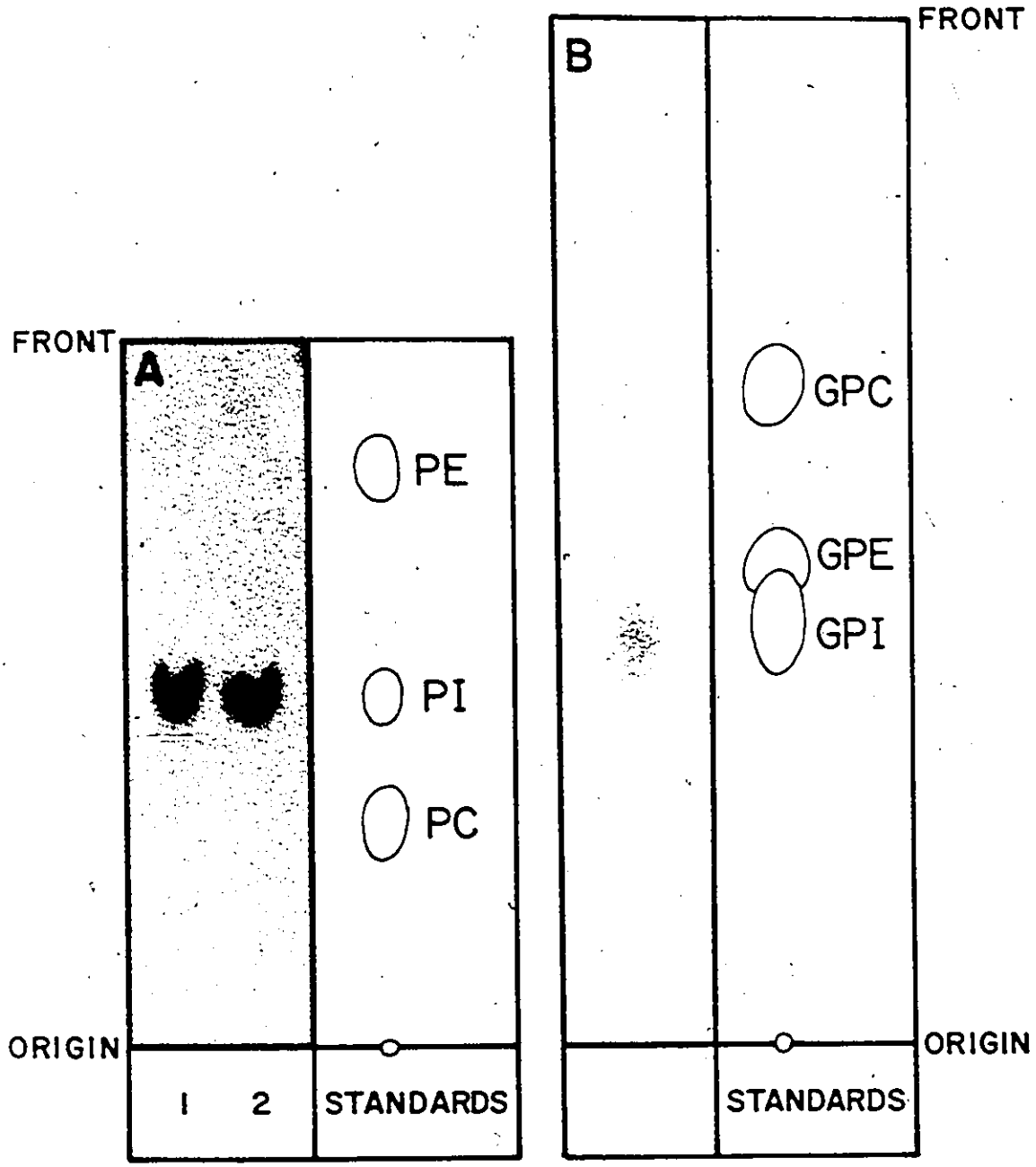
Autoradiogram of chromatograms of phosphatidyl [^{14}C]inositol and its deacylation product labelled from [U-^{14}C]myo-inositol in the presence and absence of CDP-diglyceride.

A. Phosphatidyl [^{14}C]inositol product from assay systems described in Table 9.
   1. in the presence of CDP-diglyceride
   2. in the absence of CDP-diglyceride

   Chromatography was on silica gel H plates in chloroform-methanol-acetic acid-water (65:25:8:4)

B. Deacylation product of combined phosphatidyl [^{14}C]inositol products from assays in presence and absence of CDP-diglyceride. GPI, GPE and GPC were prepared by deacylation of standard PI, PE and PC respectively.

Chromatography was on Whatman No. 1 paper in methanol-98% formic acid-water (80:13:7).


FRONT
A
PE
PI
PC
ORIGIN
1
2
STANDARDS
B
FRONT
GPC
GPE
GPI
ORIGIN
STANDARDS

(Tables 9 and 10) most likely represents an exchange reaction of the type described in Introduction III.2.d, whereas the CDP-diglyceride stimulated synthesis of PI most likely arises from CDP-diglyceride: inositol phosphatidyl transferase activity.

III. Biosynthesis of Nitrogenous Phospholipids. The Incorporation of ^{14}C Activity from [U-^{14}C]L-Serine, [1,2-^{14}C]Ethanolamine, [Me-^{14}C]L-Methionine and [1,2-^{14}C]Choline into Phospholipids by Whole Leaves and Leaf Slices

The results of this study are shown in Tables 11 and 12, and in Figures 16 and 17. They can be summarized as follows:

1) ^{14}C-Labelling of PS

In both whole leaves and leaf slices, a small proportion of ^{14}C from [^{14}C]serine was incorporated into a component chromatographically identical to PS (Fig. 16 and Table 11).

2) ^{14}C-Labelling of PE and PE-Me

a) With both whole leaves and leaf slices a high proportion of the ^{14}C in lipids labelled from [^{14}C]ethanolamine was associated with the "PE+PE-Me" fraction (Table 11 and Fig. 16). With the whole leaf, all of the ^{14}C in this fraction was localized in the methanol-water soluble products

FIGURE 16

Autoradiogram of a chromatogram of lipids labelled from [^{14}C]methionine, [^{14}C]ethanolamine and [^{14}C]serine by whole leaves.

1. [Me-^{14}C]methionine
2. [1,2-^{14}C]ethanolamine
3. [1,2-^{14}C]ethanolamine + unlabelled methionine
4. [U-^{14}C]L-serine

Chromatography was on a silica gel H plate in chloroform-methanol-acetic acid-water (65:25:8:4). Mobility of standard lipids not shown in figure were: PG (R_f 0.60), DGD (R_f 0.75) and MGD (R_f 0.90).

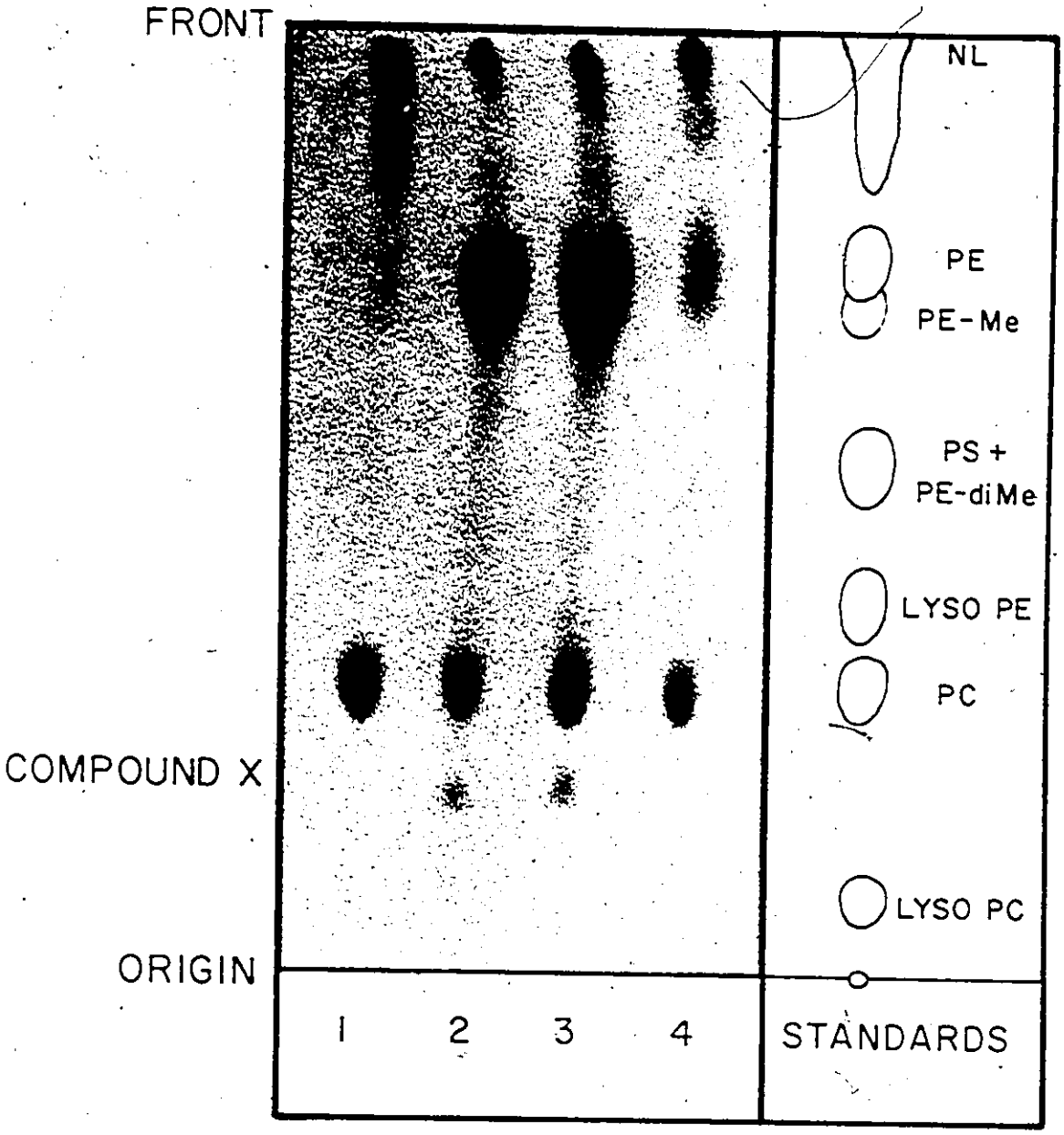

FRONT
NL
PE
PE-Me
PS +
PE-diMe
LYSO PE
PC
COMPOUND X
LYSO PC
ORIGIN
1
2
3
4
STANDARDS

TABLE 11. THE INCORPORATION OF ^{14}C LABELLED SUBSTRATES INTO LIPID BY WHOLE LEAVES AND LEAF SLICES

Substrate[a]	^{14}C-Incorporation into lipid[b] per whole leaf or g. of leaf slice, dpm x 10^{-3}							
	Total Lipid	PC	lyso PC	PE + PE-Me	lyso PE	PS + PE-diMe	NL	Compound X
Whole Leaf								
[U-^{14}C]L-serine (2.4 µCi)	116	22(19)	0	33(28)	0	4(3)	57(50)	0
[1,2-^{14}C]ethanolamine (1.7 µCi)	301	33(11)	0	233(78)	0	0	30(10)	5(1)
[1,2-^{14}C]ethanolamine (2.2 µCi) + cold L-methionine (10 mM)	615	41(7)	0	519(85)	0	0	40(6)	15(2)
[Me-^{14}C]L-methionine (1.9 µCi)	167	41(25)	0	7(4)	0	0	119(71)	0
Leaf Slices								
[U-^{14}C]L-serine (10 µCi)	51	2(4)	0	10(20)	2(4)	3(6)	34(66)	0
[1,2-^{14}C]ethanolamine (10 µCi)	401	5(1)	0	266(66)	31(8)	0	63(16)	36(9)
[Me-^{14}C]L-methionine (10 µCi)	169	13(8)	0	8(5)	0	0	148(87)	0
[1,2-^{14}C]choline (10 µCi)	102	92(90)	10(10)	0	0	0	0	0

[a] For experimental details see Experimental I.1. The figures in parenthesis denote amount of ^{14}C activity taken up by each whole leaf, or supplied to the leaf slices.

[b] ^{14}C labelled lipids were those migrating with the corresponding standard on TLC, see Fig. 16, numbers in parenthesis denote distribution as % of total lipid ^{14}C activity, calculated as described in Experimental I.1.a.

FIGURE 17

Autoradiogram of a chromatogram of methanol-water soluble products from deacylation of lipids labelled with ^{14}C by whole leaves.

A. PE + PE-Me labelled from [1,2-^{14}C]ethanolamine.

Chromatography on Whatman No. 1 paper in isopropanol-water-conc. ammonia (7:2:1).

B. 1. PC labelled from [Me-^{14}C]methionine

2. PC labelled from [1,2-^{14}C]ethanolamine

3. PE labelled from [U-^{14}C]serine

4. PC labelled from [U-^{14}C]serine

Chromatography on Whatman No. 1 paper in n-butanol-acetic acid-water (5:3:1).

Standard GPE, GPE-Me, GPE-diMe and GPC obtained by deacylation of corresponding standard phospholipid. Spots b and c refer to the unidentified ^{14}C-labelled spots in the PE fraction labelled from [U-^{14}C]serine.

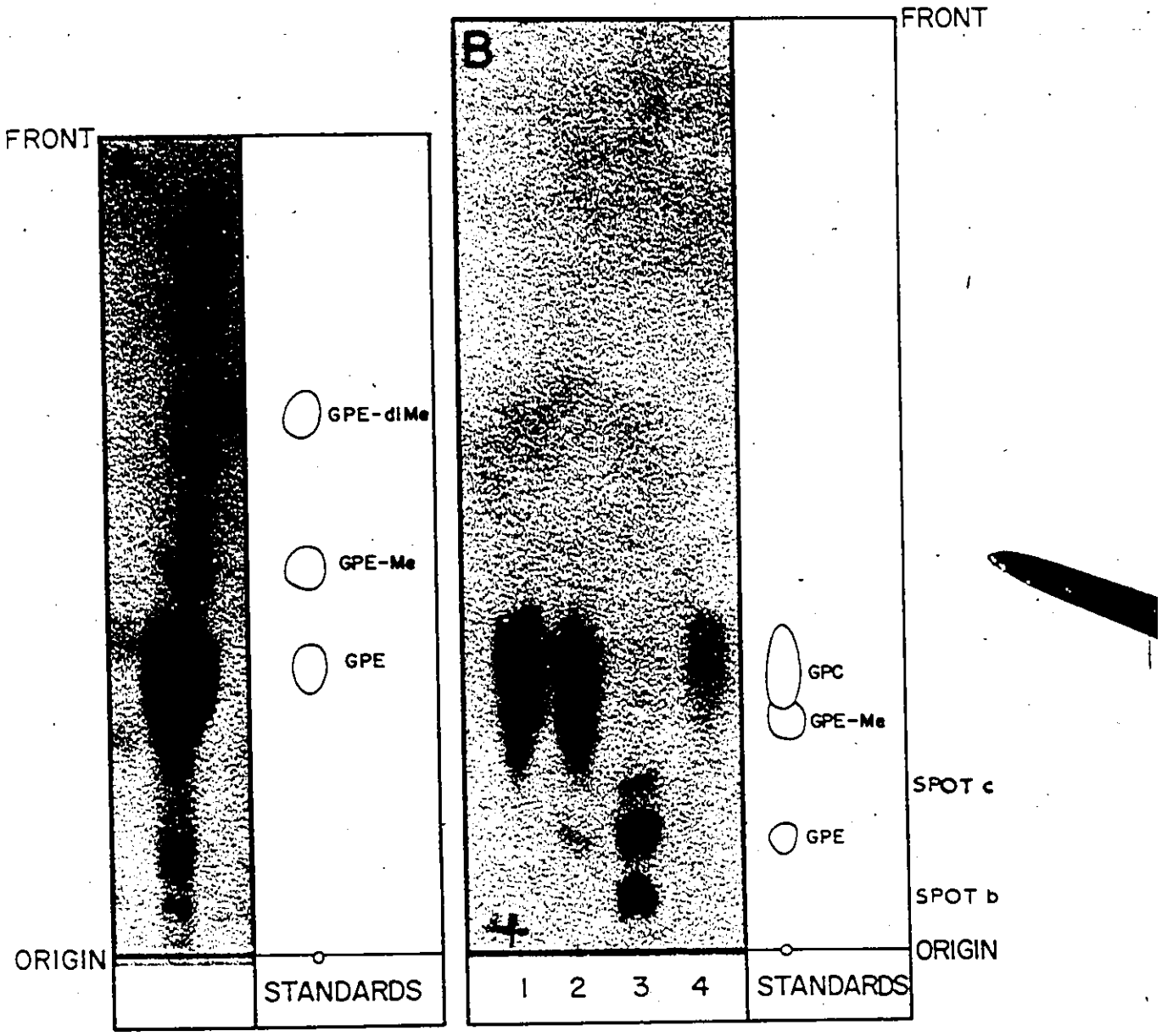

FRONT
GPE-diMe
GPE-Me
GPE
ORIGIN
STANDARDS
B
FRONT
GPC
GPE-Me
SPOT c
GPE
SPOT b
ORIGIN
1 2 3 4
STANDARDS

TABLE 12. THE INCORPORATION OF ^{14}C LABELLED SUBSTRATES INTO DEACYLATED MOIETIES OF PC, PE AND PE-Me BY WHOLE LEAVES AND LEAF SLICES

Substrate	^{14}C-Incorporation into deacylated lipid[a] dpmx10^{-3}		
	GPC	GPE	GPE-Me
Whole leaf			
[U-^{14}C]L-serine	5.1(23)	10[b](-)	0
[1,2-^{14}C]ethanolamine	32(97)	226[c](97)	4[c](-)
[Me-^{14}C]L-methionine	36(88)	0	0
Leaf slices			
[U-^{14}C]L-serine	-	-	-
[1,2-^{14}C]ethanolamine	-	245(92)	-
[Me-^{14}C]methionine	8.7(67)	-	-
[1,2-^{14}C]choline	91(99)	-	-

[a]Deacylation was carried out on the individual lipids described in Table 11 isolated by preparative TLC in chloroform-methanol-acetic acid-water (65:25:8:4). The identity of deacylated products was confirmed by paper chromatography, as described in Fig. 17 and text. Numbers in parenthesis denoted % of lipid ^{14}C in deacylated moiety. Dash denotes data was unavailable.

[b]Determined from the relative ^{14}C in the GPE spot (Fig. 17B)

[c]Determined from the relative ^{14}C in the GPE and GPE-Me spots (see Fig. 17A).

after deacylation, the major component of which was chromatographically identical with standard GPE; a minor component containing 2% of the ^{14}C was identified as [^{14}C]GPE-Me (Fig. 17A and Table 12). Incubation of the whole leaf with [1,2-^{14}C]ethanolamine in the presence of unlabelled L-methionine resulted in a two-fold stimulation of incorporation of ^{14}C into the "PE+PE-Me" fraction. This was probably a reflection of the greater uptake of [1,2-^{14}C]ethanolamine by this particular leaf rather than any specific effect of methionine itself. Since the "PE+PE-Me" fraction labelled in this system was not deacylated, it is not known whether PE-Me was actually labelled in this experiment. With leaf slices, all of the ^{14}C in the deacylated "PE+PE-Me" fraction was localized in GPE, and no [^{14}C]GPE-Me was detected (Table 12). However, failure to detect [^{14}C]GPE-Me may have resulted from insufficient exposure of the chromatogram of deacylated "PE+PE-Me" to X-ray film.

b) The "PE+PE-Me" fraction in whole leaf and leaf slices was also labelled with ^{14}C from [U-^{14}C]L-serine (Table 11 and Fig. 16). With the whole leaf, 53% of the ^{14}C activity in this fraction was localized in the water-soluble deacylated products and 47% in the chloroform soluble products. The identity of the latter will be discussed in Results IV.7. The water-soluble products contained two major components, one chromatographically identical to standard GPE (R_f, 0.12),

and the other with low mobility (spot b, R_f, 0.06), and a trace component (spot c, R_f, 0.18) (Fig. 17B). Spot b was not cyclic 1,2-glycerophosphate, which would be obtained by over hydrolysis, since the latter migrates with R_f 0.27 (Kates, 1972) in the solvent system used. It is likely that spot b is digalactosylglycerol, since the mobility of both compounds is about half that of GPE (Kates, 1972). Furthermore, digalactosyl diglyceride was found to have the same mobility as PE on TLC in chloroform-methanol-acetic acid-water (65:25:8:4, by vol.), (see legend Fig. 16), and would be isolated with the ^{14}C-labelled "PE+PE-Me" fraction.

c) The "PE+PE-Me" fraction both in the whole leaf and leaf slices was slightly labelled with ^{14}C from [Me-^{14}C] methionine. With the whole leaf, 36% of the ^{14}C activity in this fraction was localized in the water soluble products after deacylation and the remainder in the chloroform soluble products. The identity of the latter will be discussed in Results IV.7. The water-soluble product, containing 2000 dpm of ^{14}C, was chromatographed on Whatman No. 1 paper in isopropanol-28% ammonia-water (7:1:2, by vol.). However, the faint ^{14}C spot (R_f, 0.7) detected on the autoradiogram obtained by exposure of the chromatogram to X-ray film for six weeks did not correspond to GPE-Me. Since this exposure time is long enough for detection of ^{14}C activity as low as 500 dpm (see Methods V.3), it was concluded that little or no ^{14}C from [Me-^{14}C] methionine was incorporated into the GPE-Me moiety of PE-Me.

3) ^{14}C-Labelling of PC

a) Both whole leaves and leaf slices incorporated ^{14}C from ^{14}C-labelled ethanolamine, methionine and serine into PC (Fig. 16 and Table 11). However, with all three substrates the proportion of total lipid ^{14}C in PC was considerably greater in whole leaves than in leaf slices, but little difference was observed in the proportion of ^{14}C in the other components. With whole leaves, 97% and 88% of the total ^{14}C in PC labelled from [^{14}C] ethanolamine and [^{14}C] methionine respectively was localized in the GPC moiety (Table 12 and Fig. 17B). In contrast, with [^{14}C] serine, only 23% of the ^{14}C was localized in the GPC moiety (Table 12 and Fig. 17B). The labelling of the fatty acid moiety of PC by [^{14}C] serine and [^{14}C] methionine will be discussed in Results IV.7.

With leaf slices, 67% of the ^{14}C in PC labelled from [^{14}C] methionine was localized in the GPC moiety (Table 12). However, the distribution of ^{14}C between the fatty acid and H_2O-soluble moieties of PC labelled from [^{14}C] serine and [^{14}C] ethanolamine was not determined because of insufficient ^{14}C activity in the PC.

b) Over 90% of the ^{14}C incorporated into total lipid by leaf slices from [1,2-^{14}C]choline was associated with PC (table 11), all of the activity of which was localized in the GPC moiety (Table 12).

4) ^{14}C Labelling of Lyso PE and Lyso PC

In leaf slices, considerable amounts of ^{14}C from [^{14}C]serine and [^{14}C] ethanolamine were incorporated into lyso PE (Table 11). This lipid was identified by its ^{14}C-labelled product from deacylation which migrated with standard GPE on Whatman No. 1 paper in n-butanol-acetic acid-water (5:3:1 by vol.). In contrast, in the whole leaf, no ^{14}C from either substrate was incorporated into lyso PE even though the incorporation of ^{14}C into PE, the probable precursor of lyso PE, was as high or even higher than that in leaf slices (Table 11). Leaf slices also incorporated ^{14}C activity from [^{14}C] choline into lyso PC (Table 11); however, in whole leaves, in which PC was highly labelled from [^{14}C] methionine or [^{14}C] ethanolamine, no lyso PC was detected.

5) ^{14}C-Labelling of Other Lipids

Both whole leaves and leaf slices incorporated considerable amounts of ^{14}C from all substrates except [^{14}C] choline into the "neutral lipid" fraction, and traces of ^{14}C from [^{14}C] ethanolamine into an unknown compound X (Fig. 16 and Table 11). The identity of these products will be discussed below.

The significance of the above results in connection with the pathways operating in phospholipid and neutral lipid biosynthesis will be discussed below.

