Short Title: PHOSPHOLIPID BIOSYNTHESIS IN OAT COLEOPTILE MICROSOMES.

Ph.D. Biology

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Biosynthesis of Microsomal Nitrogenous Phospholipids and Development of the Oat Coleoptile.

ABSTRACT

A study of ^{14}C-labelled serine, ethanolamine and choline incorporation into oat coleoptile microsomal phospholipids, and some aspects of the development of coleoptiles and coleoptile microsomal fractions, is presented. The results have been interpreted to mean: a) Three distinct sites exist for the incorporation of L-serine, ethanolamine and choline, into their respective phospholipids, by calcium stimulated reactions; incorporation is not mediated by the transphosphatidylase action of phospholipase D, b) choline is also incorporated by a mechanism that is apparently independent of divalent cations and c) phosphatidylserine is decarboxylated to form phosphatidylethanolamine; the results cannot be explained as decarboxylation of serine and subsequent incorporation of ethanolamine. Developmental changes in protein, RNA and lipid phosphorus content of coleoptiles and coleoptile microsomes, in addition to changes in rate of incorporation of serine and ethanolamine into microsomal phospholipids, are discussed in relation to the development of the endoplasmic reticulum.

BIOSYNTHESIS OF MICROSOMAL NITROGENOUS PHOSPHOLIPIDS AND DEVELOPMENT OF THE OAT COLEOPTILE

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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October, 1971

ACKNOWLEDGEMENTS

I am most grateful to Dr. W.G. Boll for the help he has given me and the patience he has shown throughout the course of this study. Without his encouragement this work could never have been accomplished.

My first introduction to some of the techniques involved in lipid research was at the laboratory of Dr. M. Kates. Throughout the course of this work Dr. Kates, Dr. D. Rubenstein and Dr. G.M. Maclachlan, among others, provided fruitful discussion on the various technical problems that arose. I gratefully acknowledge the help of these gentlemen.

I owe thanks to many members of the former Department of Botany and present Department of Biology at McGill University. I am especially grateful to Marcy Fewkes, whose sensible and knowledgeable assessment of my research at critical stages was most helpful and encouraging; to Don Gerson, who spent many hours attempting to impart an understanding of enzyme kinetics to me, and to Dr. R.D. Gibbs, whose knowledge of the plant kingdom and colorful character made his teaching and conversation a pleasure and an inspiration.

I gratefully acknowledge the generous fellowship awarded to me by the McConnell Memorial Foundation and the research aid provided by the National Research Council of Canada.

Many thanks to Pat and Kate.

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ABBREVIATIONS

Acetic Acid	A
Adenosine diphosphate	ADP
Adenosine triphosphate	ATP
Chloroform	C
p-Chloromercuribenzoate	PCMB
Cytidine diphosphate	CDP
Cytidine monophosphate	CMP
Cytidine triphosphate	CTP
Diisobutylketone	D
Endoplasmic reticulum	ER
Ethylene diamine tetraacetic acid	EDTA
N-ethylmaleimide	NEM
Generally Labelled	G
Methanol	M
Millicurie per millimole	mCi/mM
Millimolar	mM
Phosphatidic acid	PA
Phosphatidylcholine	PC
Phosphatidylethanolamine	PE
Phosphatidylglycerol	PG
Phosphatidylinositol	PI
Phosphatidylserine	PS
Pyridoxal phosphate	PALP
Pyrophosphate	PP_i
Ribonucleic acid	RNA
Uniformly labelled	U
Water	W

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INTRODUCTION

Cell growth and development clearly involve changes in cellular membranes. It is most probable that other morphogenetic changes are controlled by, or at least related to the biogenesis of these membranes. The study of membrane biogenesis requires, amongst other things, the study of phospholipid biosynthesis during the development of a membrane system, since phospholipids are almost exclusively found in membranes and play a major role in their structure.

The oat coleoptile (Avena sativa L.) should be excellent material for such a study. Much is known about the growth and development of the oat coleoptile from growth regulator work. Studies on the fine structure of the coleoptile have been made (O'Brien, 1967b; O'Brien and Thimann, 1967a, 1967b). Changes in ER membranes and membrane-bound ribosomes with development have been investigated (Setterfield, 1961; Masuda et al., 1966). A report (Zimmerer and Hamilton, 1965) noting the phospholipid composition of oat coleoptiles has been published.