IV. Biosynthesis of Nitrogenous Phospholipids. In Vitro Studies

1. Biosynthesis of Phosphatidyl Serine by the Nucleotide Pathway

Assays for biosynthesis of phosphatidyl serine (PS) by the nucleotide pathway were carried out using a particulate fraction (1200-100,000xg pellet) incubated at pH 8.0 with [U-^{14}C]L-serine, either in the presence or in the absence of CDP-diglyceride (see Table 13 for details). In the system containing CDP-diglyceride, a small but significant amount (0.25%) of [U-^{14}C]-L-serine was incorporated into a lipid product which was assumed to be PS on the basis of its mobility on TLC (Table 13). ^{14}C activity was also present in the "PE" spot but at a very low level. However, in the absence of CDP-diglyceride, ^{14}C incorporation into PS was reduced to about 15% of that obtained in the presence of CDP-diglyceride. Thus, PS synthesis by the particulate fraction was largely dependent on exogenous CDP-diglyceride, suggesting the presence of a CDP-diglyceride: serine phosphatidyl transferase ("PS synthetase"). The PS synthetase in E.coli has been shown to depend for activity on the presence of Cutscum (2 mg/ml) and Na_2SO_4 (0.1M) in the assay system (Kanfer and Kennedy, 1964). However, in the present study, the addition of these components to the assay system was found to almost completely inhibit PS synthesis (Table 13).

TABLE 13. THE EFFECT OF CDP-DIGLYCERIDE ON THE INCORPORATION OF [U-^{14}C]L-SERINE INTO PHOSPHATIDYL SERINE BY A TOTAL PARTICULATE FRACTION

Assay System[a]	^{14}C-Incorporation[b], dpm	
	PS	PE
Complete system	5711	126
Complete system, minus CDP-dig.	781	0
Complete system, + cutscum + Na_2SO_4	194	0

[a] Complete assay system (vol. 1 ml): 7.5 µM [U-^{14}C]L-serine (2.1×10^6 dpm), 0.13 mM CDP-diglyceride (prepared as described in Methods I.2.b.i), 50 mM sodium phosphate (pH 8.0), 1.5 mM β-mercaptoethanol and 0.4 ml of 1200-100,000xg pellet suspended in water (1.3 mg protein). Cutscum (0.5 mg) and Na_2SO_4 (80 mM) were added as indicated. Incubation, 1h at 30°C.

[b] ^{14}C-labelled lipids were separated by TLC in chloroform-methanol-acetic acid-water (65:25:8:4 by vol. Rfs of authentic PS and PE, 0.53 and 0.72 respectively). Values of ^{14}C incorporation were corrected for boiled enzyme controls (PS, 303 dpm; PE, 308 dpm).

2. Biosynthesis of Phosphatidyl Ethanolamine by Decarboxylation of [^{14}C] Phosphatidyl Serine

a) Subcellular distribution and identification of ^{14}C-labelled products

The biosynthesis of PE by the decarboxylation of PS was investigated in systems containing [^{14}C] PS and different subcellular fractions. Results of this study are shown in Fig. 18 and Table 14. A high proportion (74%) of the total enzyme activity recovered was localized in the 100,000xg supernatant fraction, while of the particulate fractions the 15,000xg pellet contained the most activity. However, because of the low overall recovery (46%) of enzyme activity, these results on subcellular distribution of PE synthesis can only be considered approximate. It should be pointed out that in the boiled enzyme control no distinct [^{14}C]PE spot was observed on the autoradiogram showing ^{14}C-labelled lipid products (lane 4, Fig. 18). However, a small amount of ^{14}C (ca. 1% of ^{14}C in PS substrate) was detected in this spot by scintillation counting; all data in Table 14 are corrected for ^{14}C in this "boiled enzyme" control.

Unexpectedly, with all cell fractions, ^{14}C was also found in the "neutral lipids" (compound Y, Fig. 18 and Table 14). Though the recovery of activity was very high under the conditions of this experiment, it is clear that almost all of the activity was localized in the 100,000xg supernatant.

FIGURE 18

Autoradiogram of a chromatogram of lipids labelled from [^{14}C]PS by cell fractions.

1. cell free homogenate
2. 1200xg
3. 15,000xg
4. 15,000xg, boiled enzyme
5. 100,000xg
6. 100,000xg supernatant
7. [^{14}C]PS substrate

Assay system as described in Table 14. Chromatography on silica gel H plates in chloroform-methanol-conc. ammonia (65:25:5).

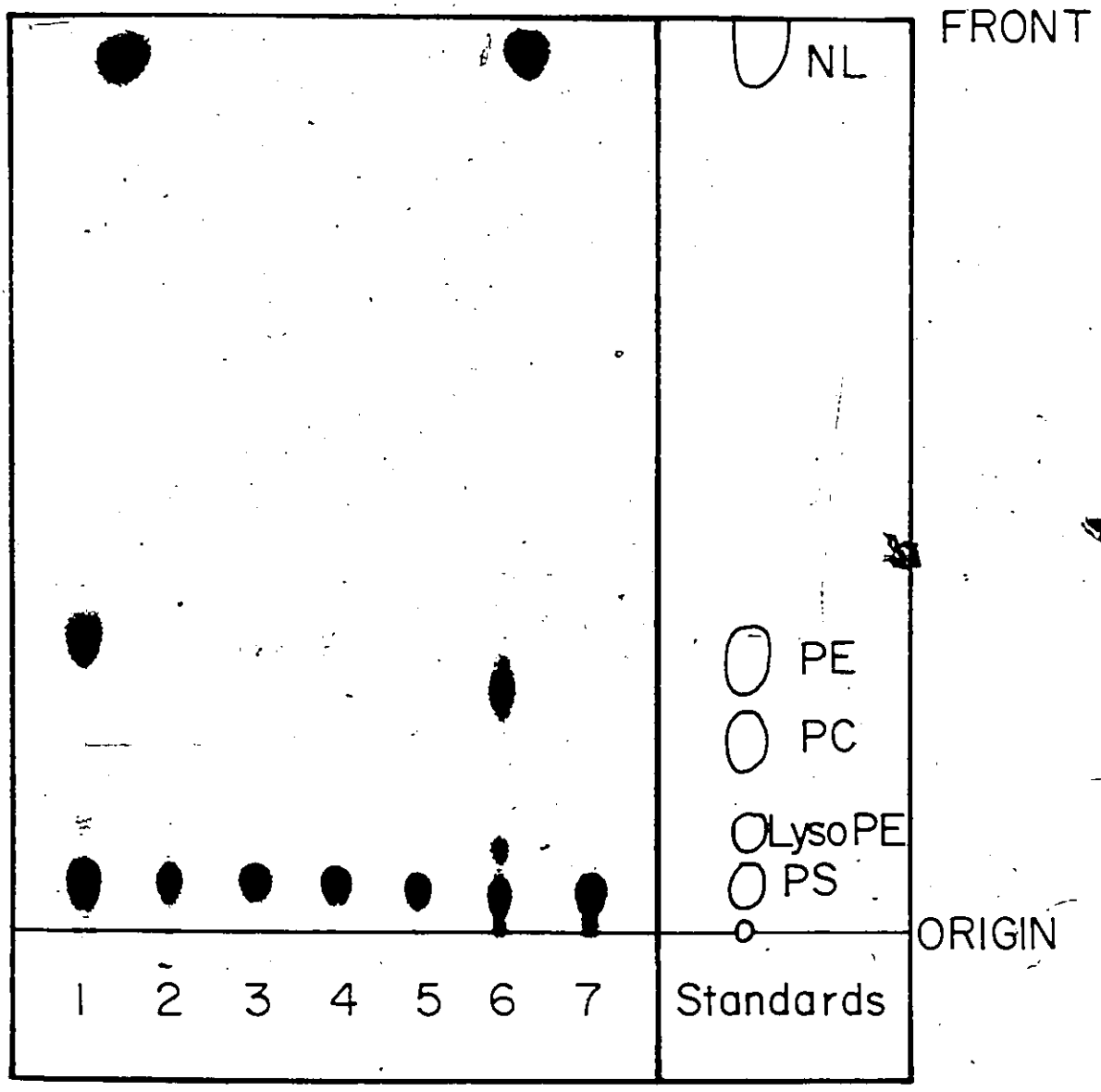

FRONT
NL
PE
PC
LysoPE
PS
ORIGIN
1
2
3
4
5
6
7
Standards

TABLE 14. SUBCELLULAR DISTRIBUTION OF PHOSPHATIDYL SERINE DECARBOXYLASE

Cell Fraction	Formation[a] of PE		Formation[a] of Compound Y	
	Activity dpmx10^{-5}	%[b]	Activity dpmx10^{-5}	%[b]
Cell-free homogenate	21.1		16.3	
1200xg	0.5	5	0.7	2
15,000xg	1.4	14	1.1	3
100,000xg	0.7	7	0.4	1
100,000xg supernatant	7.2	74	36.2	94
Recovery	9.8(46%)		38.4(235%)	

[a]Assay system (vol. 0.5 ml): [^{14}C]phosphatidyl serine (1.7 x 10^4 dpm, prepared as described in Methods V.1.b.vi) and 0.1 ml of cell fraction suspended in homogenizing medium. pH of cell fractions adjusted to 7.0 with dil. HCl prior to assay. Incubation, 20 min. at 30°C. ^{14}C-Labelled lipid products were separated as shown in Fig. 18, and ^{14}C incorporation into each lipid was calculated as described in Experimental II.3.a. Values of activity are corrected for boiled enzyme control.

[b]refers to % of recovered activity.

Among the ^{14}C labelled lipid products from all cell fractions, a small amount of ^{14}C was associated with a component having an R_f identical to that of standard lyso PE (Fig. 18). Consistent with a lyso PE structure for this component was the finding that it was most heavily labelled by those cell fractions, namely the cell-free homogenate and 100,000xg supernatant, most actively synthesizing [^{14}C] PE (Fig. 18).

^{14}C has also been found to be associated with products distributed in the methanol-water phase following the Bligh and Dyer extraction step in the enzyme assay procedure. That this ^{14}C activity was not due to traces of unextracted ^{14}C labelled lipid was confirmed by the finding that no ^{14}C activity was extracted from the methanol-water phase upon repeated washing with chloroform. The identity of the methanol-water products will be discussed below.

The incorporation of ^{14}C from [^{14}C] PS into PE strongly suggested the direct formation of [^{14}C] PE from [^{14}C] PS by the action of PS-decarboxylase. This was confirmed by comparing the ratio of ^{14}C in the water soluble moiety to the ^{14}C in the fatty acid moiety of both [^{14}C] PE and [^{14}C] PS. It was found that this ratio was lower in [^{14}C] PE than in [^{14}C] PS by an amount close to that calculated for the formation of [^{14}C] PE by the decarboxylation of [^{14}C] PS (see Table 15). It should be noted that essentially all of the ^{14}C in the water-soluble mild alkaline hydrolysis

TABLE 15. DISTRIBUTION OF ^{14}C ACTIVITY BETWEEN WATER SOLUBLE AND CHLOROFORM SOLUBLE MOIETIES OF [^{14}C] PHOSPHATIDYL SERINE AND [^{14}C] PHOSPHATIDYL ETHANOLAMINE

Compound[a]	Initial Activity dpm	Distribution of ^{14}C[b], dpm		% recovery	Ratio ^{14}C in H_2O sol. / ^{14}C in $CHCl_3$ sol.
		$CHCl_3$ sol.	H_2O sol.		
[^{14}C]PS	13,000	5040(39)	8460(65)	104	1.67
[^{14}C]PE	5930	2750(46)	2970(50)	96	1.08[c]

[a] [^{14}C]PS was prepared as described in Methods V.1.b.vi. [^{14}C]PE was formed by a 15,000xg fraction in a reaction mixture essentially as described in Table 14, and isolated by preparative TLC in chloroform-methanol-28% ammonia (65:25:5 by vol.)

[b] Determined by the mild alkaline hydrolysis procedure described in Methods III.1. Values in parenthesis are % of ^{14}C in intact lipid.

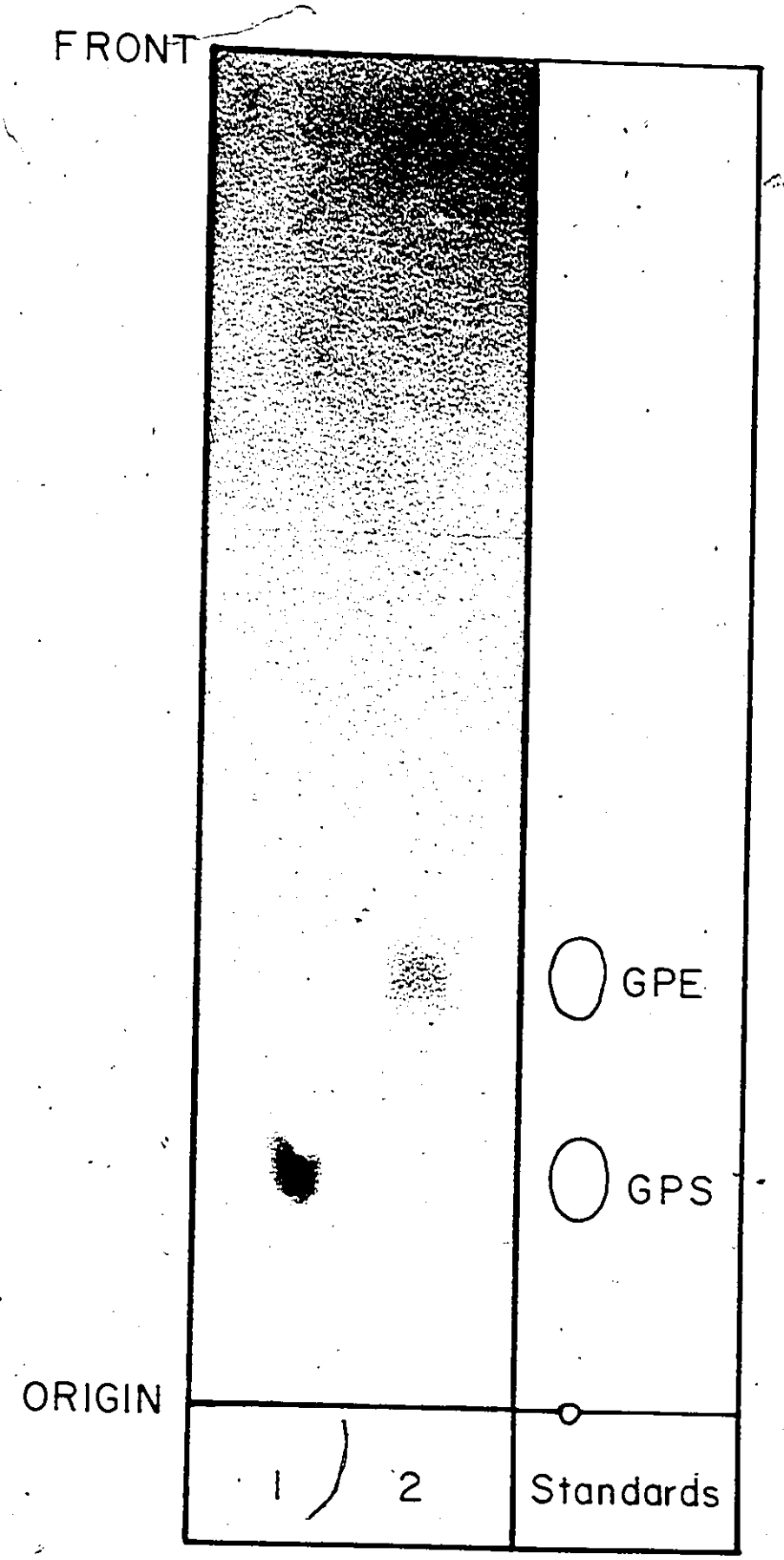
[c] Calculated for [^{14}C]PE, 1.11, assuming all ^{14}C activity in GPS is localized in the serine moiety.

FIGURE 19

Autoradiogram of a chromatogram of methanol-water soluble products from deacylation of [^{14}C]PS and [^{14}C]PE.

1. [^{14}C]PS
2. [^{14}C]PE

The labelled lipids are those described in Table 15. Chromatography was on Whatman No. 1 paper in isopropanol-water-conc. ammonia (7:2:1). Standard GPS and GPE obtained by deacylation of standard PS and PE respectively.


FRONT
GPE
GPS
ORIGIN
1
2
Standards

products from [^{14}C]PE and [^{14}C]PS was associated with GPE and GPS respectively (Fig. 19), and all of the ^{14}C in the chloroform soluble products was associated with fatty acid methyl esters (R_f, 0.70 on TLC in petroleum ether-diethyl-ether-acetic acid (90:10:1, by vol.)).

Concerning the structure of compound Y, it was identified as an ester derivative of ^{14}C-labelled fatty acids since on hydrolysis with mild alkali (see Methods III.1) it gave a single ^{14}C labelled product with R_f (0.72, R_f intact lipid, 0.0) on TLC in petroleum ether-diethylether-acetic acid (90:10:1, by vol.) identical to that of standard fatty acid methyl ester. The biosynthetic pathway in the formation of this compound will be discussed below.

b) Characteristics of the Particulate PS decarboxylase

^{14}C-Incorporation into PE was strongly inhibited by the detergents sodium lauryl sulphate and Triton X-100 (Experiment 1, Table 16). In the presence of Triton X-100, PE synthesis was not significantly stimulated by the presence of Mg^{2+} and Mn^{2+}, suggesting indirectly that PS decarboxylase is not dependent on these metal ions for activity (Table 16, Exp. 1). It should be pointed out that the ^{14}C in the boiled enzyme or zero time controls was due to ^{14}C labelled contaminants present in the [^{14}C]PS substrate migrating with PE and compound Y in the TLC solvent system used.

PE synthesis was strongly inhibited by hydroxylamine (Table 16, Exp. 2), indicating that PS decarboxylase contains

TABLE 16. THE INCORPORATION OF ^{14}C FROM [^{14}C]PS INTO PE AND COMPOUND Y BY A PARTICULATE FRACTION

Assay System	Incorporation[a] of ^{14}C, dpm	
	PE	Compound Y
Exp. 1		
Complete[b]	7000	11,250
" + sod. lauryl sulphate	2100	3360
" + Triton X-100	4530	4150
" + Triton + Mg^{2+} + Mn^{2+}	4900	3900
" boiled enzyme	1050	390
" zero time	1000	500
Exp. 2		
Complete[c]	1413	1049
" + hydroxylamine	352	1317

[a]Determined as described in Table 14.

[b]Complete assay system (vol. 0.5 ml): [^{14}C]PS (1.1×10^5 dpm), 50 mM Tris HCl (pH 7.0) and 0.2 ml (3 mg protein) of 1200-100,000xg pellet suspended in 2 mM Tris HCl (pH 7.0). Additions were made as indicated to give the following concentrations: sodium lauryl sulphate (0.5 mg/ml), Triton X-100 (0.5 mg/ml), $MgCl_2$ (10 mM) and $MnCl_2$ (10 mM). Incubation, 1h at 30°C.

[c]Complete assay system as described in Table 14, using 15,000xg pellet suspension as enzyme source. Hydroxylamine hydrochloride was added where indicated to a final concentration of 30 mM. Values are corrected for boiled enzyme control.

TABLE 17. THE INCORPORATION OF ^{14}C ACTIVITY INTO PE, COMPOUND Y AND METHANOL-WATER SOLUBLE PRODUCTS FROM [^{14}C] PS AS A FUNCTION OF REACTION TIME

Time	^{14}C Activity[a], dpmx10^{-3}				% Recovery[b]
	PS	PE	Y	MeOH-H_2O sol	
0	94.4	0	0	0	100
32	86.1	2.3	3.7	1.3	97
60	74.3	2.7	5.5	3.8	89
120	68.6	3.6	7.0	6.1	87

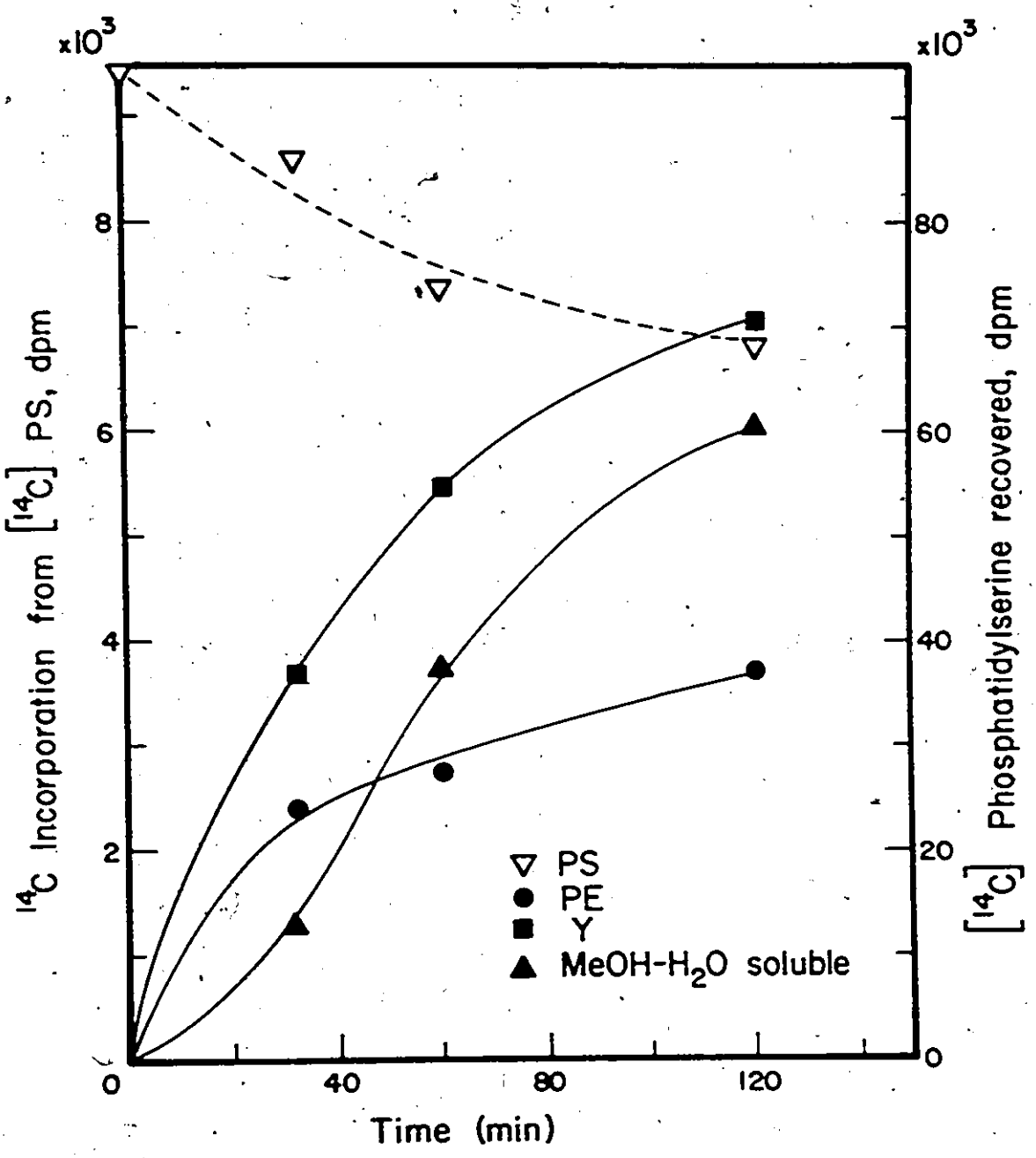
[a] Assay system (vol. 0.5 ml): [^{14}C]phosphatidyl serine (96 x 10^3 dpm), 50 mM Tris HCl (pH 7.0), and 0.3 ml of 1200-100,000xg pellet suspended in 2 mM Tris HCl (pH 7.0). Incubation at 37°C for varying lengths of time as indicated. ^{14}C-Activity in each lipid determined as described in Table 14. ^{14}C-Activity in MeOH-H_2O soluble products determined by counting MeOH-H_2O phase following Bligh and Dyer extraction of lipids. Values are corrected for 60 min. boiled enzyme control (PE, 722 dpm; Y, 600 dpm; MeOH-H_2O soluble, 3900 dpm).

[b] Includes ^{14}C activity in CO_2, calculated as 24% of ^{14}C activity in [^{14}C]PE.

FIGURE 20

The effect of reaction time on the incorporation of ^{14}C activity into PE, compound Y and methanol-water soluble products from [^{14}C]PS by a-particulate fraction.

Data obtained from Table 17.


x10³
x10³
8
6
4
2
0
80
60
40
20
0
40
80
120
Time (min)
¹⁴C Incorporation from [¹⁴C] PS, dpm
[¹⁴C] Phosphatidylserine recovered, dpm
PS
PE
Y
MeOH-H₂O soluble

pyridoxal phosphate as co-enzyme (Clark, 1963). It is interesting that the ^{14}C incorporation into compound Y was actually increased by the presence of hydroxylamine, in contrast to the inhibition of the PS decarboxylase. This observation was verified in subsequent experiments and will be discussed below.

The formation of [^{14}C]PE as a function of reaction time was anomolous in that [^{14}C]PE formation fell off markedly after 30 min (Table 17 and Fig. 20). In contrast, the decrease after 30 min in the rate of ^{14}C-incorporation into compound Y was more gradual. Included in Table 17 and Fig. 20 are data on the time course of formation of ^{14}C-labelled methanol-water soluble enzyme products. The time course showed an apparent lag phase during the first 30 min after which it paralleled that of the ^{14}C-incorporation into compound Y.

3. Biosynthesis of Phosphatidyl ethanolamine by the Nucleotide Pathway

a) The formation of [^{14}C]phosphatidyl ethanolamine from CDP-[1,2-^{14}C]ethanolamine

A very high incorporation (43%) of ^{14}C into PE was found with a system at pH 8.0 containing CDP-[1,2-^{14}C] ethanolamine, Mn^{2+}, a 100,000xg fraction, and no added diglyceride (Table 18; Fig. 21, lane 1). Small amounts of ^{14}C were also associated with lyso PE and an unidentified "neutral lipid" fraction. The addition of S-adenosylmethionine, alone

or in the presence of Triton X-100 or the 100,000xg supernatant fraction, did not affect the relative proportion of ^{14}C in the lipid products (Fig. 21, lanes 2-4, and Table 18), and this will be discussed below in Results IV.5.

The identity of the [^{14}C]PE and [^{14}C]lyso PE products was confirmed as follows: Each component was isolated from the combined total lipids of assay systems 1-4 (Fig. 21) and deacylated to give a single ^{14}C-labelled water-soluble product chromatographically identical to GPE (Fig. 22).

PE synthetase was found to require Mn^{2+} or Mg^{2+} as co-factors. The effect of varying concentrations of added metal ions on PE synthesis by a dialyzed 100,000xg fraction is shown in Fig. 23A. The optimum concentration of Mg^{2+} at which PE synthesis was maximal was 8 mM, while that for Mn^{2+} was 2 mM. It should be pointed out that the optimum for Mn^{2+} was only an upper estimate since PE synthesis at lower concentrations was not determined. Both metal ions appeared to inhibit PE synthesis at concentrations higher than their respective optimum values (Fig. 23A), but the most marked inhibition was with Mn^{2+}. However, it is noteworthy that at high Mn^{2+} concentrations a precipitate, possibly of Mn^{2+} salts, appeared in the assay mixtures during incubation and may have been responsible for the observed decrease in [^{14}C]PE synthesis.

The formation of PE at 20 mM Mg^{2+} as a function of pH is shown in Fig. 23B. The optimum pH in Tris HCl was found to be 7.0.

FIGURE 21

Autoradiogram of a chromatogram of lipids labelled from CDP-[1,2-^{14}C]ethanolamine by a 100,000xg fraction.

Assay System 1. Complete system[a]

Assay System 2. Complete system[a] + SAM (53 µM)

Assay System 3. Complete system[a] + SAM (53 µM) + Triton (0.5 mg)

Assay System 4. Complete system[a] + SAM (53 µM) + 100,000xg supernatant (4 mg protein).

[a]Complete assay system (vol. 1 ml): 4.14 µM CDP-[1,2-^{14}C] ethanolamine (5.5×10^5 dpm), 50 mM Tris HCl (pH 8.0), 2 mM $MnCl_2$ and 0.3 ml of 100,000xg pellet suspension (1.3 mg protein). All tubes initially contained the complete system alone. After 15 mins incubation at 30°C, in this system, additions were made as described above, and incubations continued a further 60 min.

Chromatography on silica gel H plate in chloroform-methanol-acetic acid-water (65:25:8:4).

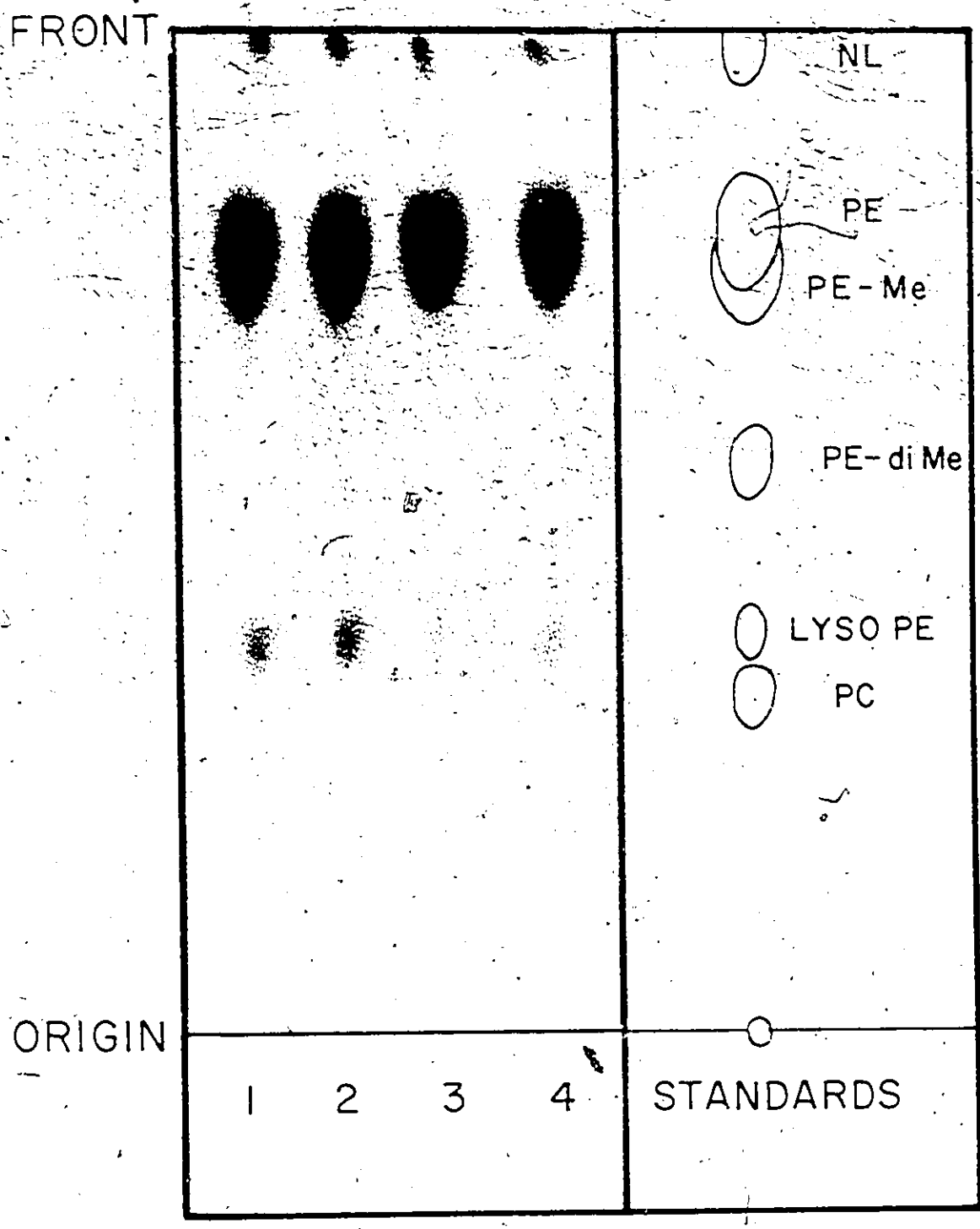

FRONT
NL
PE
PE-Me
PE-di Me
LYSO PE
PC
ORIGIN
1
2
3
4
STANDARDS

TABLE 18. THE INCORPORATION OF ^{14}C FROM CDP-[1,2-^{14}C] ETHANOLAMINE INTO LIPID BY THE 100,000xg FRACTION

Assay System[a]	^{14}C-Incorporation[b], dpmx10^{-3}			
	PE	Lyso PE	PC	Neutral Lipid
Complete	246	16	0	16
" + SAM	246	16	0	16
" " + Triton X-100	223	7	0	14
" " + 100,000xg supernatant	239	12	0	15

[a] As described in legend to Fig. 21.

[b] ^{14}C-Labelled lipids are those shown in Fig. 21, and ^{14}C-incorporation into each lipid calculated as described in Experimental II.3.a.

FIGURE 22

Autoradiogram of a chromatogram of methanol-water soluble products from deacylation of PE and lyso PE labelled from CDP-[1,2-^{14}C] ethanolamine.

1. [^{14}C]PE
2. [^{14}C]Lyso PE

Chromatography was on Whatman No. 1 paper in methanol-98% formic acid-water (80:30:7 by vol.).

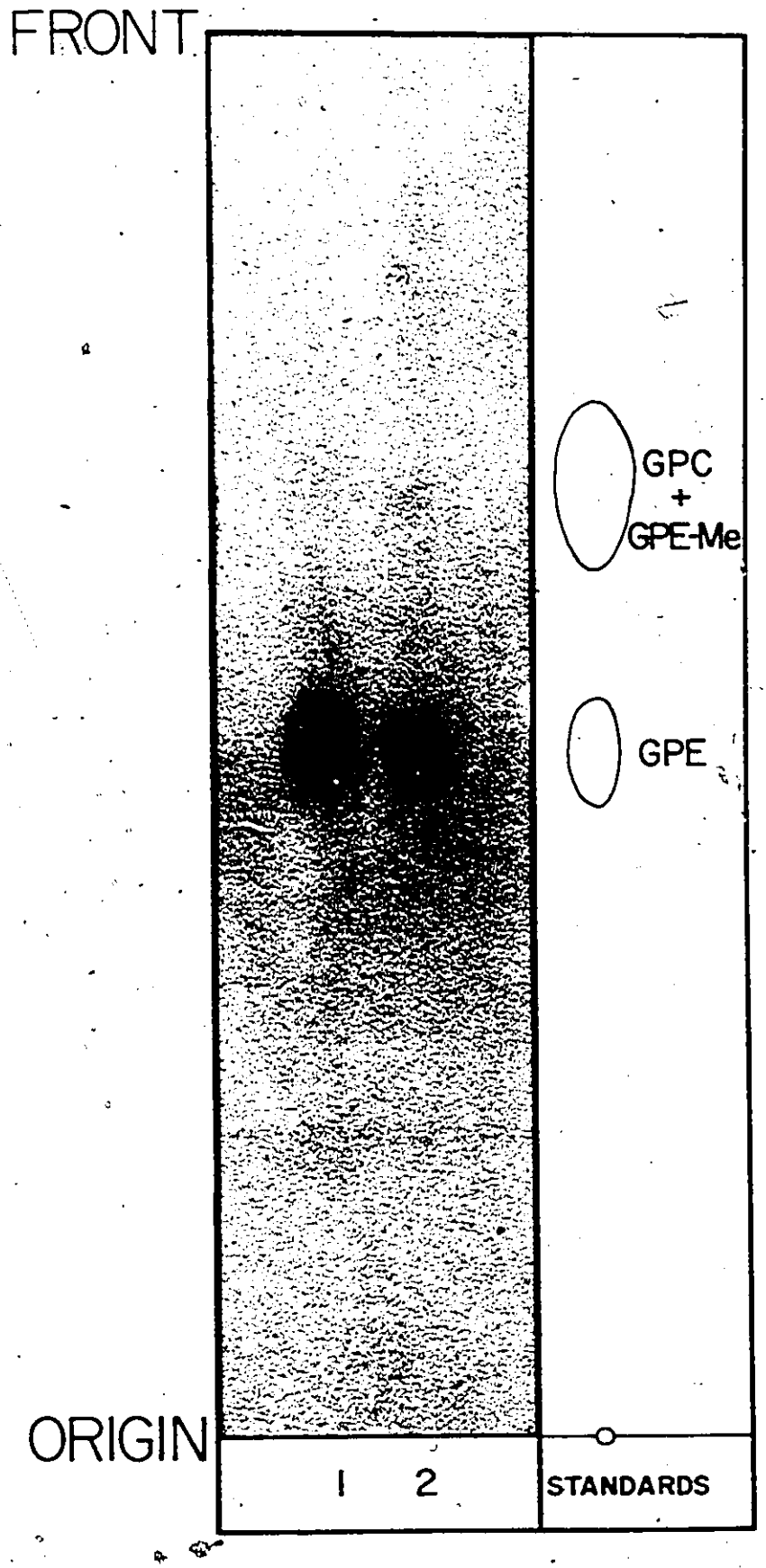

FRONT
GPC
+
GPE-Me
GPE
ORIGIN
1
2
STANDARDS

FIGURE 23

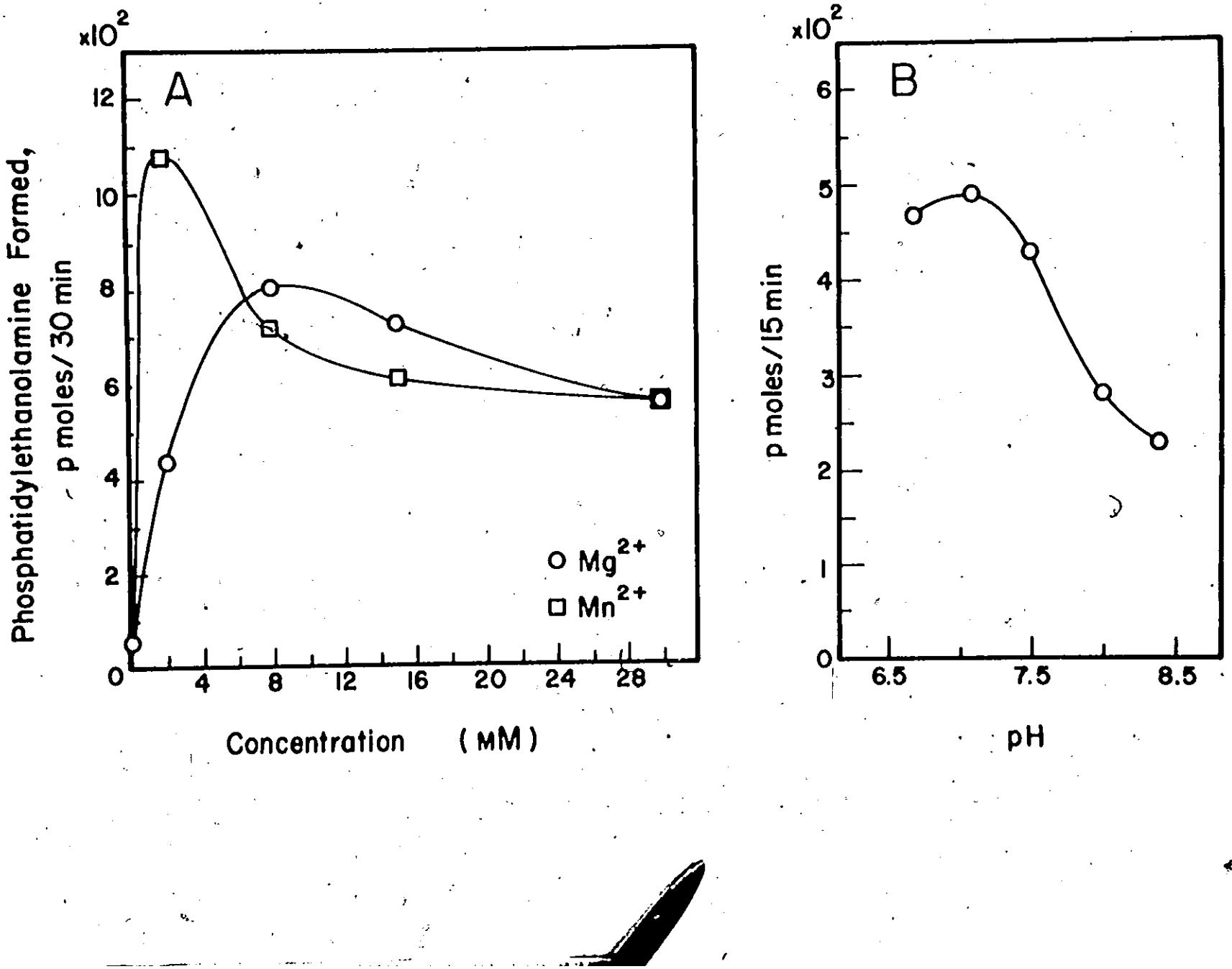
The effect of divalent metal ion concentration and pH on PE synthesis from CDP-[1,2-^{14}C]ethanolamine by a 100,000xg fraction.

A. Divalent metal ion concentration

Assay system (vol. 1 ml): 4.14 µM CDP-[1,2-^{14}C] ethanolamine (5.5×10^5 dpm), 50 mM Tris HCl (pH 7.5), 0.5 ml of 100,000xg pellet suspension dialyzed for 1-1/2 h prior to assay against 20 mM Tris HCl (pH 8.0), and varying amounts of $MnCl_2$ or $MgCl_2$ as indicated. Incubation, 30 min at 30°C. PE synthesis calculated from ^{14}C incorporation into total lipids. Values were corrected for boiled enzyme control (0.05 pmoles PE/30 min) carried out in assay system containing 15 mM Mn^{2+} plus 15 mM Mg^{2+}. TLC of ^{14}C labelled products from assays containing 2 mM or 30 mM Mn^{2+}, or 8 mM Mg^{2+}, showed over 90% of ^{14}C localized in PE.

B. pH

Assay system (vol. 1 ml): 2.05 µM CDP-[1,2-^{14}C] ethanolamine (2.86 x 10^5 dpm), 20 mM $MgCl_2$, 0.2 ml (2.1 mg protein) of 100,000xg pellet suspended in 2 mM Tris HCl (pH 7.5), and 100 mM Tris HCl of varying pH as indicated. Incubation, 15 min at 30°C. PE synthesis calculated from ^{14}C incorporation into total lipids.


A
B
x10²
Phosphatidylethanolamine Formed, p moles/30 min
p moles/15 min
Concentration (mM)
pH
Mg²⁺
Mn²⁺
0
2
4
6
8
10
12
16
20
24
28
1
5
6.5
7.5
8.5

The formation of [^{14}C]PE labelled in the GPE moiety from CDP-[1,2-^{14}C]ethanolamine by a 100,000xg fraction established the operation of a CDP-ethanolamine: sn-1,2-diglyceride phosphorylethanolamine transferase ("PE synthetase") utilizing endogenous diglyceride. The involvement of diglyceride was demonstrated by the finding that PE synthesis in the 15,000xg and 100,000xg fractions was stimulated 36% and 42% respectively by the addition of unsaturated sn-1,2-diglyceride (Table 19).

Results on the subcellular distribution of PE synthetase are shown in Table 19. In the absence of exogenous diglyceride, 94% of the total PE synthetase activity was evenly distributed between the 15,000xg pellet, 100,000xg pellet, and the 100,000xg supernatant, whereas only 12% of the total activity was present in the 1200xg pellet fraction. In the presence of diglyceride, PE synthetase activity was stimulated in all fractions except the 100,000xg supernatant in which activity was slightly inhibited, and was distributed mainly in the 15,000xg and 100,000xg pellet fractions. Though the effect of enzyme concentration on PE synthetase in each fraction both in the absence and presence of diglyceride was not investigated, the recovery of activity was quantitative, suggesting that the enzyme concentration in each fraction used in the assays was in the linear range of the enzyme activity vs. concentration plot.

Though the distribution of the mitochondrial marker enzyme cytochrome c oxidase among the cell fractions described

TABLE 19. SUBCELLULAR DISTRIBUTION OF PHOSPHATIDYL ETHANOLAMINE SYNTHESIS FROM CDP-[1,2-^{14}C]ETHANOLAMINE

Cell Fractions	Protein		Chlorophyll		Phosphatidyl ethanolamine synthesis[a]					
					- diglyceride			+ diglyceride[b]		
	mg	%	mg	%	Activity (nmoles/30 min)	%	Specific Activity	Activity (nmoles/30 min)	%	Specific Activity
Cell-free homogenate	770	100	11.2	100	35.4	100	0.05	43.2	100	0.06
1200xg	140	12	5.3	47	4.1	12	0.03	4.5	10	0.03
15,000xg	130	11	4.7	42	12.1	34	0.09	16.5	37	0.13
100,000xg	50	4	0.1	1	10.7	30	0.21	15.2	35	0.30
100,000xg supernatant	740	64	0	0	10.7	30	0.01	8.5	20	0.01
% recovery		91		90		106			102	

[a]Assay system (vol. 1 ml): 1.5 μM CDP-[1,2-^{14}C]ethanolamine (2x10^5 dpm), 100 mM Tris HCl (pH 7.0), 20 mM $MnCl_2$ and an aliquot of cell free homogenate (0.05 ml) or cell fraction (0.1 ml) suspended in homogenizing medium. Prior to their addition to assay mixture, the pH of each fraction was adjusted to 7.0 with 0.2N HCl. Incubation, 30 min at 30°C. PE synthesis was calculated from the incorporation of ^{14}C into total lipid.

[b]*sn*-1,2-Diglyceride was prepared as described in Methods I.2.b.ii, and 300 μg suspended in above assay mixture minus CDP-ethanolamine and cell fraction by sonication for 2 min.

in Table 19 was not determined, it was found in a separate experiment using an identical cell fractionation procedure that cytochrome c oxidase activity was distributed as follows: 1200xg, 29%; 15,000xg, 61%; 100,000xg, 7% and 100,000 supernatant, 3%. Since the 100,000xg microsomal fraction showed the highest specific activity when assayed both in the presence and absence of diglyceride (Table 19) it can clearly be concluded that the PE synthetase activity in this fraction must be due to a microsomal enzyme system. Whether the activity in the 1200xg and 15,000xg fractions arose from separate chloroplast and mitochondrial systems, or to microsomal contamination, will be discussed below.

b) Formation of Phosphoryl[^{14}C]ethanolamine from [^{14}C]Ethanolamine by Ethanolamine Kinase

Attempts were made to demonstrate the first enzyme in the nucleotide pathway in PE biosynthesis, namely ethanolamine kinase. The results of this study are shown in Table 20.

A small but significant incorporation (2%) of ^{14}C into phosphorylethanolamine was found with a system using a 15,000xg supernatant fraction at pH 7.5 containing [1,2-^{14}C] ethanolamine and ATP. When ATP was omitted, incorporation was reduced eight fold. The residual low activity observed was presumably due to endogenous ATP. Results were found to be unaffected by prior dialysis of the 15,000xg supernatant fraction. Thus the dependence of phosphoryl[^{14}C]ethanolamine

TABLE 20. ETHANOLAMINE KINASE ACTIVITY IN THE 15,000xg SUPERNATANT FRACTION

Assay System[a]	^{14}C-Incorporation into phosphorylethanolamine, dpm
Complete system	21,650
" " minus ATP	2,800

[a] Complete system (vol. 1 ml): 0.2 mM [1,2-^{14}C]ethanolamine (1.2 x 10^6 dpm), 0.2M Tris HCl (pH 7.5), 0.5 mM ATP, 2 mM $MgCl_2$ and 0.4 ml of 15,000xg supernatant fraction (6 mg protein). Incubation, 1h at 30°C. Lipids were extracted by the Bligh and Dyer procedure (see Methods VI.1) and counted for ^{14}C. An aliquot (ca. 50,000 dpm) of the methanol-water phase was chromatographed on Whatman No. 1 paper in n-butanol-acetic acid-water (5:2:3 by vol.), and radioactive spots corresponding to standard ethanolamine (Rf, 0.44), phosphorylethanolamine (Rf, 0.25) and CDP-ethanolamine (Rf, 0.13) were located by autoradiography, and cut out and counted. Total incorporation of ^{14}C into phosphorylethanolamine was calculated from the relative distribution of ^{14}C in the phosphorylethanolamine spot multiplied by the total ^{14}C in the methanol-water phase.

formation from [^{14}C]ethanolamine on exogenous ATP suggests the presence of a cytoplasmic ethanolamine kinase.

c) Formation of CDP[-^{14}C]ethanolamine from phosphoryl [1,2-^{14}C]ethanolamine

Attempts to demonstrate the second step in the nucleotide pathway in PE biosynthesis, in which phosphoryl-ethanolamine is converted to CDP-ethanolamine, consistently failed. In a typical experiment no ^{14}C could be detected in CDP-ethanolamine when phosphoryl[1,2-^{14}C]ethanolamine (final conc. 0.3 mM) was incubated in the above assay system described in Table 20 supplemented with 0.5 mM CTP. Furthermore, no ^{14}C was incorporated from phosphoryl[^{14}C]ethanolamine into PE, even though the assay system contained the necessary co-factors and endogenous diglyceride substrate for conversion of any CDP-[1,2-^{14}C]ethanolamine formed into PE. These results were unaffected by addition to the system of NaF, an inhibitor of Mg^{2+} dependent nucleotidase (Heppel and Hilmoe, 1951), or by dialyzing the 15,000xg supernatant prior to assay for 4 h at 4°C against homogenizing medium.