The main purpose of the work presented here was to investigate the biosynthesis of oat coleoptile microsomal phospholipids. Characterizing and establishing the pathways of biosynthesis of such lipids must precede the developmental study mentioned above. As a consequence of previous studies carried out in this laboratory (Willemot and Boll, 1967) there was a particular interest in PS, PE and PC.

This thesis describes a study of the fate of ^{14}C-labelled serine, ethanolamine and choline fed to microsomal preparations

from oat coleoptiles. Conclusions are drawn regarding the mechanism of their incorporation into phospholipid. Preliminary work on the development of the oat coleoptile, and oat coleoptile microsomal fraction, is also presented.

I. REVIEW OF THE SUBJECT

A. DEVELOPMENTAL CHANGES IN THE ENDOPLASMIC RETICULUM AND MICROSOMAL FRACTION

A brief review of the literature concerning developmental changes in the endoplasmic reticulum and microsomal fraction of plants will be presented. This review is not intended to be complete but only to show what provided the basis for the preliminary developmental work that is presented in this thesis.

Developmental changes in the ER and microsomal fraction have been reported by many workers using various tissues, for example, corn roots (Lund et al., 1958), pea and wheat embryos (Setterfield et al., 1959), corn mesocotyl (Key et al., 1961), pea seedling roots (Loening, 1961), corn roots (Whaley et al., 1964), cultured cells of Acer sp. (Sutton-Jones and Street, 1968) and bean cotyledons (Payne and Boulter, 1969a, 1969b). It should be emphasized that caution must be used in making comparisons of developmental changes in these different organs as one is never certain that an equivalent developmental stage is being compared (Whaley et al., 1964). However, the results of each of these studies is certainly in agreement with the following statement: It is generally accepted that the development of the ER parallels the degree of cell differentiation (see Porter, 1961).

Developmental changes in the ER and microsomal fraction of oat coleoptiles have been studied by Setterfield (1961). Electron micrographs showed that cells of 2 mm coleoptiles

contained mainly free ribosomes and very little ER. As growth proceeded free ribosomes were diluted out due to the increase in cell volume. An increase in ER membranes only takes place in older cells; at about the same time an increase in membrane-bound ribosomes also occurs. Setterfield noted that these changes in the cytoplasm of elongating coleoptiles were in structures that would enter a microsomal fraction obtained by cell fractionation, in fact, the observations from the electron microscope were confirmed by ultracentrifugation studies of microsomal pellets in the presence and absence of deoxycholate. Masuda et al.(1966) extended these findings by studying ribonucleic acid metabolism in the elongating coleoptile. They concluded that young coleoptiles have a full complement of ribosomes necessary for subsequent growth during which new ribosomes are not synthesized. Messenger-type RNA synthesis, however, is required throughout normal growth. They also noted that in fully grown coleoptiles free ribosomes decline markedly while membrane-bound ribosomes increase.

Investigations into the fine structure of the oat coleoptile have also been carried out by O'Brien (1967b) and O'Brien and Thimann (1967a, 1967b). These studies were not concerned with developmental changes.

Studies of developmental changes in microsomal membranes by assaying lipid biosynthetic pathways during development have been carried out using animal material (see Getz, 1970). No comparable studies have been done on plant microsomes. The only report, known to myself, of studies on incorporation of a lipid precursor specifically into microsomes in a changing

plant system is that of Abdelkader (1969). He studied the incorporation of acetate-1-^{14}C into microsomes of fresh and so called "aged" potato slices, i.e., slices held for 24 hr in buffer.

Elongating oat coleoptile sections have been shown to incorporate ^{32}P-phosphate into lipid (Zimmerer and Hamilton, 1965). Changes in incorporation throughout development were not studied. Mitochondria were suggested as the possible site for this incorporation.

B. BIOSYNTHESIS OF NITROGENOUS PHOSPHOLIPIDS IN PLANTS

The pathways of biosynthesis of PS, PE and PC have been well studied in animal and bacterial systems (see reviews of Kennedy (1961), Dawson (1966), Kates (1966) and Hill and Lands (1970)). In plants, comparatively little is known (Kates, 1970). As an introduction, an overall picture of these pathways and their interrelationships will be presented. In the individual sections, of the review below, the evidence for the existence of the various pathways is given. This review covers the literature up until August, 1971.