The possibility that any CDP-[1,2-^{14}C]ethanolamine product was hydrolyzed to phosphoryl[1,2-^{14}C]ethanolamine by a pyrophosphatase was ruled out in a separate experiment in which CDP-[1,2-^{14}C]ethanolamine was incubated in the above assay system (Table 20), without any formation of phosphoryl [^{14}C]ethanolamine. Also, on incubation of a cell free homo-

genate with phosphoryl[1,2-^{14}C]ethanolamine in the presence of Mg^{2+} and CTP, at pH 7.5, no CDP-[^{14}C]ethanolamine was formed suggesting that CTP:phosphorylethanolamine cytidyl transferase was absent in other cell fractions.

4. Biosynthesis of Phosphatidyl choline by the Nucleotide Pathway

The presence in spinach leaves of CDP-choline:sn-1,2-diglyceride phosphorylcholine transferase, which was first demonstrated by Devor and Mudd (1971), was confirmed in the present studies using assay mixtures containing CDP-[1,2-^{14}C] choline, Mn^{2+}, exogenous diglyceride, and different subcellular fractions. The results of this study are shown in Table 21. Though the effect of enzyme concentration on PC synthesis in each fraction was not investigated, the quantitative recovery of activity after fractionation suggests the enzyme concentration in each fraction was in the linear range. It was found that the fraction with the highest specific activity and proportion of the total activity was the 100,000xg microsomal pellet, which as discussed in Results IV.3.a. was essentially free of mitochondria. The subcellular origin of the activity in other cell fractions is not clear but will be discussed further below.

It should be pointed out that in the above experiment, PC synthesis was calculated from the incorporation of ^{14}C into total lipids. TLC analysis of the ^{14}C labelled lipid products

TABLE 21. SUBCELLULAR DISTRIBUTION OF PHOSPHATIDYL CHOLINE SYNTHESIS FROM CDP-[1,2-^{14}C]CHOLINE

Cell Fraction	Phosphatidyl choline synthesis[a]		
	Activity (nmoles/30 min)	%	Specific Activity
Cell free homogenate	76.5	100	0.10
1200xg	8.3	11	0.06
15,000xg	19.2	25	0.15
100,000xg	36.0	47	0.72
100,000xg supernatant	12.4	16	0.02
% recovery		99	

[a]Assay system (vol. 1 ml): 52 µM CDP-[1,2-^{14}C]choline (1.1 x 10^6 dpm), 100 mM Tris HCl (pH 8.0), 2 mM $MnCl_2$, sn-1,2-diglyceride (300 µg), and an aliquot of cell-free homogenate (0.05 ml) or cell fraction (0.1 ml) suspended in homogenizing medium. Cell fractions were those used for assay of PE synthesis described in Table 19 after storage at pH 7.0 for 24h at -15°C. The diglyceride was prepared and dispersed in the assay mixture as described in Table 19. Incubation, 30 min. at 30°C. PC synthesis was calculated from the incorporation of ^{14}C into total lipids.

from the 100,000xg fraction in chloroform-methanol-acetic acid-water (65:25:8:4, by vol.) showed that 95% of the total lipid ^{14}C was associated with PC (R_f, 0.30) and 5% with lyso PC (R_f, 0.07). The identity of these lipids was confirmed by the formation of GPC as the only H_2O soluble product after deacylation (R_f 0.31 on Whatman No. 1 paper in n-butanol-acetic acid-water (5:3:1, by vol.), identical to that of standard GPC).

The optimum pH for CDP-choline:sn-1,2-diglyceride-phosphorylcholine transferase was found to be 7.0, identical to that for CDP-ethanolamine:sn-1,2-diglyceride phosphoryl-ethanolamine transferase (Fig. 23B), using the same assay system.

5. The Biosynthesis of Phosphatidyl choline by the Methylation Pathway

The methylation pathway was investigated using phosphatidyl[1,2-^{14}C]ethanolamine or S-adenosyl[Me-^{14}C] methionine as labelled substrate.

a) Phosphatidyl[1,2-^{14}C]ethanolamine as labelled substrate

[^{14}C]PE was synthesized in situ by pre-incubating a 100,000xg fraction in a system at pH 8.0 containing CDP-[1,2-^{14}C]ethanolamine and Mn^{2+}. The mixture was then further incubated with unlabelled S-adenosyl methionine (SAM), either alone or in the presence of Triton X-100 or the 100,000xg

supernatant fraction. Details of each assay system and the distribution of ^{14}C among lipid products are shown in Fig. 21 and Table 18. No [^{14}C]PC was detected in any of the incubation mixtures containing SAM. These results suggested the absence of a PE:SAM methyltransferase capable of methylating PE generated in situ. Since no [^{14}C]PC was formed in the presence of Triton X-100 or the 100,000xg supernatant (see assays 3-4, Fig. 21, Table 18), the negative results were not due to inadequate dispersion of PE substrate nor to lack of soluble cofactors.

b) S-adenosyl[Me-^{14}C]methionine as labelled substrate

i) Individual steps in the Methylation Pathway

Assays were carried out in which a 100,000xg fraction was incubated with [^{14}C]SAM both in the absence and presence of exogenous PE, phosphatidyl monomethylethanolamine (PE-Me) or phosphatidyl dimethylethanolamine (PE-diMe). It should be pointed out that under the conditions of assay used, a small amount (0.5%) of [^{14}C]SAM was found to break down non-enzymatically to produce two chloroform soluble ^{14}C-labelled compounds which migrated on TLC in the solvent system described in Fig. 24 with R_fs of 0.40 and 0.70. A similar observation was made by Smith and Law (1970) who partially identified these products as [Me-^{14}C]methylthioadenosine and [Me-^{14}C]methylthioribose respectively. However, in all studies reported below these breakdown products were completely removed from

total lipid extracts by the following acid washing procedure: the combined chloroform phases (vol. 4.5 ml) following Bligh and Dyer extraction of ^{14}C-labelled lipid products from assay mixtures (see Methods VI.1) was washed twice with 1 ml of methanol-0.2N HCl (10:9 by vol.), and finally once with 1 ml of methanol-water (10:9); the chloroform phase was then neutralized immediately with 0.2N methanolic ammonia, diluted with benzene and concentrated to dryness under a N_2 stream.

The results of studies with [^{14}C]SAM are given in Figs. 24-27 and Tables 22-25. It was found that only a small amount (1390 dpm) of ^{14}C was incorporated into PC by [^{14}C]SAM in the absence of exogenous PE, PE-Me or PE-diMe, and of which only 13% was localized in the H_2O soluble GPC moiety (Table 22). The ^{14}C-incorporation into PC, and therefore presumably into its GPC moiety, was not stimulated by the presence of added sonicated spinach PE. Also, in neither of these two assay systems were significant amounts of ^{14}C incorporated into either the PE(+PE-Me) or PE-diMe fractions. These results (Table 22) were consistent with the findings from the experiments using [^{14}C]PE described above (section a) in showing the inability of the 100,000xg fraction to methylate significant quantities of either endogenous or exogenous PE.

However, in the presence of exogenous PE-Me or PE-diMe, the incorporation of ^{14}C into the GPC moiety of PC was stimulated 20-fold and 200-fold respectively (Table 22). Furthermore, in the presence of PE-Me, ^{14}C was also incorporated into PE-diMe in which 83% of the ^{14}C was localized in the

FIGURE 24

Autoradiogram of a chromatogram of lipids labelled with ^{14}C from [^{14}C]SAM by a 100,000xg fraction in the absence and presence of exogenous PE, PE-Me and PE-diMe.

1. Complete assay system
2. Complete assay system + PE
3. Complete assay system + PE-Me
4. Complete assay system + PE-diMe

Assay conditions as described in Table 22. Chromatography was on silica gel H plate in chloroform-methanol-acetic acid-water (65:25:8:4).

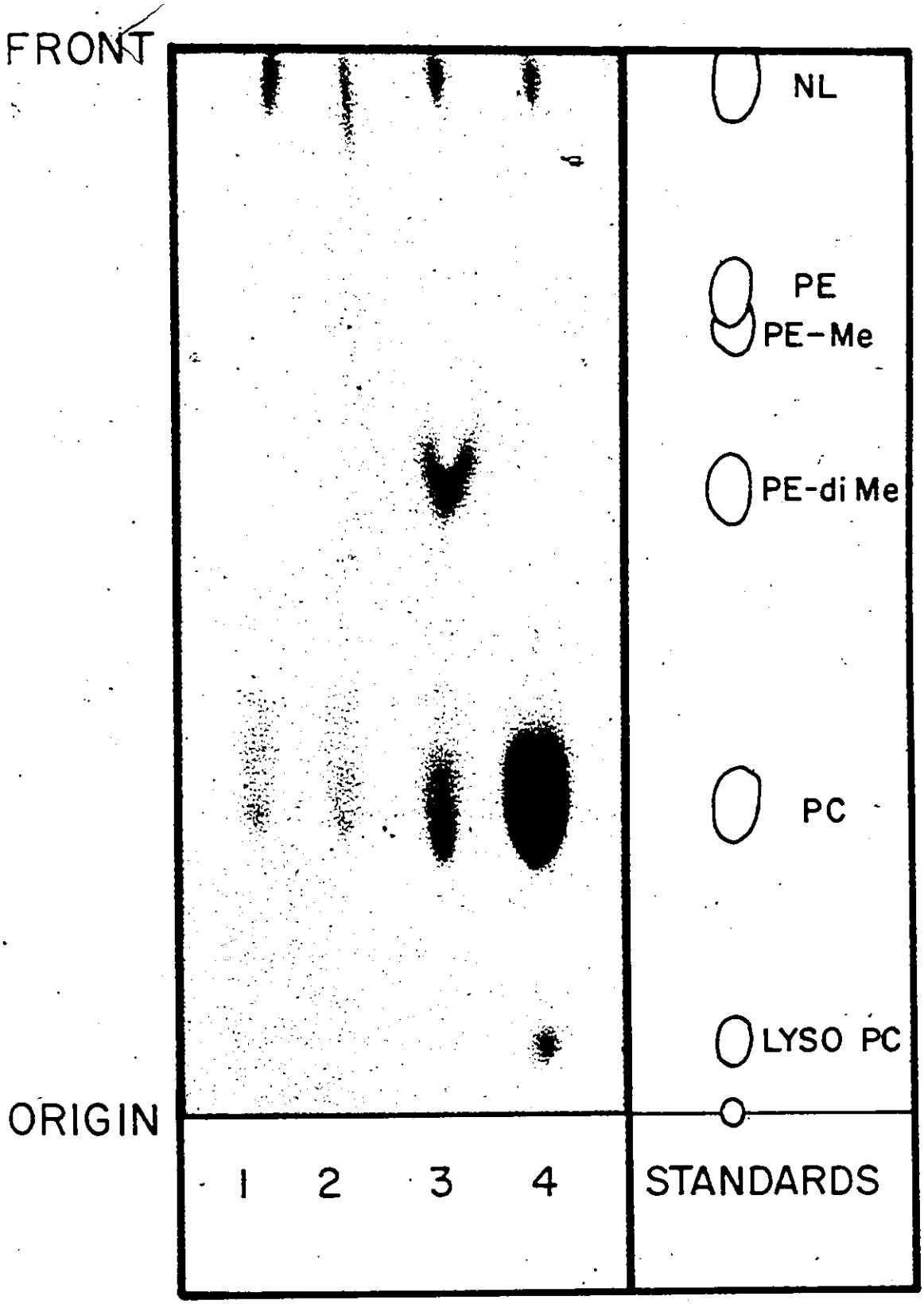


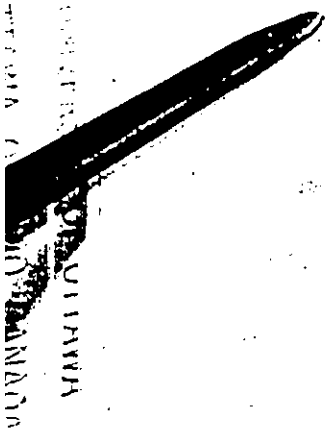
TABLE 22. THE EFFECT OF PE, PE-Me and PE-diMe ON THE BIOSYNTHESIS OF PHOSPHATIDYL CHOLINE BY THE METHYLATION PATHWAY IN SPINACH MICROSOMES

Assay System[a]	^{14}C-Incorporation[b], dpm x 10^{-3}									
	PC			PE			PE-diMe			Neutral Lipid
	Total	H_2O sol	$CHCl_3$ sol	Total	H_2O sol	$CHCl_3$ sol	Total	H_2O sol	$CHCl_3$ sol	Total
Complete	1.39	0.18(13)	1.21(86)	0.37	-	-	0	-	-	1.73
" + PE (0.2 mg)	1.23	-	-	0.49	-	-	0	-	-	1.78
" + PE-Me (0.4 mg)	4.85	3.40(70)	1.45(30)	0.43	-	-	3.36	2.79(83)	0.57(17)	1.68
" + PE-diMe (0.4 mg)	32.73	30.77(94)	1.96(6)	0.38	-	-	0	-	-	0.94
" + 100,000xg supernatant (3 mg protein)	0.90	-	-	0.49	-	-	0	-	-	4.99

[a] Complete assay system (total vol. 1 ml): 8 μM [^{14}C]SAM (9.5 x 10^5 dpm), 50 mM Tris HCl (pH 8.5) and 0.47 ml of 100,000xg pellet suspension (1.7 mg protein). Incubation, 30 min at 30°C. Spinach PE (isolated as described in Methods I.2.b.iii) and PE-Me and PE-diMe were dispersed by sonication (Experimental II.3.b.ii) and added where indicated.

[b] Lipid products are those shown in Fig. 24; ^{14}C incorporation into each lipid calculated as described in Experimental II.3.a. Numbers in parentheses denote % distribution of ^{14}C activity between H_2O soluble and $CHCl_3$ soluble moieties after mild alkaline hydrolysis (see Methods III.1). Dash (-) denotes data unavailable.

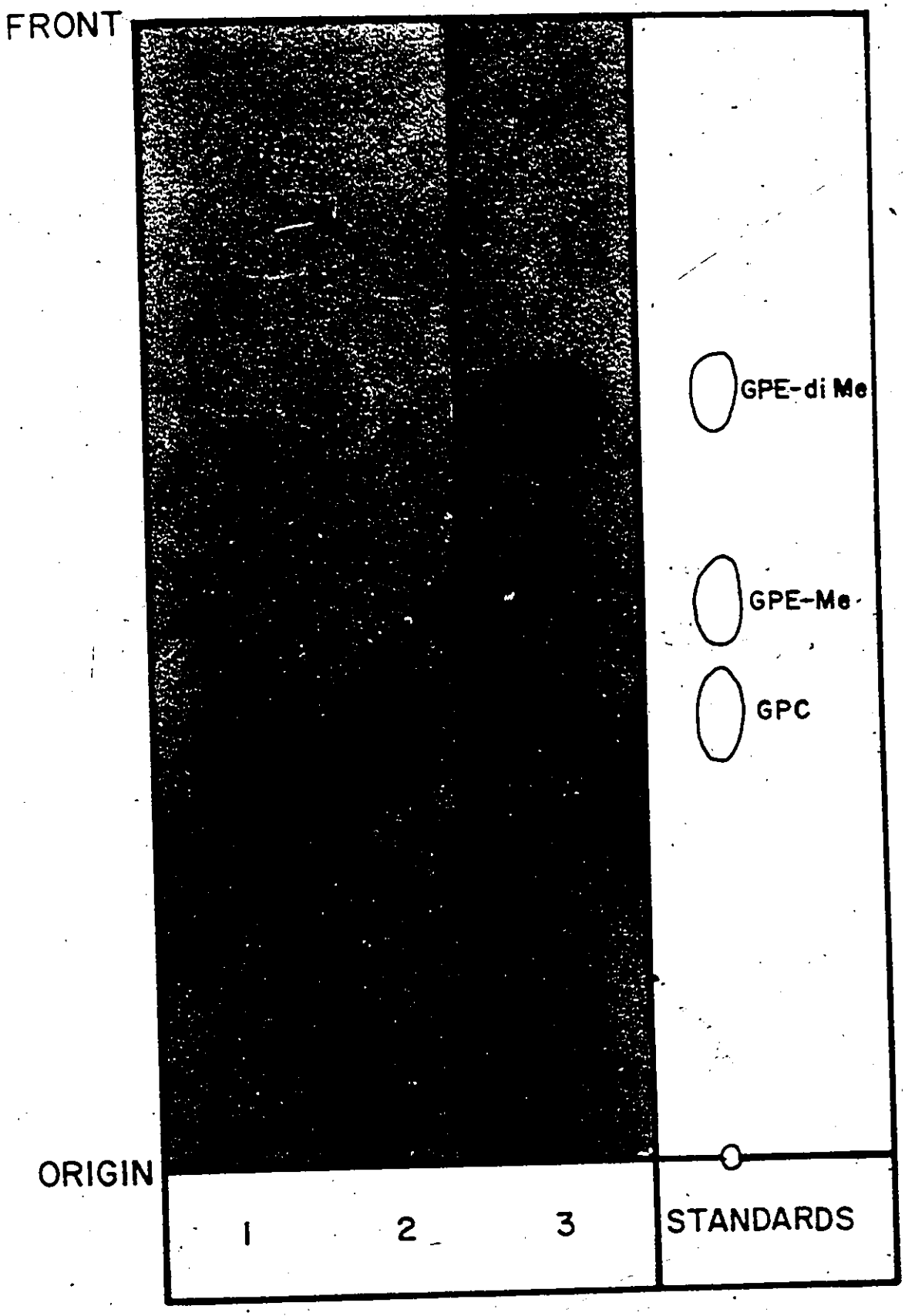
FIGURE 25



Autoradiogram of a chromatogram of methanol-water soluble products from deacylation of PC and PE-diMe labelled with ^{14}C from [^{14}C]SAM.

1. [^{14}C]PC labelled in the presence of PE-Me
2. [^{14}C]PC labelled in the presence of PE-diMe
3. [^{14}C]PE-diMe labelled in the presence of PE-Me

Labelled lipids are those described in Table 22. Chromatography was on Whatman No. 1 paper in isopropanol-water-conc. ammonia (7:2:1 by vol.).


FRONT
GPE-di Me
GPE-Me
GPC
ORIGIN
1
2
3
STANDARDS

GPE-diMe moiety. [^{14}C]GPC and [^{14}C]GPE-diMe were identified by paper chromatography as shown in Fig. 25. These results therefore established the existence of an enzyme system in the 100,000xg fraction catalyzing the methylation of PE-Me to PC via the intermediate formation of PE-diMe.

It should be pointed out that both in the absence and presence of added phospholipid, ^{14}C was incorporated into the chloroform soluble fatty acid moiety of PC (Table 22). The nature of the reactions leading to this incorporation will be described in more detail in Results IV.7. Also, a small amount of ^{14}C was incorporated from [^{14}C]SAM into the neutral lipid fraction and was fairly independent of added phospholipid (see Fig. 24 and Table 22). The identity of ^{14}C-labelled components comprising this neutral lipid fraction will be described in Results V.1.

The results described above (Figs. 21, 24 and Tables 18, 22) raised the question of the role of PE in the methylation pathway. Though a very small amount of ^{14}C was incorporated into the GPC moiety of PC in the absence of added PE-diMe (Table 22), this may have arisen from methylation of trace amounts of PE-Me and PE-diMe that may be present in the 100,000xg fraction. As mentioned earlier, the addition of exogenous PE dispersed by sonication did not stimulate ^{14}C-incorporation into the GPC moiety of PC (Table 22). An experiment was then carried out with a 100,000xg ("heavy microsomal") pellet using spinach PE dispersed by the dialysis

procedure of Fleischer and Klouwen (see Experimental II.3.b.iii). However, incorporation of ^{14}C into the water-soluble (GPC) moiety of PC was increased only by about 60% (Table 23 and Fig. 26). The small amount of ^{14}C incorporated into the PE(+PE-Me) fraction by the 100,000xg pellet, alone or in the presence of the 250,000xg pellet, was also increased, but the ^{14}C was concentrated largely (82%) in the fatty acid ($CHCl_3$ soluble) moieties. Only 18% of the total ^{14}C in this fraction was localized in the H_2O soluble moiety, but no [^{14}C]GPE-Me could be detected by paper chromatography. Since insignificant amounts of [^{14}C]PC, and no [^{14}C]PE-Me, were formed by the addition of dialyzed-dispersed PE, it was concluded therefore that demonstration of PE:SAM methyltransferase was not dependent on the mode of dispersion of exogenous PE.

The possibility that PE:SAM methyltransferase was present in another cell fraction was then investigated. Neither the 1200xg nor the 15,000xg fractions in assay systems at pH 8.5 containing [^{14}C]SAM and dialyzed-dispersed PE were found to incorporate significant amounts of ^{14}C into PE-Me or the GPC moiety of PC. The 250,000xg ("light microsomal") fraction also incorporated only traces of ^{14}C into PC; under the same assay conditions this fraction was capable of converting added PE-diMe to PC, and presumably also PE-Me to PC ("light microsomes" exp., Table 23). Furthermore, addition of the 250,000xg pellet to an assay system containing the 100,000xg pellet did not stimulate ^{14}C-incorporation into PC

TABLE 23. THE BIOSYNTHESIS OF PHOSPHATIDYL CHOLINE BY THE METHYLATION PATHWAY IN "HEAVY" AND "LIGHT" SPINACH MICROSOMES

Assay System[a]	^{14}C-Incorporation[b], dpm x 10^{-3}							
	PC			PE			PE-diMe	Neutral Lipid
	Total	H_2O sol	$CHCl_3$ sol	Total	H_2O sol	$CHCl_3$ sol	Total	Total
"Heavy microsomes" (100,000xg)								
Complete	3.14	0.99(32)	2.15(68)	0.60	-	-	0.35	3.08
" + dialyzed PE	9.12	1.57(17)	7.56(83)	1.89	0.67(18)	3.02(82)	0.47	17.74
" + dialyzed PE + "light microsomes"	9.75	1.74(18)	8.01(82)	1.80			0.46	18.98
" + PE-diMe	154.0	-	-	0.58	-	-	0.31	2.33
"Light microsomes" (250,000xg)								
Complete + dialyzed PE	0.41	-	-	0.31	-	-	0	0.62
" + PE-diMe	22.0	-	-	0.31	-	-	0	0.51

[a]Complete assay system (vol. 1 ml): 16 μM [^{14}C]SAM (1.9 x 10^6 dpm), 50 mM Tris HCl (pH 8.5) and 0.55 ml of either "heavy microsome" fraction (100,000xg pellet, 3.5 mg protein) or "light microsome" fraction (250,000xg pellet, 2.3 mg protein). 0.3 mg of spinach PE, dispersed by dialysis as described in Experimental II.3.b.iii, and 0.5 mg of PE-diMe dispersed by sonication as described in Experimental II.3.b.ii, were added where indicated.

[b]^{14}C-Labelled lipids were separated by TLC in chloroform-methanol-acetic acid-water (65:25:8:4) and ^{14}C-incorporation into each lipid calculated as described in Experimental II.3.a. Numbers in parentheses denote % distribution of ^{14}C activity between $CHCl_3$ soluble and H_2O soluble moieties after mild alkaline hydrolysis. Dash (-) denotes data unavailable.

FIGURE 26

Autoradiogram of a chromatogram of the methanol-water soluble product from deacylation of PC labelled with ^{14}C from [^{14}C]SAM by a 100,000xg fraction in the presence of exogenous PE. The [^{14}C]PC sample deacylated was that described in Table 23.

Chromatography was on Whatman No. 1 paper in isopropanol-water-conc. ammonia (7:2:1 by vol.).

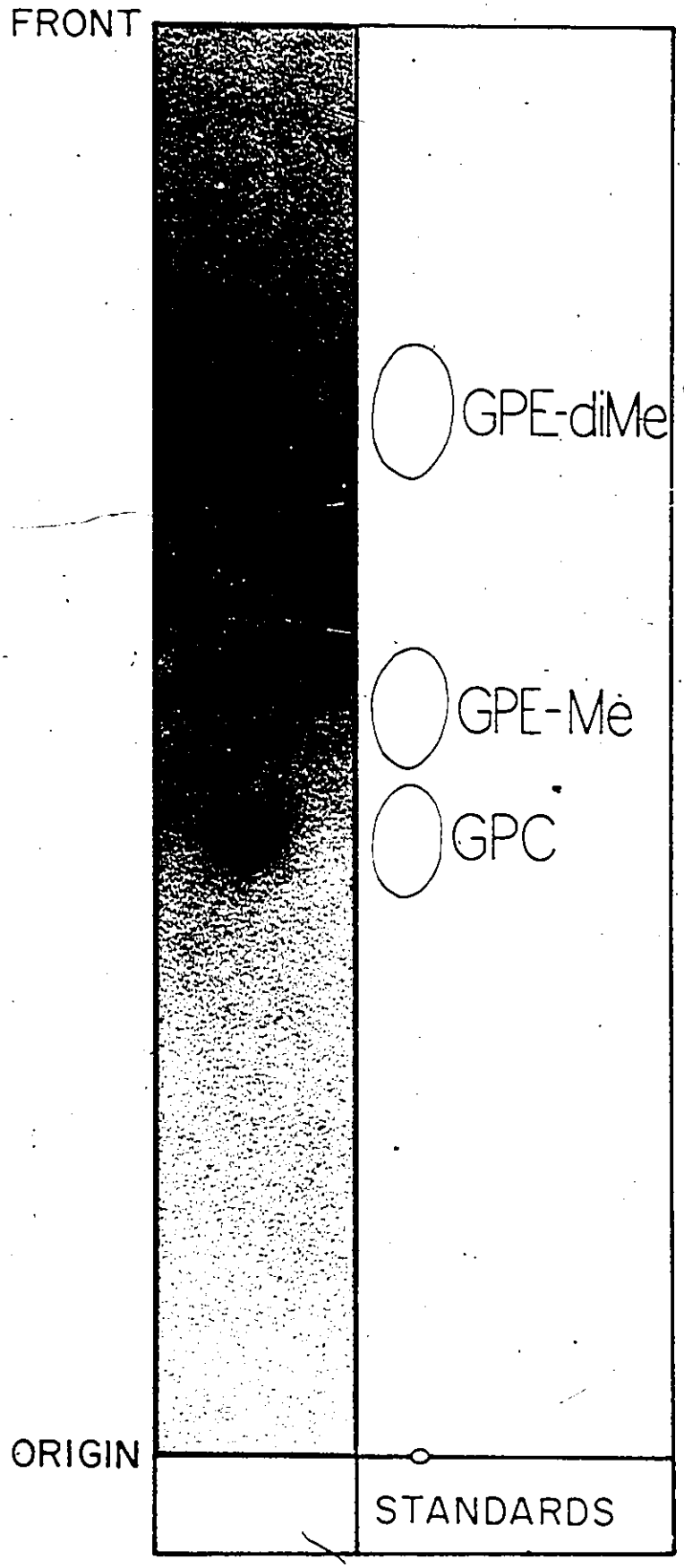

FRONT
GPE-diMe
GPE-Me
GPC
ORIGIN
STANDARDS

TABLE 24. THE EFFECT OF METAL IONS ON ^{14}C-INCORPORATION FROM [^{14}C]SAM INTO PC AND NEUTRAL LIPIDS

Assay System[a]	^{14}C-Incorporation[b], dpm	
	PC	Neutral Lipids
Complete system	1020	4400
" + 1 mM $MgCl_2$	1020	4030
" + 10 mM $MgCl_2$	840	2360
" + 1 mM $MnCl_2$	1080	1420
" + 10 mM $MnCl_2$	430	280
" + 5 mM NaCl + 5 mM KCl	1160	2860

[a] Complete assay system (vol. 1 ml): 8 µM [^{14}C]SAM (9.5×10^5 dpm), 100 mM Tris HCl (pH 8.0) and 0.3 ml of 1200 - 100,000xg pellet suspension (3 mg protein). Incubation, 30 min at 30°C.

[b] ^{14}C-labelled lipids were separated by TLC in chloroform-methanol-acetic acid-water (65:25:8:4 by vol), and ^{14}C-incorporation into PC (Rf, 0.31) and neutral lipid (Rf, 1.0) calculated as described in Experimental II.3.a.

from dialyzed-dispersed PE substrate ("heavy microsomes" exp., Table 23). When the 100,000xg supernatant fraction was tested, it was found that no ^{14}C was incorporated into PE-Me or into the GPC moiety of PC. Also the addition of this fraction to an assay system containing the 100,000xg pellet did not increase ^{14}C incorporation into PC regardless of whether the ^{14}C-labelled substrate was SAM (Table 22), or PE (Fig. 21 and Table 18). It was therefore concluded that PE:SAM methyltransferase was not present either in the 1200 xg, 15,000xg and 250,000xg pellets, or the 100,000xg supernatant fraction.

The possibility was considered that failure to detect PE:SAM methyltransferase in the above experiments might have been due to the absence of possible metal ion cofactors. However, it was found that the presence of either Mn^{2+}, Mg^{2+}, or Na^{+} plus K^{+}, failed to stimulate incorporation of ^{14}C into PC by a 1200-100,000xg fraction, and in fact Mg^{2+} and Mn^{2+} were strongly inhibitory at 10 mM concentrations (Table 24). It was concluded therefore that the detection of PE:SAM methyltransferase was not dependent on the presence of mono- or divalent metal ions. It should be noted that the metal ions at all concentrations strongly inhibited ^{14}C-incorporation into neutral lipid, and the significance of this will be discussed further below.

The possibility that PE:SAM methyltransferase was inactivated during subcellular fractionation or assay by air

oxidation of active site cysteine residues was ruled out by the finding that ^{14}C incorporation into PC by a 100,000xg fraction was unaffected by the presence of β-mercaptoethanol in the homogenizing medium or assay mixtures. Also, it was found that ^{14}C-incorporation into PC was unaffected by extensive dialysis of the 100,000xg fraction prior to assay, which largely ruled out the possibility that low molecular weight inhibitors might be loosely bound to the enzyme.

Since all attempts to demonstrate the methylation of PE by SAM failed, other pathways for the biosynthesis of PE-Me were considered. A possibility is that PE is methylated by another methyl donor. 5-[Me-^{14}C]tetrahydrofolate ([^{14}C]THF) was tested in an assay system identical to that described in Table 22 except that [^{14}C]SAM was replaced by [^{14}C]THF (final conc. 8 μM). However, no ^{14}C was found to be incorporated into lipid, thus ruling out the possibility of [^{14}C]THF as a methyl donor. Other methyl donors were not tested. Another possibility for the biosynthesis of PE-Me is N-methylation of PS by SAM followed by decarboxylation. This was tested by incubation of the 100,000xg fraction at pH 7.5 either with unlabelled PS plus [^{14}C]SAM, or with [^{14}C]PS plus unlabelled SAM. TLC in chloroform-methanol-acetic acid-water (65:25:8:4) of the labelled lipid products from the former assay showed that no ^{14}C was incorporated into PE-Me, nor was there any increase in the ^{14}C -incorporation into PC above that from a control assay in which unlabelled PS was omitted.

TLC in the same solvent system of the labelled lipid products from the latter assay also showed that no ^{14}C was incorporated into PC from [^{14}C]PS. These results therefore indicate that PE-Me is not biosynthesized by a PS methylation and decarboxylation pathway.

ii) Subcellular localization and characteristics of PE-diMe:SAM methyltransferase

Results on the subcellular localization are shown in Table 25. The cell fraction with the highest percentage of total activity, and highest specific activity, was the 100,000xg microsomal pellet, which was essentially free of mitochondria (see Results IV.3.a). The low activity in the 1200xg fraction, in which a high proportion of the total chlorophyll was localized, strongly suggests the absence of this enzyme in chloroplasts. Appreciable activity was found in the 15,000xg fraction, but it is not known whether it arose from microsomal contamination or from a separate mitochondrial enzyme system.

It should be pointed out that the high recovery of activity (156%) may have been due to assay of the cell free homogenate at protein levels above the linear range of the activity-enzyme concentration plot, resulting in a low apparent activity for this fraction.

The methyltransferase has a pH optimum in Tris HCl buffer of 8.0 (Fig. 27). The enzyme is apparently specific for SAM since no ^{14}C was found to be incorporated into lipid when [^{14}C]SAM was replaced by 5-[Me-^{14}C]tetrahydrofolate in an assay system identical to that described in Table 22.

TABLE 25. SUBCELLULAR DISTRIBUTION OF PHOSPHATIDYL DIMETHYLETHANOLAMINE: S-ADENOSYLMETHIONINE-METHYL TRANSFERASE

Cell Fraction	Protein		Chlorophyll		Phosphatidyl choline synthesis[a]		
	mg	%	mg	%	Activity nmoles/15 min	%	Specific Activity
Cell free homogenate	1230	100	19.1	100	40.6	100	0.03
1200xg	140	11	7.9	41	2.1	5	0.02
15,000xg	180	15	7.9	41	17.2	42	0.09
100,000xg	80	7	0.9	5	37.4	92	0.47
100,000xg supernatant	730	59	0	0	6.9	17	0.01
% recovery		92		87		156	

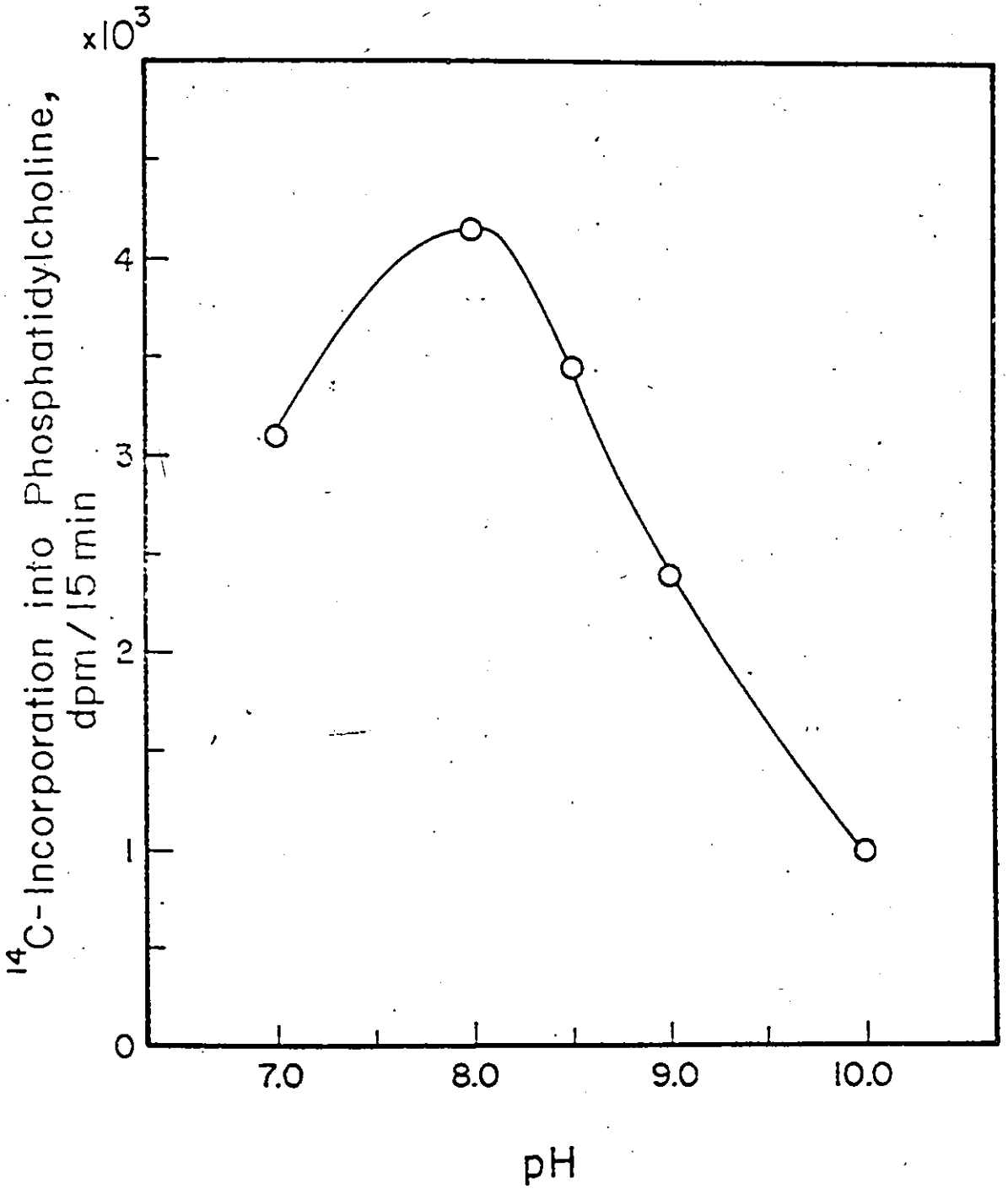
[a]Assay system (vol. 0.5 ml): 2.7 μM SAM (3.1×10^5 dpm), 100 mM Tris HCl (pH 8.0), dipalmitoyl PE-diMe (0.4 mg, dispersed prior to addition to assay mixture as described in Experimental II.3.b.ii), and 0.1 ml of cell free homogenate or cell fraction dispersed in homogenizing medium. Incubation, 15 min at 30°C. The ^{14}C-incorporation into total lipids was measured. Control assays were carried out for each fraction in the above assay mixture minus PE-diMe. PC synthesis activity was then defined as:

$$\text{PC synthesis (n moles)} = \frac{\text{dpm } ^{14}\text{C-incorporation in presence of PE-diMe} - ^{14}\text{C dpm of control}}{\text{specific activity of } [^{14}\text{C}]\text{SAM (dpm/n mole)}}$$

FIGURE 27

The effect of pH on the incorporation of ^{14}C into PC synthesized by methylation of PE-diMe by [^{14}C]SAM.

Assay system as described in Table 22 except that pH of Tris HCl buffer varied as indicated. ^{14}C-Incorporation into PC calculated as the difference between ^{14}C-incorporation into total lipid from assays containing PE-diMe and control assays in which PE-diMe was absent.


x10³
4
3
2
1
0
14C- Incorporation into Phosphatidylcholine, dpm/15 min
7.0
8.0
9.0
10.0
pH

6. The Biosynthesis of Phosphatidyl Serine, Phosphatidyl Ethanolamine and Phosphatidyl Choline by Exchange Reactions

The results of this study are shown in Table 26. In the presence of EDTA, a particulate (1200-100,000xg) fraction at pH 8.5 incorporated very small amounts of [1,2-^{14}C] ethanolamine and [1,2-^{14}C]choline into PE and PC respectively, but no [U-^{14}C]L-serine into either PS or PE. However, in the presence of Ca^{2+} and absence of EDTA, the incorporation of [1,2-^{14}C]ethanolamine into PE and of [1,2-^{14}C]choline into PC was stimulated eight fold and four fold respectively, while again no [U-^{14}C]serine was incorporated into either PS or the PS decarboxylation product, PE. Thus, the observed Ca^{2+} stimulation suggests that the incorporation of ethanolamine and choline into lipids in the absence of added nucleotides occurred via exchange reactions of the type described in Introduction III.1.j. However, it should be pointed out that the observed activities were extremely low, i.e., only 0.18% of [1,2-^{14}C]ethanolamine and 0.12% of [1,2-^{14}C]choline were incorporated into PE and PC respectively.

7. The Incorporation of ^{14}C Activity from S-adenosyl [Me-^{14}C]methionine into the Fatty Acid Moieties of Phosphatidyl Choline and Phosphatidyl Ethanolamine. In vitro and in vivo studies

As described above in Results IV.5, a 100,000xg fraction at pH 8.5 incorporated ^{14}C from [^{14}C]SAM into the

TABLE 26. THE INCORPORATION OF [U-^{14}C]L-SERINE, [1,2-^{14}C]ETHANOLAMINE AND [1,2-^{14}C]CHOLINE INTO PHOSPHOLIPIDS BY SPINACH PARTICULATE FRACTION

Assay System[a]	^{14}C-Incorporation[b], dpm		
	PS	PE	PC
Complete + [U-^{14}C]L-serine + EDTA	0	0	0
" " + Ca^{2+}	0	0	0
Complete + [1,2-^{14}C]ethanolamine + EDTA	0	498	0
" " + Ca^{2+}	0	4066	0
Complete + [1,2-^{14}C]choline + EDTA	0	0	385
" " + Ca^{2+}	0	0	1622

[a]Complete assay system (vol. 1 ml): 50 mM Tris HCl (pH 8.5), 1.5 mM β-mercaptoethanol, 0.4 ml of 1200-100,000xg pellet suspended in H_2O (1.3 mg), and either 7.4 μM [U-^{14}C]L-serine (2.1 x 10^6 dpm), 0.4 mM [1,2-^{14}C]-ethanolamine (2.3 x 10^6 dpm) or 0.1 mM [1,2-^{14}C]choline chloride (1.4 x 10^6 dpm). Where applicable, assay systems contained either 2 mM EDTA Na_2 or 5 mM $CaCl_2$. Incubation, 1 h at 30°C. The pellet assayed was that used in the experiment described in Table 13.

[b]^{14}C labelled lipids were separated by TLC in chloroform-methanol-acetic-acid-water (65:25:8:4, by vol.; Rfs of PE, PS and PC, 0.72, 0.53 and 0.31 respectively), and the ^{14}C incorporation into each lipid calculated as described in Experimental II.3.a.

chloroform soluble fraction of deacylated PC (Tables 22 and 23). TLC of this fraction showed the major component to be fatty acid methyl esters, together with traces of free fatty acids, (Fig. 28B). Since individual molecular species of fatty acid methyl esters cannot be separated on silica gel H plates, the structure of the fatty acids in PC labelled from [^{14}C]SAM is not known, but most likely they are cyclopropane acids. This question will be further discussed below.

The incorporation of ^{14}C into PC fatty acid was linear with time of incubation up to 30 min (Fig. 29A), and had a pH optimum of 8.5 (Fig. 29B). At these values of incubation time and pH, half maximal ^{14}C-incorporation into PC fatty acid was obtained at a [^{14}C]SAM concentration of approximately 15 µM (Fig. 29C). It should be pointed out that the results in Fig. 29 are expressed as the total ^{14}C-incorporation into PC, and it is assumed that essentially all of the ^{14}C is localized in the PC fatty acid moiety for each of the reaction mixtures assayed. This assumption is most probably valid since on deacylation of the combined [^{14}C]PC products from the time course and pH experiments (Fig. 29A and B), over 90% of the ^{14}C was found to be localized in the fatty acid moieties.

^{14}C-Incorporation from [^{14}C]SAM into the fatty acid moieties of PE was also demonstrated (Fig. 28B), but in a reaction mixture containing exogenous dialyzed PE which probably contained some deoxycholate (see Experimental II.3.b.iii). However, the effect of deoxycholate itself on the ^{14}C-incorporation into PE fatty acids is not known.

Evidence for ^{14}C-incorporation from [^{14}C]SAM into the fatty acid moieties of PC and PE was obtained also in the *in vivo* experiment with whole leaves. It will be recalled that ^{14}C from [Me-^{14}C]methionine was incorporated in equal amounts into the chloroform soluble products of both the deacylated PC and PE fractions (see Results III.2.c. and III.3.a). A major component of these chloroform soluble products from both lipid fractions was shown to be ^{14}C-labelled fatty acid methyl esters (Fig. 28A). It should be noted that a significant amount of ^{14}C from the PE fraction was associated with a component migrating on TLC just slower than fatty acid methyl ester (Fig. 28A), and which was not further identified. These results suggest the presence in whole leaves of enzymes catalyzing the incorporation of Me-^{14}C from [Me-^{14}C]methionine, presumably via the intermediate formation of [^{14}C]SAM, into cyclopropane fatty acids localized in PC and PE.

It is of interest that the PC and PE fractions were also labelled in about equal amounts by whole leaves from [U-^{14}C]serine, but to a much greater extent than from [Me-^{14}C] methionine (see Results III.2.b. and III.3.a). The main ^{14}C-labelled component in the chloroform soluble products was again fatty acid methyl ester (see Fig. 28A).

FIGURE 28

Autoradiogram of a chromatogram of chloroform soluble products from deacylation of PE and PC labelled *in vivo* from [U-^{14}C]serine and [Me-^{14}C] methionine and *in vitro* from [^{14}C]SAM.

A. Lipids[a] labelled by whole leaves as described in Table 11.
   1. [^{14}C]PE labelled from [Me-^{14}C]methionine
   2. [^{14}C]PC labelled from [Me-^{14}C]methionine
   3. [^{14}C]PE labelled from [U-^{14}C]serine
   4. [^{14}C]PC labelled from [U-^{14}C]serine

B. Lipids[a] labelled by 100,000xg fraction as described in Tables 22 and 23.
   5. [^{14}C]PC labelled from [^{14}C]SAM
   6. [^{14}C]PE labelled from [^{14}C]SAM in the presence of dialyzed-dispersed PE.

Chromatography was on silica gel H plates in pet. ether-diethylether-acetic acid acid (90:10:1). Abbreviations: a, methyl stearate plus methyl dihydrosterculate; b, stearic acid.