The de novo generation of the monophosphate diester linkage always involves cytidine-containing coenzymes (Kennedy, 1961). CTP is utilized in the formation of phospholipids either by reaction with PA to form CDP-diglyceride or reaction with a phosphorylated base to produce CDP-"base", with the subsequent release of pyrophosphate in both cases. CDP-diglyceride is used in the formation of PI, PG or PS, whereas CDP-choline

or CDP-ethanolamine is used in the formation of PC or PE, respectively. Evidence for these reactions in plants will be dealt with below (Sect I.B.1.).

Various pathways exist for the interconversion of phospholipids, PS may be formed at the expense of PE, and vice versa, via the exchange reaction of Borkenhagen et al. (1961). PS can be converted to PE via the enzyme PS decarboxylase. The stepwise methylation of PE by S-adenosylmethionine converts this lipid into PC. Transphosphatidylation by phospholipase D (phosphatidylcholine phosphatidohydrolase, E.C. 3.1.4.4) can convert PC into PE, PG or PS, as well as producing various artificial phospholipids. Transphosphatidylase action can convert PG into cardiolipin. The evidence for each of these reactions in plants is presented below. Transacylations will not be dealt with (see recent review of Kates, 1970).

It should be emphasized that pathways for the interconversion of phospholipids can provide means for the de novo generation of any one phospholipid class. In fact, it is very likely that in many systems this is so, since no pathway may exist for the direct synthesis of a particular phospholipid class by means of cytidine-containing nucleotides. Pathways involving cytidine-containing nucleotides can, however, provide for the de novo generation of any one lipid class as well as phospholipids in general.

Where more than one pathway may exist for the synthesis of a phospholipid there is the possibility that each pathway may have a separate function. By way of example, in animal systems PC biosynthesis via methylation of PE seems to produce

PC mainly with polyunsaturated fatty acids, whereas the CDP-choline pathway produces PC mainly with saturated fatty acids Rytter et al. (1968). Evidence for such differences in plants must first wait for a more careful elucidation of phospholipid biosynthetic pathways.

1. Energy dependent incorporation into phospholipids

Only evidence for energy dependent pathways which are involved in the biosynthesis of nitrogenous containing phospholipids, and lipids that are biosynthetically closely related to them, will be reviewed in this section. The reviews of Kennedy (1961), Kates (1966), Dawson (1966) and Hill and Lands (1970) cover this field for microorganisms and animals. Pathways involving the synthesis of galactolipids, sulfolipid and steryl glucosides have been covered by Kates (1970). The pathways involving CDP-diglyceride will be reviewed first.

Douce (1968) showed that isolated mitochondria of cauliflower incorporated CTP-α-^{32}P, but not CTP-β,γ-^{32}P, into CDP-diglyceride, probably via a pathway identical to that shown by Paulus and Kennedy (1960) for animal tissue; namely:

$$PA + CTP \longrightarrow CDP\text{-diglyceride} + PP_i$$

The synthesis of CDP-diglyceride was also shown in cell free extracts of yeast (Hutchison and Cronan, 1968).

Sumida and Mudd (1968) showed that activity of CTP was incorporated into lipid mainly by a mitochondrial fraction of cauliflower.

Sumida and Mudd (1970b) studied an enzyme preparation

containing CTP-phosphatidic acid cytidyl transferase which is the enzyme that mediates the synthesis of CDP-diglyceride. Optimum activity was obtained at pH 5.6, which was considerably lower than that found by other workers. The fact that PA was not observed to stimulate this reaction was discussed and one possibility suggested was that sufficient PA is present due to the action of endogenous phospholipase D. A requirement for PA has been shown in animal systems (Hill and Lands, 1970). Recently Douce (1971) has shown incorporation of PA into CDP-diglyceride by isolated cauliflower mitochondria. As in the case with the animal enzyme the plant enzyme always requires Mn^{++} or Mg^{++} for activity. $CaCl_2$ gave no enhancement of activity.

Bahl et al. (1970) showed the presence of CTP-phosphatidic acid cytidyl transferase in a number of different plant tissues. The activity was highest in the microsomal fraction. Mg^{++} was required for activity.