[a] ^{14}C-Labelled PE and PC were isolated from total labelled lipids (see Tables 11, 22 and 23) by preparative TLC in chloroform-methanol-acetic acid-water (65:25:8:4)

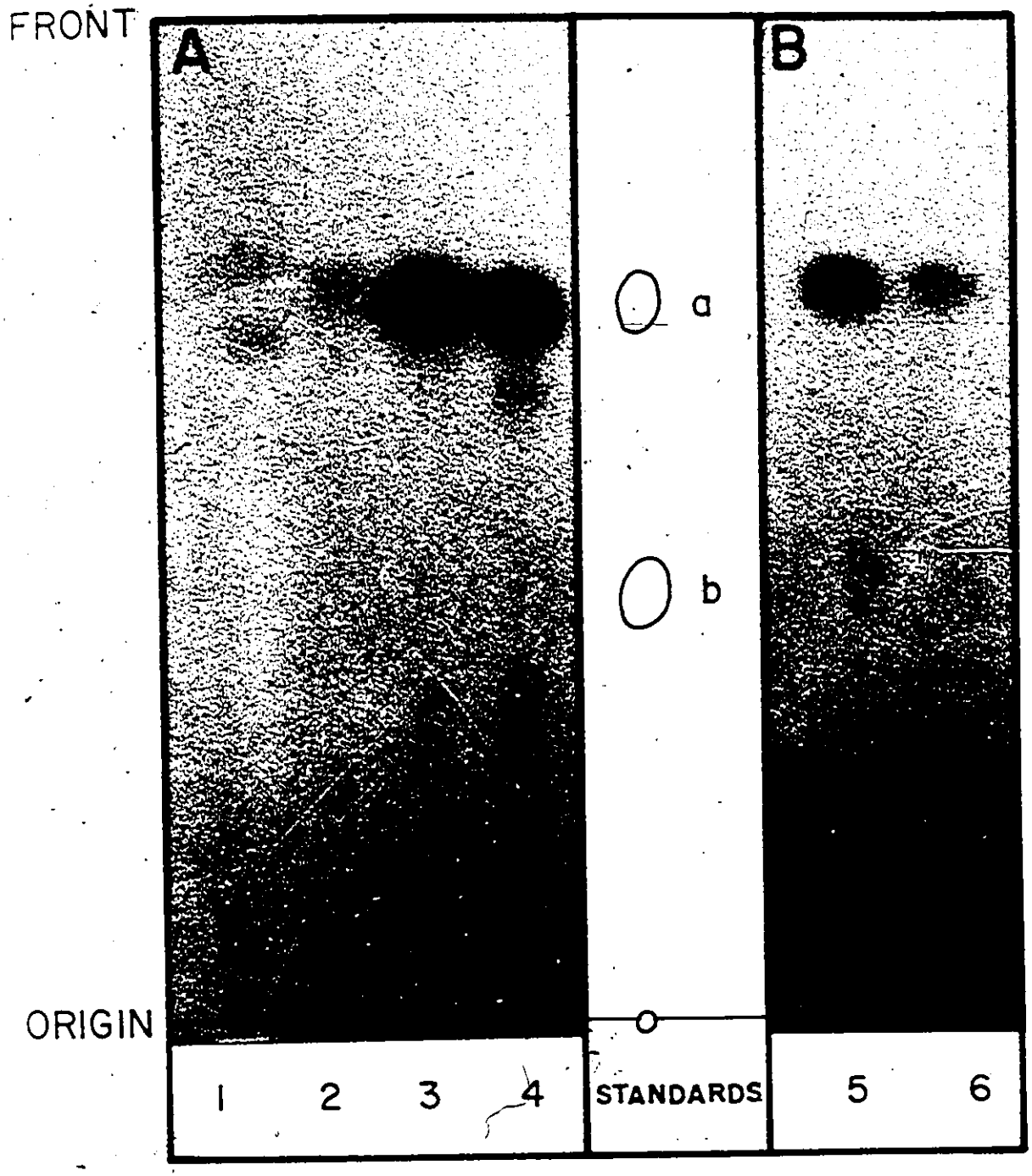

FRONT
A
B
a
b
ORIGIN
1
2
3
4
STANDARDS
5
6

FIGURE 29

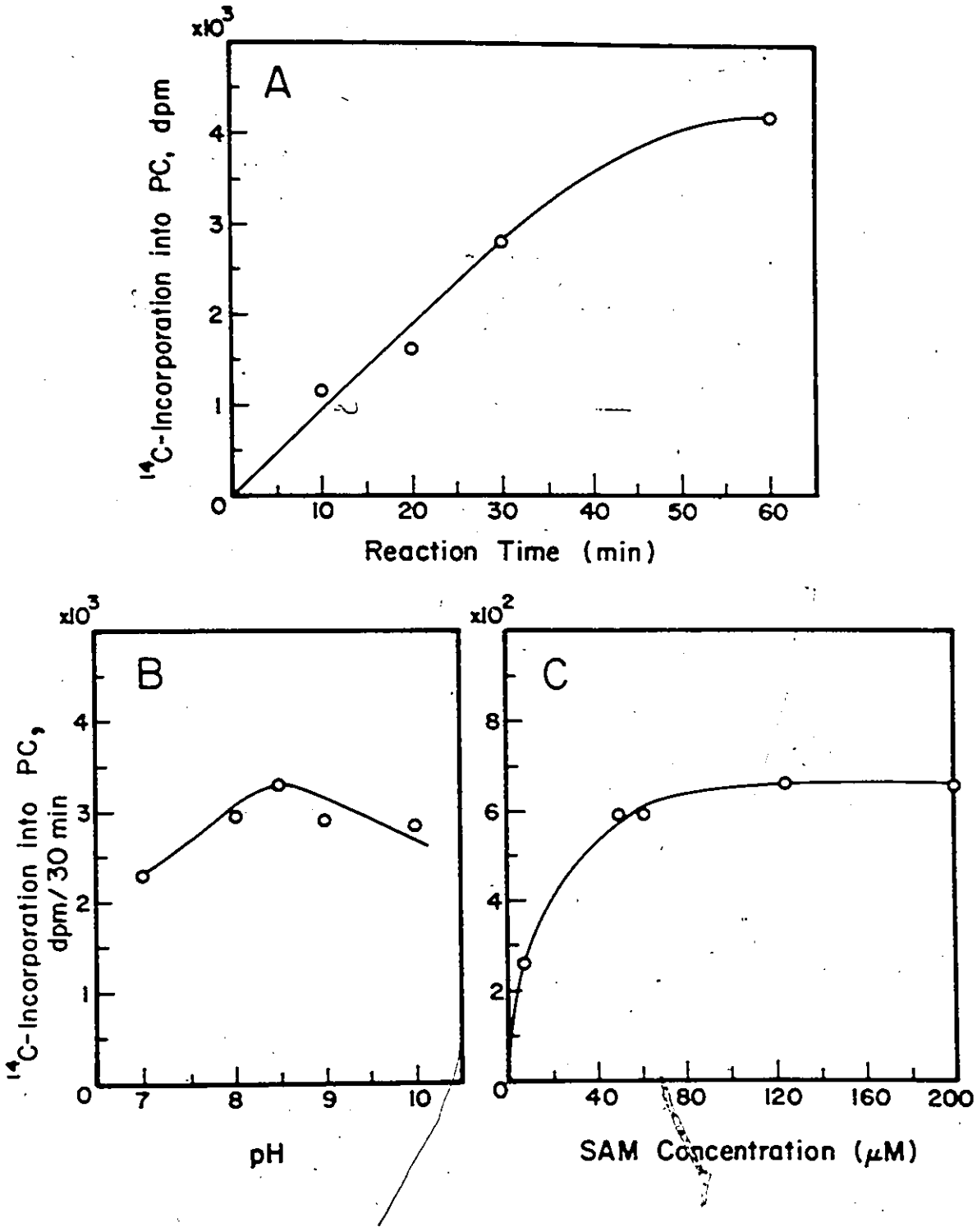
The effect of reaction time, pH and SAM concentration on the incorporation of ^{14}C from [^{14}C]SAM into fatty acid moieties of PC by a 100,000xg fraction.

Assay system as described in Table 22 except that following assay conditions varied as indicated.

A. Reaction time. Enzyme source: 100,000xg fraction (2.5 mg protein)

B. pH. Enzyme source: 100,000xg fraction (2.5 mg protein)

C. [^{14}C]SAM concentration. Specific activity of [^{14}C]SAM was 1.9×10^4 dpm/nmole, and values of ^{14}C-incorporation into PC are the mean of duplicate experiments (deviation from mean, < ±5%). Enzyme source: 100,000xg fraction (1.7 mg protein).


A
x10³
¹⁴C-Incorporation into PC, dpm
0
1
2
3
4
10
20
30
40
50
60
Reaction Time (min)
B
x10³
¹⁴C-Incorporation into PC, dpm/30 min
0
1
2
3
4
7
8
9
10
pH
C
x10²
0
2
4
6
8
40
80
120
160
200
SAM Concentration (μM)

V. The Incorporation of ^{14}C Activity from S-adenosyl [Me-^{14}C] methionine into Neutral Lipids

1) In vitro studies

During the studies on the biosynthesis of PC by the methylation pathway (Results IV.5), it was observed that ^{14}C was incorporated from [^{14}C]SAM by the 100,000xg pellet and supernatant fractions into neutral lipid (see Tables 22-23 and Fig. 24). Experiments on the subcellular distribution of the enzymes catalyzing this incorporation were then carried out, the results of which are shown in Table 27. The fractions most actively incorporating ^{14}C into neutral lipid were found to be the chloroplast plus mitochondria (600-15,000xg) pellet, and the 100,000xg supernatant, while comparatively little activity was found in the 100,000xg pellet fraction. The incorporation of ^{14}C into neutral lipid by the 100,000xg pellet was stimulated 4-fold by dialyzed-dispersed PE. This stimulation was unlikely caused by PE itself, but possibly by the small amounts of deoxycholate which may have been present in the PE dispersion (see Experimental II.3.b.iii). The percentage recovery of neutral lipid synthesizing activity after cell fractionation was 79%.

The TLC separation of ^{14}C labelled neutral lipid products from each cell fraction is shown in Fig. 30. The products from the cell-free homogenate consisted of two major components (spots Q and S) and two minor components (spots P and R). Components S and R migrated with R_fs identical

TABLE 27. SUBCELLULAR DISTRIBUTION OF ENZYMES INCORPORATING ^{14}C FROM S-ADENOSYL [$^{14}CH_3$]METHIONINE INTO NEUTRAL LIPIDS

Cell Fraction	^{14}Incorporation into Neutral Lipid[a]	
	Activity dpmx10^{-6}	%
Cell-free homogenate	2.40	
600-15,000xg	0.94	39
100,000xg	0.03[b]	1
100,000xg supernatant	0.92	39
% recovery		79

[a]Assay system (vol. 1.0 ml): 58 µM SAM (1.9×10^6 dpm), 50 mM Tris HCl (pH 8.5) and 0.55 ml of cell fraction suspended in 2 mM Tris HCl (pH 8.0). Incubation, 1 h at 30°C. Lipid extract was freed of [^{14}C]SAM breakdown products as described in Results IV.5. ^{14}C-Labelled lipids were separated by TLC in chloroform-methanol-acetic-acid-water (65:25:8:4 by vol.) and the ^{14}C-incorporation into neutral lipid (Rf, 1.0) determined as described in Experimental II.3.a.

[b]Increased to 0.12 in the presence of dialyzed-dispersed PE (0.23 mg/ml).

FIGURE 30

Autoradiogram of a chromatogram of neutral lipids labelled from [^{14}C]SAM by subcellular fractions and from [Me-^{14}C]methionine by leaf slices.

From [^{14}C]SAM. Assay system as described in Table 27.

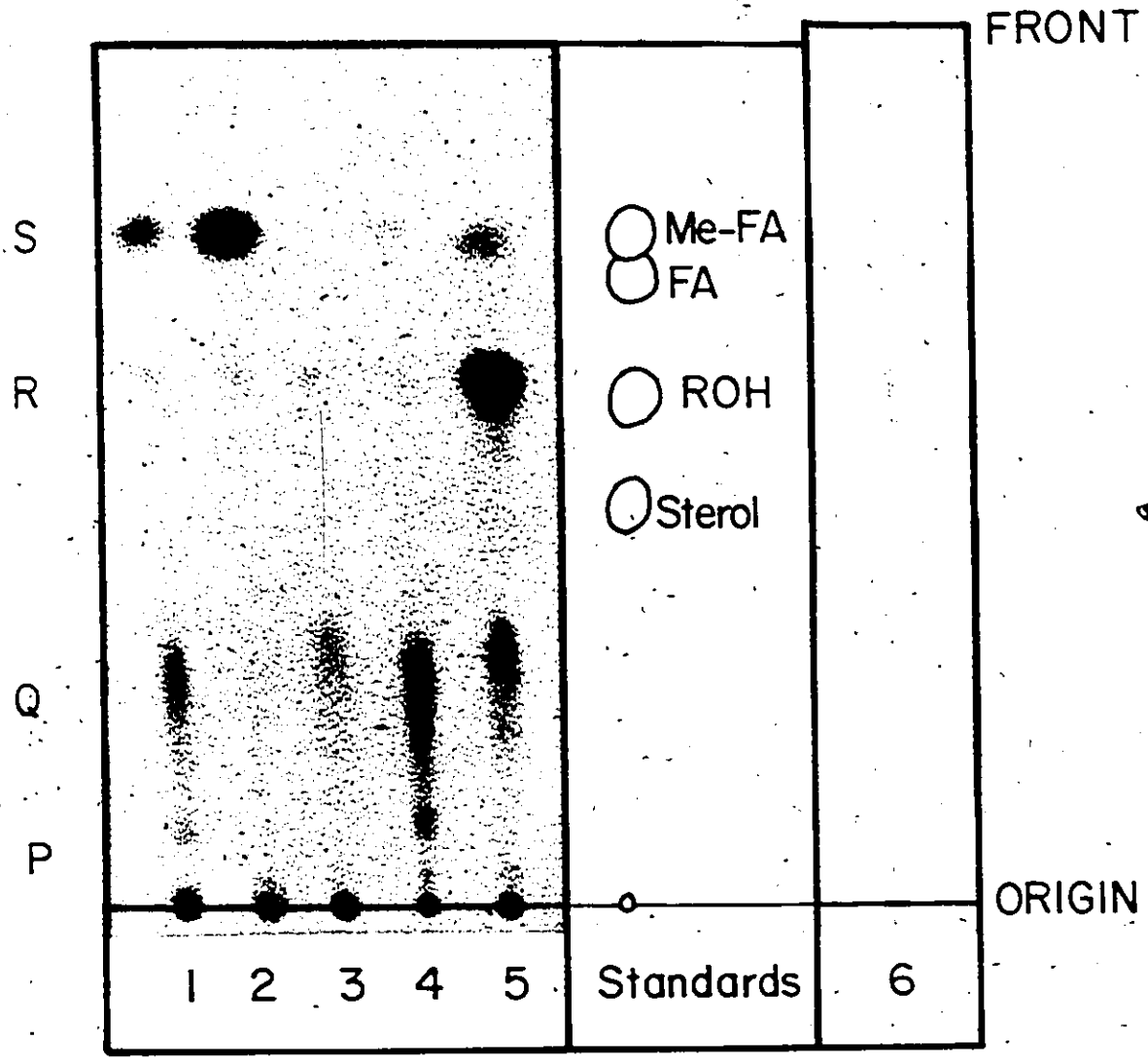
1. cell free homogenate
2. 600-15,000xg
3. 100,000xg
4. 100,000xg supernatant
5. 100,000xg pellet + dialyzed-dispersed PE.

From [Me-^{14}C]methionine. For experimental details see Table 11.

6. by leaf slices

Chromatography was on silica gel H plate in diisopropylether-acetic acid (96:4).

Abbreviations: Me-FA, fatty acid methyl ester; FA, fatty acid; ROH, long chain alcohol.


FRONT
S
R
Q
P
Me-FA
FA
ROH
Sterol
ORIGIN
1 2 3 4 5
Standards
6

to those of standard fatty acid methyl ester and long chain alcohol respectively, but the components P and Q migrated more slowly than sterol and were not further investigated.

Component S was the major ^{14}C-product from the 600-15,000xg fraction but was only slightly labelled by the 100,000xg pellet and supernatant fractions. Component Q was the major product of all fractions except the 600-15,000xg pellet, while component P was a product of the 100,000xg supernatant fraction only. Component R appeared to be a major product from the 100,000xg pellet fraction fortified with PE, but was only slightly labelled in the other fractions.

Taking the results of Table 27 and Fig. 30 together, the following conclusions can be made:

i) The enzyme system catalyzing synthesis of component S was localized in the chloroplast plus mitochondrial (600-15,000xg) fraction.

ii) The enzyme systems catalyzing synthesis of components P and Q were localized in the 100,000xg supernatant.

iii) The enzyme system(s) catalyzing synthesis of component R was distributed approximately evenly between the chloroplast plus mitochondrial fraction and the 100,000xg supernatant fraction.

Components S and R were further identified as follows. Both components were isolated from the neutral lipid products labelled by the 600-15,000xg fraction using preparative TLC in diisopropylether-acetic acid (96:4, by vol). The R_f (0.67) of component S was identical to that of standard fatty acid methyl

ester in petroleum ether-diethylether-acetic acid (90:10:1, by vol.). Furthermore, all of the ^{14}C was localized in the methanol distillate following saponification as described in Methods III.2. Component S is therefore a fatty acid methyl ester, labelled in the methyl group of the methyl ester moiety. After treatment of component R by the saponification procedure (Methods III.2), 54% of the ^{14}C was found to be unsaponifiable and had R_f on TLC in diisopropylether-acetic acid (96:4) identical to that of the untreated component R; the remaining 46% of ^{14}C was methanol water soluble. Component R may consist of two unresolved compounds, namely an unsaponifiable lipid which migrates on TLC like a long chain alcohol (see Fig. 30) and a saponifiable lipid. The nature of these lipids was not further investigated.

It was possible that the labelled fatty acid methyl ester observed (Fig. 30) may have been formed non-enzymatically by reaction of endogenous free fatty acids with [^{14}C]methanol released from [^{14}C]SAM. The experiment described above (Table 27) was repeated using a cell free homogenate as enzyme source together with a boiled enzyme control. The incorporation of ^{14}C into neutral lipid by the cell free homogenate (0.27 ml aliquot) was seven times greater than that by an equal aliquot of boiled homogenate (Table 28). Doubling the aliquot assayed increased the ^{14}C-incorporation by the cell free homogenate by 55% but did not affect that by the boiled homogenate. When the ^{14}C-neutral lipid products were chromatographed on TLC in diisopropylether-acetic acid (96:4) all of the ^{14}C in the boiled homogenate controls remained at the origin, whereas the

TABLE 28. ENZYMATIC AND NON-ENZYMATIC INCORPORATION OF ^{14}C FROM [^{14}C]SAM INTO "NEUTRAL LIPID"

Assay System[a]	Aliquot ml	Incorporation of ^{14}C into Neutral Lipid[b] dpm
Cell-free homogenate	0.27	6450
" "	0.55	9960
" " , boiled	0.27	920
" " , boiled	0.55	1000
Homogenizing medium	0.27	200
" " , zerotime	0.27	190

[a]Assay system (vol. 1.0 ml): 57.1 µM [^{14}C]SAM (8.4×10^5 dpm), 50 mM Tris HCl (pH 8.5) and different aliquots of cell free homogenate, boiled homogenate, or homogenzing medium, as indicated. In the boiled homogenate assays, the aliquot of homogenate was boiled in the reaction tube for 10 min and the remaining constituents of the reaction mixture added. Incubation, 1 h at 30°C. Lipid extract freed of [^{14}C]SAM breakdown products as described in Results IV,5.b.i.

[b]Determined as described in Table 27.

^{14}C in the unboiled homogenate was found in components S, (methyl fatty acid ester), R, Q and P in a pattern similar to that shown in Fig. 30. Therefore, these results strongly suggest that the ^{14}C-labelled fatty acid methyl ester and also components P, Q and R observed in Fig. 30 are products of enzymatic action.

In the experiment reported in Table 28 a second control was carried out to determine if the ^{14}C in "neutral lipid" from the boiled homogenate was due to labelled breakdown products formed from [^{14}C]SAM during incubation. When the boiled homogenate was replaced by the homogenizing medium used in the preparation of the cell free homogenate, ^{14}C in "neutral lipid" was decreased about five times to a value which was equal to that of the zero time control, representing labelled contaminants in the [^{14}C]SAM. Thus the ^{14}C "neutral lipid" product from the boiled homogenate was formed non-enzymatically, but, as described above, was not identical to the products formed enzymatically.

2. In vivo studies

It will be recalled that a very high proportion of the ^{14}C incorporated by whole leaf or leaf slices from [Me-^{14}C] methionine or [U-^{14}C]serine was incorporated into the neutral lipid fraction (Table 11 and Fig. 16). The fraction labelled from [Me-^{14}C]methionine by leaf slices consisted of the same four components P, Q, R and S labelled in the in vitro experiment (see Fig. 30). The major labelled product was component R;

this is in contrast to the labelling pattern from the cell free homogenate in which component R was only slightly labelled (Fig. 30). Though not shown, the same labelling pattern as that from [Me-^{14}C]methionine was obtained for the neutral lipid fraction labelled by leaf slices from [U-^{14}C] serine.

E. DISCUSSION

1. Pathways of Biosynthesis of Phospholipids in Spinach Leaves

a) Phosphatidyl glycerol

Evidence was obtained which showed that PG in spinach leaves is biosynthesized from sn-glycerol-3-phosphate and CDP-diglyceride, the first step being catalyzed by CDP-diglyceride: sn-glycerol-3-phosphate phosphatidyltransferase to form PGP (eqn. 7) and the second by PGP phosphatase (eqn. 8). The role of glycerophosphate as substrate was demonstrated in numerous experiments by the incorporation of ^{14}C from [1,3-^{14}C]sn-glycerol-3-phosphate into PG by a 40,000xg fraction in the presence of CDP-diglyceride (Figs. 5-14 and Tables 3-8). Furthermore, [1-^{14}C]glycerol was inactive as substrate (Table 3), which ruled out the possibility that the observed ^{14}C-incorporation from [^{14}C]glycerophosphate into PG occurred via prior dephosphorylation followed by an exchange reaction of the [^{14}C]glycerol formed with endogenous lipid. The role of CDP-diglyceride as substrate was indicated by the finding that the incorporation of ^{14}C from [^{14}C]glycerophosphate into PG was absolutely dependent upon the presence of CDP-diglyceride (Table 4 and Fig. 8).

The amount of [^{14}C]PG synthesized by the 40,000xg fraction under optimum conditions (0.1 mM CDP-diglyceride, 1 mM [^{14}C]glycerophosphate, 30 mM Mg^{2+}, pH 7.3, 37°C) was consistently high in the range 14-17 nmoles/h/0.4 mg protein

(Tables 4-5, Figs. 8-14), representing a 14-17% conversion of the limiting substrate CDP-diglyceride to PG.

The intermediate formation of PGP was demonstrated by the labelling of this compound with both ^{32}P and ^{14}C when a 40,000xg fraction was incubated with [1,3-^{14}C]sn-glycerol-3-[^{32}P]phosphate in the presence of CDP-diglyceride (Fig. 5). Furthermore, PGP formation increased with increasing GP concentration (Fig. 14A, Table 5). A more direct demonstration would have involved isolation of the [^{14}C+^{32}P]PGP and using it as substrate for the subsequent dephosphorylation reaction. However, insufficient material was available for this experiment.

In all experiments, the accumulation of the PGP intermediate was either low or below the limits of detection. The formation of this intermediate is therefore the rate determining step in PG synthesis, and all the cofactor requirements and kinetic data observed for PG formation are really characteristic of the phosphatidyltransferase catalyzing this first step. It follows that the phosphatidyl transferase has a pH optimum of 7.3 (Fig. 11A), requires Mg^{2+} (half max act., 2 mM) or Mn^{2+} for activity (Fig. 13), and has a Michaelis-Menten constant (Km) for GP of 0.25 mM (Fig. 14 and Table 5). The Km for CDP-diglyceride could not be obtained from the plot of PG formation vs CDP-diglyceride concentration (Fig. 8) because of insufficient data at low CDP-diglyceride concentrations. The cause of the apparent inhibition of PG formation

and thus of the phosphatidyltransferase by high CDP-diglyceride concentrations is not clear. A similar inhibition was observed for the E.coli and chicken liver enzymes (Chang and Kennedy, 1967a; Kiyasu et al., 1963), but in all cases it is probably an artifact arising from a lowering of Mg^{2+} concentration below the saturating level by the formation of insoluble CDP-diglyceride Mg^{2+} salts.

The phosphatidyltransferase resembles the bacterial enzyme rather than the corresponding enzyme in animal tissue. Thus the transferase purified from E.coli (Chang and Kennedy, 1967a) also requires Mg^{2+} or Mn^{2+} for activity, though the half maximal activity with Mg^{2+} (6-8 mM) is somewhat higher than the corresponding value (2 mM) for the spinach enzyme. On the other hand, the enzyme in liver (Kiyasu et al., 1963) and brain (Davidson and Stanacev, 1970), does not require any metal ions for activity. Also the Km (0.25 mM) of glycerophosphate for the spinach enzyme is identical to that for the purified E.coli enzyme, but is somewhat higher than the corresponding Km (0.15 mM) for the liver enzyme. A PG synthesizing system has been demonstrated in a cauliflower inflorescence mitochondrial fraction by Douce and Dupont (1969) but its properties were not described.

An unexpected property of the spinach 40,000xg PG synthesizing system is its apparent resistance to a number of sulfydryl group reacting reagents (Tables 6 and 8). This is in striking contrast to the observed inhibition by sulfydryl

reagents of the PGP phosphatase in crude particulate fractions from liver (Kiyasu et al., 1963) and cauliflower inflorescence (Douce and Dupont, 1969). Thus, while the liver and cauliflower phosphatases were found to be completely inhibited by 5 mM $HgCl_2$ respectively, no significant inhibition of the spinach phosphatase was obtained with either 10 mM or 40 mM $HgCl_2$. The spinach PGP phosphatase apparently resembles the PGP phosphatase from a mutant of B.megaterium which has also been reported to be uninhibited by $HgCl_2$ (Patterson and Lennarz, 1971).

b) Phosphatidyl inositol

[^{14}C]PI was synthesized from [U-^{14}C]myo-inositol by a 1200-80,000xg particulate fraction both in the absence and presence of CDP-diglyceride (Tables 9 and 10), indicating the existence of both an exchange reaction and a reaction catalyzed by CDP-diglyceride:inositol phosphatidyltransferase. It is significant that both reactions specifically required Mn^{2+} as divalent metal ion cofactor (Table 9). The inositol exchange reaction is clearly different from the type of exchange reaction involving serine, ethanolamine and choline found in pea seedlings (Vandor and Richardson, 1968) and animal tissue (Borkenhagen et al., 1957; Kanfer, 1972) which specifically requires Ca^{2+}.

A possible mechanism which explains both the phosphatidyltransferase and the "exchange" reaction and also the common Mn^{2+} requirement, is that shown in equations 5-6. This

mechanism was first suggested by Paulus and Kennedy (1960) to explain similar phosphatidyltransferase and inositol "exchange" reactions in liver extracts. In this mechanism, the CDP-diglyceride:inositol phosphatidyltransferase enzyme can form a phosphatidyl-enzyme complex in reversible reactions with either CDP-diglyceride or PI. However, only when the phosphatidyl-enzyme is derived from CDP-diglyceride will a net synthesis of PI result.

It should be emphasized that this mechanism is only a tentative suggestion. The observed increase in [^{14}C]PI synthesis in the presence of CDP-diglyceride (Tables 9 and 10) does not by itself establish the existence of CDP-diglyceride:inositol phosphatidyltransferase. The direct involvement of CDP-diglyceride as substrate can only be conclusively demonstrated by experiments to determine if ^{14}C from CDP-[^{14}C]diglyceride can be incorporated directly into PI. However, the existence of CDP-diglyceride:inositol phosphatidyltransferase in spinach leaves is most probable in view of the work of Sastry and Kates (1966) who observed incorporation of ^{32}P into PI from sn-glycerol-[^{32}P]phosphate in the presence of CTP and Mn^{2+} by spinach cell fractions actively synthesizing [^{32}P]PA. Presumably the [^{32}P]PA was converted to [β-^{32}P]CDP-diglyceride which reacted with endogenous inositol to form [^{32}P]PI. Direct involvement of CDP-diglyceride in the biosynthesis of PI has been well demonstrated by Sumida and Mudd (1970a) in cell fractions from another plant tissue, namely cauliflower inflorescence.

c) Phosphatidyl serine

Whole leaf and leaf slices were found to incorporate small amounts of ^{14}C from [U-^{14}C]L-serine into PS (Table 11). Though the distribution of ^{14}C between the fatty acid, glycerol and serine moieties of [^{14}C]PS was not determined, a high proportion of the ^{14}C was most likely localized in the serine moiety incorporated intact into PS. This conclusion is supported by the evidence presented below that the ^{14}C-incorporation into the GPE moiety of PE from [U-^{14}C]serine by whole leaves arose by decarboxylation of phosphatidyl [^{14}C] serine.

Concerning the pathway of PS biosynthesis in spinach leaves, it was found that the incorporation of [U-^{14}C]L-serine into PS by a leaf 12,000-100,000xg particulate fraction was almost totally dependent upon the presence of CDP-diglyceride (Table 13), thus suggesting a reaction (eqn. 12) catalyzed by CDP-diglyceride: L-serine phosphatidyltransferase (PS synthetase). Unlike the corresponding enzyme in E.coli (Kanfer and Kennedy, 1964) the spinach phosphatidyltransferase does not require cutscum or Na_2SO_4 for activity, in fact these compounds at the concentrations tested were greatly inhibitory (Table 13). The phosphatidyltransferase may also exist in other photosynthetic tissues as suggested by the results of in vivo studies with Chlorella vulgaris by Sastry and Kates (1965) (see Introduction III.1.g).

In connection with the alternative pathway for PS biosynthesis, namely a serine exchange reaction with phospholipid, e.g., PE (eqn. 23), the low residual incorporation of [^{14}C]serine into PS in the absence of CDP-diglyceride (Table 13) could possibly have arisen via such a pathway. On the other hand, according to the results shown in Table 26 the same particulate fraction was unable to catalyze any [^{14}C] serine incorporation into PS, either in the absence or presence of Ca^{2+}, even though an active Ca^{2+}-stimulated incorporation of [^{14}C]ethanolamine and [^{14}C]choline by exchange reactions into PE and PC respectively was observed. The only difference in procedure between the experiments described in Tables 13 and 26 was the type of buffer used, i.e., potassium phosphate was used in the former experiment and Tris HCl in the latter experiment. It is therefore possible that a serine exchange enzyme system does function in phosphate buffer but is inhibited by Tris HCl buffer. In this connection, Tris HCl has been reported to inhibit serine, ethanolamine and choline exchange reactions in brain particulate fractions by as much as 50-80% (Kanfer, 1972). Also, Vandor and Richardson (1968) have reported that certain buffers, unfortunately not specified, are inhibitory for the ethanolamine exchange system in pea seedlings, but whether this was also true for the serine exchange system was not determined.

In summary, evidence was obtained which suggested that PS in spinach leaves is biosynthesized by the pathway

characteristic of bacteria. The presence of the serine exchange system characteristic of animals is unlikely but cannot be ruled out.

d) Phosphatidyl ethanolamine

In in vivo studies with whole leaves, ^{14}C from [U-^{14}C]L-serine was incorporated not only into PS but also into the glycerol phosphorylethanolamine moiety of PE (Tables 11-12, Figs. 16-17). Though the distribution of ^{14}C between the glycerol and ethanolamine moieties of GPE was not determined, it is unlikely that the glycerol moiety was labelled in view of the lack of ^{14}C-incorporation from [U-^{14}C] serine into PG by whole leaves (see Fig. 16). Thus, assuming that all of the ^{14}C was localized in the ethanolamine moiety, these findings indicated the existence of PS decarboxylase in spinach leaves. However, a more conclusive proof was the showing of a direct conversion of [^{14}C]PS to [^{14}C]PE in vitro (Tables 14-17, Figs. 18-20).

The spinach PS decarboxylase resembles the corresponding enzyme in bacteria (Kanfer and Kennedy, 1964; Patterson and Lennarz, 1971) protozoa (Dennis and Kennedy, 1970) and liver (Borkenhagen et al., 1961) in its lack of a requirement for divalent metal ions (Table 16, Exp. 1). However, unlike the enzyme in these tissues, which is stimulated by Triton X-100, the spinach enzyme appears to be strongly inhibited by this detergent (Table 16, Exp. 1).

The inhibition of the spinach decarboxylase by hydroxylamine (Table 16, Exp. 2) suggests that the enzyme contains pyridoxal phosphate as coenzyme since inhibition of activity by hydroxylamine is a common property of enzymes containing this coenzyme (Clark, 1963). The corresponding decarboxylase in bacteria (Wichner and Kennedy, 1971; Patterson and Lennarz, 1971) and protozoa (Dennis and Kennedy, 1970) is also inhibited by hydroxylamine.

The biosynthesis of PE in spinach leaves via decarboxylation of PS is probably a de novo pathway producing a net synthesis of PE. This conclusion is supported by the evidence discussed above (see Section c) which suggested that PS is biosynthesized from CDP-diglyceride and serine rather than by an exchange reaction involving serine and endogenous PE. The complete pathway, involving the reactions shown in Eqns. 12-13 is therefore the same as that operating in bacteria, but differs from that operating in animals and protozoa in which decarboxylation of PS is not involved in the de novo synthesis of PE since PS is itself most probably derived from PE by an exchange reaction (see Introduction III.1.g). PS decarboxylase together with PS synthetase may be widely distributed in plants, as indicated by indirect evidence for their occurrence in Chlorella vulgaris (Sastry and Kates, 1965) and tomato root (Willemot and Boll, 1967) (see Introduction III.1.g).

That spinach PE can also be biosynthesized from free ethanolamine was shown by the high incorporation of ^{14}C from

[1,2-^{14}C]ethanolamine into the GPE moiety of PE by whole leaf and leaf slices (Tables 11-12, Figs. 16-17). There are two possible pathways that would give rise to this incorporation, namely the CDP-ethanolamine pathway (eqns.14-16) and the exchange pathway (eqn. 23). The strongest evidence in support of the CDP-ethanolamine pathway was the demonstration of a very active diglyceride-stimulated synthesis of [^{14}C]PE from CDP-[1,2-^{14}C]ethanolamine by a 100,000xg fraction (Figs. 21-23, Tables 18-19), thus establishing the existence of CDP-ethanolamine: sn-1,2-diglyceride phosphorylethanolaminetransferase in spinach leaves. The finding that this enzyme requires Mg^{2+} or Mn^{2+} (Fig. 23A) agrees with the preliminary results reported by Mudd and Macher (1972). However, the pH optimum found in the present study (7.0, Fig. 23B) was somewhat lower than that (pH 7.5) reported by these workers.

The existence of ethanolaminekinase catalyzing the first step in the pathway (eqn. 14) was suggested by the observed ATP-stimulated incorporation of [1,2-^{14}C]ethanolamine into phosphorylethanolamine by a 15,000xg supernatant fraction (Table 20).

The second step of the pathway (eqn. 15), catalyzed by CTP: phosphorylethanolamine cytidyltransferase, could not be demonstrated *in vitro* under a variety of experimental conditions (see Results IV.3.c). The reason for these negative results is unclear. Cytidyltransferase activity if present would be expected in the 15,000xg supernatant by anology with

the localization of the corresponding enzyme in animal tissue (Kennedy and Weiss, 1956; Schneider, 1969). The absence of activity in the spinach supernatant using the same pH (7.5) as used for the animal system was probably not due to the action of interfering phosphatases on CDP-ethanolamine or the presence of dialyzable inhibitors (see Results IV.3.c). In view of the presence in spinach leaves of the other two enzymes of the CDP-ethanolamine pathway, namely ethanolaminekinase and the phosphorylethanolaminetransferase, it is concluded that the cytidyltransferase most likely functions *in vivo* but was inactive under the conditions of assay used *in vitro*. Indeed, Kennedy and Weiss (1956) have claimed, but provided no experimental evidence for, the presence of this enzyme in carrot root extracts.

Concerning the exchange pathway, the Ca^{2+}-stimulated incorporation of [1,2-^{14}C]ethanolamine into PE by a 1200-100,000xg particulate fraction in the absence of cytidine coenzymes (Table 26) suggests the *in vitro* operation of an ethanolamine exchange reaction. It will be recalled that the ethanolamine exchange reaction in pea seedlings (Vandor and Richardson, 1968) and also in liver (Borkenhagen *et al.*, 1961) and brain (Porcellati *et al.*, 1971; Kanfer, 1972) was also stimulated by Ca^{2+} and independent of cytidine coenzymes. However, in view of the well known ability of phospholipase D to catalyze exchange reactions *in vitro* (see Introduction III.1.j) the question arises as to whether the observed ethanolamine

exchange reaction was catalyzed not by an independent exchange enzyme but by phospholipase D. Though it is now generally accepted that phospholipase D in plant tissue is localized in the cytoplasm (Quarles and Dawson, 1969; Clermont and Douce, 1970), the enzyme is known to become attached to particulate fractions during cell fractionation (Kates, 1954; Clermont and Douce, 1970) and was therefore most likely present in the 1200-100,000xg particulate fraction assayed in the present study. Though the observed ethanolamine exchange reaction occurred at pH 8.5 in the absence of any surface active agents or diethyl-ether, whereas phospholipase D-catalyzed exchange reactions have a pH optimum of 5.5-6.0 and are most active in the presence of diethylether (Yang et al., 1967), it cannot be ruled out that any phospholipase D present in the particulate fraction was sufficiently active under the assay conditions used to account for the very low ethanolamine exchange activity observed. Thus, no firm conclusions can be made concerning whether the ethanolamine exchange reaction was catalyzed by an independent exchange enzyme or was an artifact arising from a phospholipase D catalyzed exchange reaction.

The evidence discussed above strongly suggests that there are two major routes operating in the de novo biosynthesis of PE in spinach leaves, namely the PS decarboxylation pathway (eqns. 12-13) and the CDP-ethanolamine pathway (eqns. 14-16). This is the first demonstration of the operation of both pathways in a single tissue. It will be recalled that bacteria

utilize only the former pathway (Kanfer and Kennedy, 1964; Patterson and Lennarz, 1971) while animals use only the latter pathway (Paulus and Kennedy, 1960). It is not possible to determine from the results of the present study which of the two pathways predominates in the biosynthesis of PE in spinach leaves. The finding that the incorporation of ^{14}C into the GPE moiety of PE by whole leaves was about 23-fold greater from [1,2-^{14}C]ethanolamine than from [U-^{14}C]serine (Table 12) might suggest that the CDP-ethanolamine pathway predominates. However, it is not valid to compare the incorporation from [1,2-^{14}C]ethanolamine with that from [U-^{14}C]serine for the following reasons: i) the experiments with these two substrates used two different leaves which may not have been physiologically equivalent, ii) the involvement of [U-^{14}C]serine in the formation of tetrahydrofolate derivatives (see Section e), and of sugar by the glycolate pathway (Tolbert, 1971), would deplete the pool of [U-^{14}C]serine available for the PS decarboxylation pathway, whereas [1,2-^{14}C]ethanolamine might not be expected to be as metabolically active, and iii) the dilution by the pool of intracellular unlabelled substrate was likely different for each of the labelled substrates.

e) Phosphatidyl choline

Methylation pathway

The incorporation of ^{14}C into the GPC moiety of PC from [1,2-^{14}C]ethanolamine and [Me-^{14}C]methionine by whole leaves and leaf slices, and into the deacylated moieties of PE

and PE-Me from [1,2-^{14}C]ethanolamine by whole leaves (Tables 11-12, Figs. 16-17), is consistent with the occurrence of the methylation pathway for PC biosynthesis in spinach leaves (eqns. 20-22). The GPC moiety of PC was also labelled *in vivo* from [U-^{14}C]serine; this could have occurred by two metabolic routes; namely, i) formation of phosphatidyl [1,2-^{14}C]ethanolamine via decarboxylation of phosphatidyl [U-^{14}C]serine, as discussed in Section d, followed by methylation to phosphatidyl [1,2-^{14}C]choline, ii) utilization of carbon-3 of [U-^{14}C]serine for the formation of [Me-^{14}C]methionine via the operation of the tetrahydrofolate one-carbon pathway known to be present in plants (Burton and Sakami, 1969; Dodd and Cossins, 1970; Kisaki *et al.*, 1971; Clandinin and Cossins, 1972). This route is supported by the similar pattern of labelling of neutral lipids by both [U-^{14}C]serine and [Me-^{14}C]methionine *in vivo* (see Section 4 below). Although labelling of the glycerol moiety of GPC by [U-^{14}C]serine was not directly investigated, it is unlikely in view of the lack of ^{14}C-incorporation from [U-^{14}C]serine into PG by whole leaves (see Fig. 11).

Two of the intermediate steps in the methylation pathway, namely the methylation of PE-Me to PE-diMe and of PE-diMe to PC by SAM were demonstrated unambiguously *in vitro* (Tables 22-23, Figs. 24-25). However, attempts to demonstrate the methylation of either endogenous or exogenous PE by SAM *in vitro* consistently failed (see Results IV.5.b.i). Any incorporation of ^{14}C from [^{14}C]SAM into the GPC moiety of PC

by microsomal fractions was always extremely small and in fact may well have resulted from methylation of possible traces of PE-Me and PE-diMe.

The failure to demonstrate methylation of either endogenous or exogenous PE is surprising in view of the successful demonstration of such reactions in other tissues. Thus, methylation of endogenous PE has been demonstrated in microsomal preparations from the photosynthetic bacterium Rhodopseudomonas spheroides (Gorchein et al., 1968), rat liver (Bremer and Greenberg, 1961; Gibson et al., 1961; Cooksey and Greenberg, 1961), Neurospora crassa (Scarborough and Nyc, 1967), Saccharomyces cervisiae (Steiner and Lester, 1969), Ochromonas malhamensis (Lust and Daniel, 1965) and Tetrahymena pyriformis (Smith and Law, 1970). On the other hand, methylation of exogenous PE has been demonstrated only in cell free extracts from Agrobacterium tumefacians (Kaneshiro and Law, 1964) and Hyphomicrobium vulgare (Goldfine and Hagen, P-O., 1968).

The failure to observe PE:SAM methyltransferase activity in the spinach microsomal fraction was due neither to unfavourable assay conditions nor to the presence of this enzyme in other cell fractions (see Results IV.5.b.i). Concerning assay conditions (see Tables 22-24), the pH used (8.5) was found to be optimal for ^{14}C-incorporation into PC (Fig. 29B) and was close to the optimum (8.0) for methylation of PE-diMe (Fig. 27B). Though the [^{14}C]SAM concentration used was rather low (8-16 μM), raising the concentration to saturating levels

only increased the ^{14}C-incorporation into PC about 2-fold (Fig. 29C); also, it should be noted that this ^{14}C-incorporation was probably localized in the fatty acid moieties (see Results IV.7).

One of the products expected from any reaction in which [^{14}C]SAM acts as a methyl donor is S-adenosylhomocysteine (SAHC) which has been shown to be a potent inhibitor of the PE:SAM methyltransferase from rat liver (Gibson et al., 1961), T. pyriformis (Smith and Law, 1970) and A. tumefacians (Kaneshiro and Law, 1964). It will be recalled that although the spinach microsomal fraction showed negligible PE:SAM methyltransferase activity, this fraction incorporated relatively large amounts of ^{14}C into neutral lipid and PC fatty acid moieties (Tables 22-23). The question arises as to whether the concentration of SAHC produced in these side reactions was sufficient to inhibit any PE:SAM methyltransferase present. However, it was found that the ^{14}C-incorporation from [^{14}C]SAM into PC was not stimulated in an assay mixture containing 1 mM Mn^{2+} in which formation of ^{14}C-labelled neutral lipid and therefore SAHC was extremely low (Table 24). Also, it is doubtful whether the concentration of SAHC produced in these side reactions would be high enough for effective inhibition. In an assay system containing 8 μM [^{14}C]SAM (9.5×10^5 dpm), the total ^{14}C incorporated into PC fatty acid and neutral lipid was 2940 dpm (Table 22) equivalent to a concentration of SAHC of only 25 nM. The methyltransferase from A. tumefacians (Kaneshiro and Law, 1964) was completely inhibited by 20 μM SAHC in an assay system containing 30 μM SAM, while in

T.pyriformis (Smith and Law, 1970) 40 μM SAHC caused a 60% inhibition in an assay system containing 40 μM SAM. Therefore, the failure to detect PE:SAM methyltransferase in spinach microsomes could not have been due to inhibition of this enzyme by SAHC.

The two most likely explanations for the failure to demonstrate methylation of PE by SAM in vitro are either i) inactivation of PE:SAM methyltransferase during cell breakage in the leaf homogenization procedure, or ii) existence of an alternative route for the formation of PE-Me. Evidence in support of the first possibility was obtained in the in vivo experiments. It will be recalled that the proportion of total lipid ^{14}C in PC labelled either from ^{14}C-labelled ethanolamine, methionine or serine was considerably greater in whole leaves than in leaf slices (Table 11). Furthermore, even though the incorporation of ^{14}C from [^{14}C]ethanolamine into PE by whole leaf and leaf slices was about the same, the incorporation from [^{14}C]ethanolamine into PC was 6-fold greater by whole leaves than by leaf slices (Table 11). It is likely that in leaf slices the PE:SAM methyltransferase may have been partially inactivated as a result of the cell breakage during preparation of the leaf slices (see Experimental I.1.b). The same inactivation due to cell breakage may have occurred during preparation of the leaf homogenates used in the in vitro experiments. This inactivation could not have arisen from air oxidation of active cysteine residues in the enzyme since the

presence of β-mercaptoethanol in the homogenizing medium had no effect on the enzyme activity (see Results IV.5.b.i).

Concerning the second possibility of an alternative pathway for formation of PE-Me, clearly the precursor of PE-Me must be a derivative of ethanolamine since PE-Me was found to be labelled from [^{14}C]ethanolamine by whole leaves (Table 12, Fig. 17A). One feasible pathway would be the methylation of CDP-ethanolamine by SAM to form CDP-monomethylethanolamine, followed by a phosphorylmonomethylethanolaminetransferase reaction with diglyceride to form PE-Me. However, such a pathway is very unlikely in view of the lack of [^{14}C]PC formation on incubation of CDP-[1,2-^{14}C]ethanolamine with SAM and a microsomal fraction under conditions of high phosphorylethanolaminetransferase activity (Table 18, Fig. 21). A second alternative pathway might be methylation of free ethanolamine by SAM followed by conversion of monomethylethanolamine to CDP-monomethylethanolamine and then into PE-Me. However, this pathway was not tested.

A third alternative pathway might be methylation of PE by a methyl donor other than SAM. This possibility is consistent with the finding that in whole leaves ^{14}C was incorporated into PE-Me from [^{14}C]ethanolamine but not from [^{14}C] methionine, whereas the incorporation into the GPC moiety of PC by these two substrates was about the same (Tables 11-12).

Possible methyl donors might be the tetrahydrofolate (THF) derivatives, 5-methyl THF, 5,10-methylene THF or 10-formyl

THF, all of which are naturally occurring in leaves (Roos et al., 1968; Roos and Cossins, 1971; Cossins and Shah, 1972; Clandinin and Cossins, 1972). Concerning 5-methyl THF, this compound is unlikely to be a methyl donor to PE since no ^{14}C-incorporation into lipid was found on incubation of 5-[Me-^{14}C] THF with the microsomal fraction, nor was this compound capable of methylating PE-diMe (see Results IV.5.b.i and ii). 5,10-Methylene THF could possibly methylate PE to produce PE-Me and dihydrofolate in a simultaneous methylation-oxidation type of reaction analogous to that catalyzed by thymidilate synthetase in animals and bacteria (Brown, 1970), but such a reaction appears extremely unlikely.

10-Formyl THF could possibly N-formylate PE which could then be converted to PE-Me in a series of reactions involving reductions and dehydration. N-formylation of amino groups by 10-formyl THF does indeed occur in Nature, in bacteria in the synthesis of N-formylmethionyl-tRNA from methionyl-tRNA during protein synthesis, and in animals, of 5-formyl-imidazole-4-carboxamide ribonucleotide during purine synthesis (Brown, 1970). However, in view of the high metabolic energy that would be required to convert N-formyl PE to PE-Me, it is extremely unlikely that the N-formylation pathway is feasible.

In summary, PC in spinach leaves can be biosynthesized by the stepwise methylation of PE-Me. No firm conclusions can be made concerning the biosynthesis of PE-Me

except that this intermediate is derived ultimately from ethanolamine. However, in view of the unlikelihood of alternative pathways, either because of evidence obtained against them or because of their non-feasibility, it is likely that PE-Me is biosynthesized *in vivo* by the same pathway present in other tissues, namely the methylation of PE by SAM (eqn. 20). It is concluded that this pathway was not observed in spinach *in vitro* systems because of inactivation of the PE:SAM methyltransferase during the homogenization procedure.

Nucleotide and Exchange Pathways

Spinach PC can also be biosynthesized from free choline as shown by the high incorporation of ^{14}C from [1,2-^{14}C] choline into the GPC moiety of PC by leaf slices (Tables 11-12). There are two possible pathways that would give rise to this incorporation, namely the CDP-choline pathway (eqns. 17-19) and the exchange pathway (see Introduction II.1.j). The evidence obtained for the operation of the former pathway was the finding of incorporation of ^{14}C from CDP-[1,2-^{14}C]choline into PC by cell fractions in the presence of *sn*-1,2-diglyceride (Table 21). This finding strongly suggests the presence in spinach leaves of CDP-choline: *sn*-1,2-diglyceride phosphorylcholinetransferase catalyzing the reaction shown in eqn. 19, and confirms the previous demonstration of the presence of this enzyme in spinach leaves by Devor and Mudd (1971).

The presence of cholinekinase and CTP:phosphorylcholine cytidyltransferase was not investigated here, but the

former enzyme has been identified previously in spinach leaves (Tanaka et al., 1966), and the cytidyltransferase is most likely present in view of its occurrence in other plant tissue (Morré et al., 1971). The phosphorylcholinetransferase reaction is analogous to the phosphorylethanolaminetransferase reaction also operating in spinach leaves, and the question arises as to whether the two reactions are catalyzed by the same enzyme. The identical pH optimum (see Fig. 23B and Results IV.4) and similar subcellular localization (see below) of the two reactions are consistent with the idea of a single enzyme, though this question can only be answered unambiguously after purification and separation of the two respective activities.