Douce (1968) compared the addition of either glycerol or glycerol phosphate, to a reaction medium containing CTP-α-^{32}P. Only glycerol phosphate caused the production of PG with the subsequent release of CMP which is consistent with the pathway described by Kiyasu et al.(1963) from experiments with rat liver mitochondria; namely:

CDP-diglyceride + glycerol phosphate $\longrightarrow$ PG-phosphate + CMP

PG-phosphate $\longrightarrow$ PG + P_i

Further evidence for the existence of this pathway in plants has been presented by the same laboratory (Douce and Dupont, 1969).

Stanacev et al. (1967), using cell free extracts of E. coli, showed the further reaction of PG and CDP-diglyceride to form cardiolipin and CMP. This reaction has not been shown in plants.

It was noted that incorporation of ^{32}P-PA into PI was stimulated by the presence of CTP and inositol in homogenates of runner-bean leaves (Sastry and Kates, 1966).

Sumida and Mudd (1968) showed that activity from CTP-^{14}C incorporated into CDP-diglyceride, of cauliflower mitochondrial preparations, rapidly decreased probably because of the production of PG and PI. It was found that inositol incorporation was CTP-dependent. This gives support to the reaction sequence:

CDP-diglyceride + inositol ⟶ PI + CMP

as proposed by Paulus and Kennedy (1960) from results obtained with chicken liver homogenates.

The enzyme CDP-diglyceride : inositol transferase has been characterized by Sumida and Mudd (1970a). In the presence of a mitochondrial preparation from cauliflower it was shown that myo-inositol and CDP-diglyceride formed PI. Optimum pH was 9.0; Mn^{++} ions were much more effective than Mg^{++} for enzyme activity.

Cell free extracts of E. coli have been shown to synthesize PS as follows:

CDP-diglyceride + L-serine ⟶ PS + CMP

The reaction was mediated by the enzyme L-serine-CMP phosphatidyltransferase (Kanfer and Kennedy, 1964). This pathway has never been shown in plants or animals. CDP-diglyceride is the

central intermediate in the de novo synthesis of all known bacterial phospholipids (Hill and Lands, 1970).

The incorporation of choline and ethanolamine via their CDP-derivatives has been shown in animals (Kennedy, 1961). The CDP-choline and CDP-ethanolamine pathways have not been found in microorganisms (Kates, 1966; Lennarz, 1970b).

Indications that a CDP-choline pathway in plants may exist came from the work of Willemot and Boll (1967) who found that when tomato roots were fed L-serine-U-^{14}C, the presence of the inhibitors diethylethanolamine or p-chloromercuribenzoate caused most of the radioactivity to appear in PC and almost none in PE. One interpretation they gave of these results was that there was direct incorporation of choline into PC; biosynthesis of choline having occurred in the non-phosphatide form. Kates (1970) expressed the opinion that these results do not eliminate the possibility of incorporation via CDP-ethanolamine and CDP-choline into the phosphatides.

Morré et al. (1970) showed the presence of the following enzymatic activities in onion stem: choline kinase, phosphorylcholine-cytidyl transferase and phosphorylcholine-glyceride transferase and their participation in the pathways to PC biosynthesis as reported for animal systems (Borkenhagen and Kennedy, 1957; Weiss et al., 1958). The enzymes catalyze, respectively, the following reactions:

$$\text{choline} + \text{ATP} \longrightarrow \text{phosphorylcholine} + \text{ADP}$$

$$\text{CTP} + \text{phosphorylcholine} \rightleftharpoons \text{CDP-choline} + PP_i$$

$$\text{CDP-choline} + \text{D-1,2-diglyceride} \rightleftharpoons \text{PC} + \text{CMP}$$

Choline kinase was predominantly in the soluble fraction while the other two enzymes had higher activities in the particulate fractions. Phosphorylcholine-cytidyl transferase seems to be localized in the Golgi apparatus. This enzyme was inhibited by Ca^{++} ions, even in the presence of excess Mg^{++}, which by itself activates the enzyme.

Devor and Mudd (1971) have studied the incorporation of CDP-choline-1,2-^{14}C into PC of spinach leaves. The pH optimum was 8.0 and either Mn^{++} or Mg^{++} acted as cofactors. Choline phosphotransferase (called phosphorylcholine-glyceride transferase above) activity was highest in the microsomal fraction. The enzyme did not show specificity, in vitro, towards diglycerides of various fatty acid composition.

The only report that a CDP-ethanolamine system exists for the incorporation of ethanolamine in plants is that of Kennedy and Weiss (1956). They showed the presence of phosphorylethanolamine-cytidyl transferase in carrot roots as well as phosphorylcholine-cytidyl transferase.