Concerning the exchange pathway, the Ca^{2+}-stimulated incorporation of [1,2-^{14}C]choline into PC in the absence of cytidine coenzymes by a particulate fraction (Table 26) suggests the in vitro operation of a choline exchange reaction. However, in view of the possible presence of phospholipase D in the particulate fraction (see above, Section d), it cannot be ruled out that the observed low activity of choline exchange may have been catalyzed by phospholipase D and not by an independent choline exchange enzyme. This question was not further investigated.

In summary, PC in spinach leaves can be biosynthesized both by the methylation pathway and the nucleotide pathway, but whether a choline exchange pathway also operates is uncertain.

2. Evidence for Phospholipid Acylhydrolases

In the present study, ^{14}C-incorporation from CDP-[1,2-^{14}C]ethanolamine by a microsomal fraction into lyso PE as well as into PE was consistently observed (Table 18, Figs. 21-22). This most likely resulted from hydrolysis of [^{14}C]PE by a phosphatide acyl hydrolase. The enzyme appears to be "bound" to the microsomal fraction, and was clearly absent from the 100,000xg supernatant since addition of this fraction to the microsomal fraction had no effect on the amount of [^{14}C]lyso PE formed (Table 18, Fig. 21). The enzyme also acts on PC, as shown by the formation of [^{14}C]lyso PC by the microsomal fraction when PC was labelled either from CDP-[1,2-^{14}C]choline (see Results IV.4) or by reaction of [^{14}C] SAM with PE-diMe (Fig. 24). Formation of [^{14}C]lyso PE and [^{14}C]lyso PC was also observed in leaf slices but not in whole leaves (Table 11 and Results III.4). These observations suggest that the enzyme may be released during cell breakage both in the preparation of leaf slices and cell free homogenates.

It is not possible to conclude from the present study whether the acyl hydrolase is of the phospholipase A type, or the B type which hydrolyzes both acyl ester groups. Neither enzyme has yet been reported in leaves, though the results of Yagi and Benson (1962) suggest that phospholipase A is present in the green algae Scenedesmus obliquus, and Galliard (1970 and 1971) has partially purified on enzyme extract from potato tubers with phospholipase B type activity. However, lyso

phospholipids are never detected as intermediates in phospholipase B hydrolysis in either potato (Galliard, 1970), or other organisms (McMurray and Magee, 1972), suggesting that the phospholipase detected in spinach leaves is of the A type.

3. Evidence for Acyltransferase

When spinach cell-free fractions were incubated with [^{14}C]PS, not only was [^{14}C]PE formed, but also a ^{14}C-labelled fatty ester (compound Y) and a non-lipid component soluble in methanol-water (see Results IV.2, Tables 14-17, Figs. 18,20). Compound Y did not migrate on TLC in petroleumether-diethyl-ether-acetic acid (90:10:1) (see Results IV.2.a), thus eliminating the possibility that it was triglyceride (R_f, 0.35), diglyceride (R_f, 0.08) or fatty acid methyl ester (R_f, 0.65) (Kates, 1972). A likely structure for compound Y is acyl-monogalactosyldiglyceride (AMGD), which should have a high R_f in the polar solvent (see Fig. 18) and zero mobility in the non-polar solvent (see Results IV.2.a). AMGD might be formed either by release of ^{14}C-labelled fatty acids from [^{14}C]PE or [^{14}C]PS by a phospholipase type A or B followed by acyl transfer to the acceptor MGD, or alternatively, a direct transfer of ^{14}C-labelled fatty acid to MGD. The former pathway can be eliminated since it would require activation of the released fatty acid by ATP, CoA and Mg^{2+} which were absent in the assay mixture used. The latter pathway is essentially the same as that demonstrated in Vicia faba leaf extracts which involves the transfer of acyl groups to MGD, not only from MGD and DGD,

but also to a lesser extent from phospholipid (Heinz, 1967 and 1973).

Another possible structure for compound Y is esterified sterolglucoside, in which case the acyl acceptor would be sterolglucoside. In this connection, acylsterolglucosides in spinach leaves are biosynthesized from sterolglucoside (Ongun and Mudd, 1970) but the reactions involved are not known. No definite conclusions can be made concerning whether the ^{14}C-acyl group of compound Y is derived from [^{14}C]PE or [^{14}C]PS. The similar subcellular distribution of [^{14}C]PE and [^{14}C]Y synthesis (Table 14 and Fig. 18), and the simultaneous inhibition of [^{14}C]PE and [^{14}C]Y synthesis by detergents (Table 19, exp. 1) might suggest acyl transfer from [^{14}C]PE. On the other hand, this conclusion is incompatible with the inhibition of [^{14}C]PE synthesis but the stimulation of [^{14}C]Y synthesis by hydroxylamine (Table 19, exp. 2), a finding which tends to suggest that [^{14}C]PS was the acyl donor. This question was not further investigated.

The observed ^{14}C-activity found in methanol-water soluble non-lipid (Table 17, Fig. 20) is probably due to ^{14}C-labelled GPE or GPS, or both, but this was not established experimentally. Both acyl groups of either PE or PS would thus appear to be transferable. The possibility that only one acyl group was transferable is unlikely since no [^{14}C]lyso PS, and only traces of [^{14}C]lyso PE, were detected during formation of compound Y.

4. Miscellaneous Pathways Utilizing S-adenosylmethionine

Phospholipid fatty acid moieties

An unexpected finding was the incorporation of ^{14}C from [^{14}C]SAM into the fatty acid moieties of PC and PE by a microsomal fraction (see Tables 22-23 and Fig. 28B). Some comments concerning the identity of the "PE" component are however necessary at this point. Since the [^{14}C]PE isolated by preparative TLC for the deacylation study (Fig. 28) may have contained digalactosyl diglyceride (DGD) which co-chromatographs with PE (see Results III.2.b and legend to Fig. 16) it cannot be ruled out that some of the fatty acid ^{14}C in the "PE" component was localized in DGD.

The observed incorporation of ^{14}C into PC and PE from [^{14}C]SAM most likely occurred by direct reaction of [^{14}C]SAM with the fatty acid moieties of these lipids, rather than by reaction with free fatty acids followed by incorporation of labelled fatty acid into PC and PE, since the latter pathway would require CoA, Mg^{2+} and ATP which were probably absent from the microsomal fraction assayed.

Since the fatty acids of PC were labelled in the absence of exogenous phospholipid, the enzyme system can clearly utilize microsomal membrane bound PC, but it is not entirely clear whether microsomal PE can also serve as substrate. Small amounts of ^{14}C were incorporated into the "PE" component in the absence of any exogenous PE (Tables 22-23), and this activity was assumed to be localized in the fatty

acid moieties, indicating that microsomal PE can serve as substrate. This incorporation was increased in the presence of dialyzed-dispersed PE (Table 23), but this was probably due to stimulation of the reaction with microsomal PE by the deoxycholate present in the dialyzed PE preparation, since the presence of the latter also increased ^{14}C-incorporation into PC fatty acid and neutral lipid; furthermore, the ^{14}C-incorporation into PE fatty acid was not increased in the presence of sonicated PE (Table 22).

The enzyme system described above clearly functions *in vivo* as shown by the ^{14}C-incorporation into PC and PE fatty acid moieties from [Me-^{14}C]methionine by whole leaves (see Tables 11-12 and Fig. 28A). ^{14}C was also incorporated into these moieties by whole leaves from [U-^{14}C]serine (see Results III.3.a), presumably by prior incorporation of carbon-3 of [U-^{14}C]serine into the methyl group of methionine via the tetrahydrofolate one-carbon pathway (see above, Section e).

There are two possible pathways by which ^{14}C from [^{14}C]SAM can be incorporated directly into the fatty acid moieties of PC and PE. The first involves the addition of CH_2 groups across double bonds of unsaturated fatty acids in PC and PE to form phospholipid bound cyclopropane fatty acids. For PE, the reaction would be

$$\text{unsaturated fatty acyl-PE} + \text{SAM} \longrightarrow \text{cyclopropane fatty acyl-PE} + \text{SAHC} \qquad (32)$$

This pathway has been demonstrated previously by Law and co-workers in cell free extracts from Clostridium butyricum and Serratia marcescens (Zalkin et al., 1963; Chung and Law, 1964; Thomas and Law, 1966.

The second pathway involves alkylenation of unsaturated fatty acids in PC and PE by [^{14}C]SAM to form ^{14}C-methylene branched acids, followed by reduction to the corresponding phospholipid bound ^{14}C-methyl branched acids. This pathway has been demonstrated in cell free extracts of Mycobacterium phlei (Akamatsu and Law, 1970).

Since the structure of the ^{14}C-labelled fatty acids synthesized by the spinach enzyme system was not investigated, no firm conclusions can be made as to which pathway functions in spinach leaves. However, the first pathway involving synthesis of phospholipid bound cyclopropane acids appears more likely. The only methyl branched acids so far reported in plants, apart from phytanic and pristanic acids, are the iso- and anteiso acids of Antirrhinum majus (Radunz, 1965), and these are most likely formed during the de novo synthesis of the fatty acid chain, as are the corresponding acids in bacteria (see Kates, 1964). On the other hand, cyclopropane acids together with their cyclopropene analogues are more common, especially among the seed and leaf glycerolipids of several families of the order Malvales (Hitchcock and Nichols, 1971; Yano et al., 1972a). Though cyclopropane acids have not been previously reported present in spinach PC and PE (Sastry

and Kates, 1964; Allen et al., 1966; Devor and Mudd, 1971), such acids may be present in trace amounts and may therefore have escaped detection in these studies. However, in the families Malvaceae and Sterculiaceae, the methylene bridge carbon of the cyclopropane and cyclopropene fatty acids has been shown to be derived from the methyl group of methionine (Hooper and Law, 1965; Johnson et al., 1967; Yano et al., 1972b), though whether the cyclopropane synthesis takes place at the phospholipid level is not known. The present results therefore constitute the first report in plants of cyclopropane fatty acid synthesis localized at the phospholipid level.

Neutral lipids

Evidence from both in vitro and in vivo experiments established the existence of enzymes in spinach leaves which can utilize the methyl group of SAM in the synthesis of a number of neutral lipids. Thus, four neutral lipid components, (spots, P,Q,R and S, Fig. 30) were shown to be labelled both from [^{14}C]SAM by subcellular fractions, and from [Me-^{14}C] methionine by whole leaves and leaf slices. ^{14}C from [U-^{14}C] serine was also incorporated in vivo into these components, presumably by prior incorporation of carbon-3 into the methyl group of methionine via the tetrahydrofolate one-carbon pathway (see above).

Three of these neutral lipids (spots, P,Q and R) were insufficiently characterized and their labelling will not be discussed further. With regard to the fourth component,

spot S, this was identified as fatty acid methyl esters with all of the ^{14}C localized in the methyl group (see Results V.1). A possible pathway for the biosynthesis of this compound would be the direct methylation of free fatty acids by [^{14}C]SAM, as follows:

$$RCOOH + [^{14}C]SAM \longrightarrow RCO.O[^{14}C]Me + SAHC \qquad (33)$$

Akamatsu and Law (1970) have also postulated such a reaction to explain their finding of an enzymatic synthesis of fatty acid methyl ester from exogenous fatty acid and SAM by a partially purified soluble enzyme extract from Mycobacterium phlei.

Other possible pathways might be the release of [^{14}C]MeOH from [^{14}C]SAM by the action of an SAM hydrolase, followed by esterification with fatty acyl CoA or methanolysis of glyceroplipids. SAM hydrolase has been reported in certain animal tissues (Axelrod and Daly, 1965) but so far not in plants. Though no firm conclusions can be made as to which of these pathways operates, the pathway shown in eqn. 33 perhaps appears more likely.

Fatty acid methyl esters have not as yet been detected in spinach leaves, but have been detected in other plant tissues including corn pollen (Fathipour et al., 1967) and chlamydospores of Ustilago maydis (Laseter et al., 1968). They have also been detected as natural products from various animal tissues (Fischer et al., 1966; Dhopeschwarker and Mead, 1962; Kaufmann and Viswanathan, 1963; Lough and Garton, 1968).

Furthermore, in ox pancreas (Skorepa et al., 1968) and Rhizopus arrhizus (Laseter and Weete, 1971) they co-exist with the corresponding ethyl esters. The physiological role of the fatty acid methyl esters in the above tissues is not known, though Stowe (1958) has presented evidence suggesting that in plants they may be involved in the control of cell growth, together with plant growth hormones.

5. Subcellular localization of spinach phospholipid biosynthesizing enzymes

The PG synthesizing system, and the enzyme systems for the biosynthesis of PC by the nucleotide and methylation pathways, namely CDP-choline:diglyceride phosphorylcholinetransferase and PE-diMe:SAM methyltransferase, were found to be localized primarily in the microsomal cell fraction (Tables 7, 21 and 25). It is of interest that the enzyme systems for the synthesis of PA (Cheniae, 1965) the common precursor of both PG and PC, and of CDP-diglyceride (Bahl et al., 1970) the substrate for PG synthesis, are also apparently localized primarily in the microsomal fraction. PA phosphatase, which would be required for the formation of the diglyceride substrate in PC synthesis is most likely present in leaves (Kates, 1956), but its subcellular localization is not known. Comparing the subcellular localization of the spinach enzymes with that of the corresponding enzymes in animal tissue, it is of interest that in the latter the phosphorylcholinetransferase and the PE-diMe:

SAM methyltransferase are also localized in the microsomal fraction, but in contrast, the PG synthesizing system is localized in the mitochondria (see Table 2).

With regard to the PE synthesizing enzymes, the subcellular localization of CDP-ethanolamine:diglyceride phosphorylethanolaminetransferase is not clear since approximately equal amounts of enzyme were found in the 15,000xg "mitochondrial" and 100,000xg microsomal fractions (Table 19). However, since the latter fraction had the highest specific activity of this enzyme but the lowest percentage of cytochrome c oxidase activity (see Table 19 and Results IV.3.a), it is clear that the transferase activity in the 100,000xg fraction was of microsomal origin and not due to contamination by a possible independent mitochondrial enzyme system. In animal tissue the corresponding enzyme is indeed localized in the microsomal fraction (see Table 2).

Most (74%) of the PS decarboxylase activity for PE synthesis was found in a 100,000xg supernatant fraction (Table 14 and Fig. 18). This was unexpected since this enzyme in animals and bacteria has been found to be membrane bound (see Table 2 and White et al., 1971; Bell et al., 1971 and Raetz and Kennedy, 1972). It is likely that the PS decarboxylase in the supernatant fraction arose by partial solubilization of this enzyme from a particulate fraction during cell fractionation. Since the mitochondrial fraction contained the highest amount of the remaining PS decarboxylase activity, it is

therefore possible that *in vivo* PS decarboxylase was localized in mitochondria. It is of interest that in animal tissue this enzyme is indeed localized in mitochondria (see Table 2).

The phosphatidyltransferases catalyzing the biosynthesis of PS and PI were detected in a total particulate fraction (Tables 9, 10 and 13), but further studies on their localization were not carried out.

It should be pointed out that the term "microsomal fraction" is only an operative one, and describes that cell fraction centrifuged down over a particular range of centrifugal forces, in the present study 40,000-250,000xg. This fraction would be composed of a wide range of membrane types, including endoplasmic reticulum (rough and smooth), plasma and Golgi apparatus membranes (Steck, 1972; Morré *et al.*, 1970). It is also possible that fragments of chloroplast and mitochondrial outer membrane stripped from the intact organelles during cell homogenization and fractionation would also be present, but contamination from this source may assumed to be small. One might expect the primary site of phospholipid biosynthesis to be on the endoplasmic reticulum so that it could be co-ordinated with the biosynthesis of proteins intended for membrane synthesis. However, phospholipid biosynthesis may also take place on the plasma and Golgi apparatus membrane, as suggested by the findings of a PG synthetase system in rat liver plasma membrane (Victoria *et al.*, 1971), and of the cytidyltransferase and possibly other enzymes of the PC

nucleotide pathway in the Golgi apparatus from onion stem (Morré, 1970 and Morré et al., 1970).

The question arises as to whether there are other subcellular sites in spinach leaves which contain the PG, PC and PE synthetases and PE-diMe:SAM methyltransferase. Activity from these enzymes was detected in the 15,000xg mitochondrial fraction, but since this fraction was neither assayed for microsomal marker enzymes nor further purified by sucrose density centrifugation, it is not clear whether these activities were due to microsomal contamination or to a separate mitochondrial enzyme system. Concerning a possible spinach mitochondrial PG synthetase system, Douce (1968) and Douce and Dupont (1969) have presented evidence suggesting that PG synthetase is present in partially purified mitochondria from the non-photosynthetic tissue cauliflower inflorescence. However, since these workers did not report whether the enzyme was also present in the microsomal fraction, nor did they rigorously purify the mitochondria, it cannot be ruled out that their observed mitochondrial activity was due to microsomal contamination. On the other hand, it is reasonable to expect a PG synthetase in the mitochondria of photosynthetic and non-photosynthetic cells since the CDP-diglyceride substrate for this enzyme can be synthesized by mitochondria (Bahl et al., 1970; Douce et al., 1972), and mitochondrial cardiolipin biosynthesis would likely utilize PG synthesized in situ, as is the case in animal tissue (Davidson and Stanacev, 1971;

Hostetler et al., 1971). With regard to a possible spinach mitochondrial cardiolipin synthesizing system, no ^{14}C-labelled cardiolipin was observed with the 15,000xg mitochondrial fraction and [^{14}C]GP (Table 7), even though the operation of either of the two possible pathways (eqns. 10 and 11) would predict formation of cardiolipin labelled with ^{14}C from phosphatidyl [^{14}C]glycerol. However, this does not entirely rule out the presence of a mitochondrial cardiolipin synthesizing system since the amount and specific activity of the [^{14}C]PG formed was so low that any conversion to [^{14}C]cardiolipin would have been below the limits of detection.

Concerning a possible mitochondrial PC synthetase, it is of interest to compare the subcellular distribution of PC synthetase obtained in the present study with that obtained by Devor and Mudd (1971) for the same enzyme in spinach leaves. In the present study, the amount of total cell free homogenate activity associated with the 15,000xg (30 min) and 100,000xg (60 min) fractions was 25% and 47% respectively (Table 21), while the corresponding amounts in the 20,000xg (30 min) and 100,000xg (90 min) fractions isolated by Devor and Mudd from an homogenate of identical density were 4% and 53% respectively. Thus, while the two microsomal activities agree quite closely, the "mitochondrial" activity observed by Devor and Mudd is considerably less than that observed in the present study. However, this difference is explainable in terms of the different washing procedures employed. In the present study, the

mitochondrial pellet was lightly rinsed by, but not suspended in, the homogenizing medium, and the washing was combined with the mitochondrial supernatant (see Experimental II.1). In contrast, the mitochondrial pellet in Devor and Mudd's study was washed by resuspending the pellet in medium and recentrifuging at 20,000xg to yield a mitochondrial pellet which was assayed for PC synthetase, and a supernatant which was discarded. The washing procedure used by Devor and Mudd was thus more efficient and likely resulted in a purer mitochondrial fraction than that used here. Thus, the PC synthetase activity in the inefficiently washed "mitochondrial" fraction of the present study was probably due to microsomal contamination. This conclusion is supported by the difference in recoveries of total PC synthetase activity following cell fractionation. In the present study the recovery was quantitative (99%, see Table 21), whereas the low recovery obtained by Devor and Mudd (74%) was clearly due to loss of microsomal fragments in the discarded "mitochondrial" wash supernatant. A similar explanation likely applies also for the phosphorylethanolamine-transferase and PE-diMe:SAM methyltransferase activities observed in the 15,000xg fraction.

It will be recalled that PC and PE are the major mitochondrial phospholipids of non-photosynthetic, and perhaps also of photosynthetic tissue (see Introduction II.2; Schwertner and Biale, 1973; Douce et al., 1968). The results of the present study therefore suggest that mitochondrial PC

is not synthesized *in situ* by mitochondria but is derived from PC synthesized at the microsomal membrane by either the nucleotide or methylation pathways. Mitochondrial PE could also be derived from PE synthesized at the microsomal membrane by the nucleotide pathway. However, spinach mitochondria may have some capacity to synthesize PE *in situ* via the PS decarboxylase possibly present in this organelle. The process by which spinach mitochondrial PC, and possibly PE, are derived from "microsomal" synthesized lipid may involve the direct transport or exchange of these lipids from "microsomal" membrane to pre-existing mitochondria or pro-mitochondria. Such a process has indeed been demonstrated to occur in non-photosynthetic plant tissue (Abdelkadar and Mazliak, 1970) and also in rat liver and brain (Wirtz and Zilversmit, 1968; McMurray and Dawson, 1969; Jungalwala and Dawson, 1970; Miller and Dawson, 1972).

Concerning possible cytoplasmic and chloroplast sites for PG, PE and PC synthesis, low activity of all the enzymes involved in these syntheses was detected in the 100,000xg supernatant and 1200xg "chloroplast" fractions (Tables 7, 19, 21 and 25). The supernatant activities were likely due to enzyme localized in light microsomal fragments shown in Table 23 to be present in this fraction, and to enzyme "solubilized" from the heavy microsomal fraction during cell fractionation. The activities in the 1200xg "chloroplast" fraction were very likely derived from microsomal contamination,

though of course the possibility that they represent minor but independent chloroplast enzyme systems cannot be ruled out entirely. Concerning the possibility of a chloroplast PG synthetase system, low PG synthetase activity was observed in the 1200xg "chloroplast" fraction and this was unlikely due to inability of substrate to cross the chloroplast outer membrane, since a high proportion of the chloroplasts in the 1200xg fraction were likely shorn of their outer membrane (Leech, 1966). Also, when the chloroplast fraction was sonicated prior to assay, a treatment which likely disrupted the outer membrane of any intact chloroplasts present, no change in the distribution of PG synthetase was observed (see Results I.2). There was also the possibility that a PG synthetase existed in chloroplasts but was specific for CDP-diglyceride containing the unique chloroplast PG component acid, trans-3-hexadecenoic acid (Haverkate and Van Deenen, 1965a) which was not present in the CDP-diglyceride used in the present study (see Methods I.2.a). This is however unlikely, since the trans-3-acid is now known to be biosynthesized by in situ desaturation of palmitate bound to PG (Bartels et al., 1967). Thus the PG synthetase system probably does not exist in spinach chloroplasts, and this is consistent with the reported failure by Douce (1970) to detect this enzyme in other plant chloroplasts. Furthermore, the CDP-diglyceride synthesizing system has also been found absent in this organelle (Bahl et al., 1970). Thus, the present study strongly

indicates that spinach PG is synthesized by the microsomal enzyme system and then incorporated into chloroplast membranes, possibly by a mechanism involving transport from the "microsomes" similar to that discussed for the origin of mitochondrial PC and PE.

F. CLAIMS TO ORIGINAL RESEARCH

1. The pathways operating in the biosynthesis of phospholipids in spinach leaves have been elucidated by the demonstration of the following enzymes:

   a) in PG synthesis:

      i) CDP-diglyceride: sn-glycerol-3-phosphate phosphatidyltransferase, and

      ii) Phosphatidyl glycerolphosphate phosphatase

   b) in PI synthesis:

      CDP-diglyceride: myo-inositol phosphatidyltransferase

   c) in PS synthesis:

      CDP-diglyceride: L-serine phosphatidyltransferase

   d) in PE synthesis:

      i) PS decarboxylase

      ii) ethanolaminekinase and

      iii) CDP-ethanolamine: sn-1,2-diglyceride phosphorylethanolaminetransferase

   e) in PC synthesis:

      i) CDP-choline: sn-1,2-diglyceride phosphorylcholinetransferase

      ii) PE-Me (or PE-diMe): SAM methyltransferase

2. The subcellular localization of some of the above phospholipid biosynthesizing enzymes has been elucidated. Enzyme

systems a-i and ii, d-iii, e-i and e-ii are primarily localized in the microsomal fraction.

3. Evidence has been obtained for the presence of phospholipase A and an acyltransferase in spinach leaves.

4. Evidence has been obtained for the presence of microsomal-bound enzymes catalyzing the biosynthesis of cyclopropane fatty acids using S-adenosylmethionine and PC or PE as substrates.

5. The presence of enzymes utilizing S-adenosylmethionine for the biosynthesis of fatty acid methyl esters and other neutral lipids has been demonstrated.

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