Hill and Lands (1970) state that the cytidyl transferases and the phosphotransferases have not been characterized and thus the various preparations which synthesize PC also catalyze the synthesis of PE. It is therefore impossible to say whether or not the same enzymes are responsible for the synthesis of both classes of compounds.

Recent work using animal tissues indicates that CDP-choline-^{14}C is not incorporated into the phospholipids of mitochondria. This and other evidence leads to the conclusion that

synthesis of major mitochondriallipids takes place in the ER and that they are then transferred, via a soluble protein, to the mitochondria (Lennarz, 1970a). A similar exchange of phospholipids has been shown in potato tubers (Abdelkader and Mazliak, 1969).

2. Exchange reaction

The exchange reaction, as discussed here, involves the energy independent, calcium stimulated incorporation of serine, ethanolamine and choline to form their respective phospholipids, that is not mediated by phospholipase D. One pathway for such incorporation proposed by Borkenhagen et al. (1961), is as follows:

$$PE + \text{L-serine} \rightleftharpoons PS + \text{ethanolamine}$$

In early work, the calcium stimulated incorporation of serine and choline into phospholipids was attributed to the reverse action of a phospholipase (Hübscher et al., 1959). Support for this idea came from the calcium stimulated release of PA (Dils and Hübscher, 1961). The reverse action of phospholipase D is a good explanation for this incorporation but such an enzyme has never been reported to occur in animal tissues (Crone, 1967b; Dawson, 1966). In addition, Hübscher (1962) has shown that diglyceride or phosphatidic acid did not increase the amount of L-serine incorporated. Porcellati et al. (1971) also showed that cabbage phospholipase D, phosphatidic acid or diglyceride did not produce an increase in the rate of Ca^{++} stimulated serine and ethanolamine incorporation.

More recent work on the exchange reaction, using animal

tissues, is that of Koji (1967) and Crone (1967b). Dennis and Kennedy (1970), using a procedure originally suggested by Crone (1967b), obtained direct proof for the existence of the exchange reaction (of Borkenhagen et al., 1961) in cell free extracts of the protozoan Tetrahymena pyriformis. They showed that the loss of activity from PE was almost completely dependent upon the presence of L-serine; they attributed the loss of activity to a loss of ethanolamine. They did not show the converse experiment but did suggest that ethanolamine incorporation may involve a displacement of ethanolamine from PE. This is a mechanism somewhat different from that of Borkenhagen et al. (1961) who proposed that ethanolamine incorporation involves displacement of serine from PS.

Porcellati et al. (1971) studied the incorporation of serine and ethanolamine into chick brain phospholipids. Choline did not inhibit serine or ethanolamine incorporation. Both serine and ethanolamine caused a decrease in the activity of endogenous ^{14}C-labelled PE suggesting a mechanism involving exchange of serine or ethanolamine with the ethanolamine of PE.

In plants the situation is confused by the fact that the animal exchange reaction of Borkenhagen et al. (1961) and the plant transferase activity of phospholipase D, as elucidated by Yang et al. (1967), are both energy independent, calcium stimulated and both involve an exchange of alcohols with phospholipid bases. There exists some question as to whether the exchange reaction, as discussed here, and transphosphatidylation by phospholipase D are, in fact, different reactions (see

Hill and Lands, 1970). There is also uncertainty as to whether or not the exchange reaction occurs in vivo (Hill and Lands, 1970).

In a recent review on "Plant Phospholipids and Glycolipids" (Kates, 1970) no mention was made of the exchange reaction of Borkenhagen et al. (1961). This was undoubtedely due to the lack of evidence available for the existence of this reaction in plants. The only evidence for the exchange reaction in plants is that of Vandor and Richardson (1968) who showed calcium stimulated incorporation of labelled ethanolamine, L-serine and choline, into pea microsomal phospholipids, and the existence of competitive inhibition between them. They believe incorporation is catalyzed by a single enzyme system that does not distingush between PE, PS and PC in exchanging with the free bases ethanolamine, serine and choline. Vandor and Richardson (1968) feel that the transferase activity of phospholipase D is not involved; this view is based solely on the fact that certain substrates for the exchange enzyme were not also substrates for transphosphatidylation as shown by Dawson (1967).

In mammalian systems the exchange reaction most probably takes place in the microsomal fraction, other fractions showing little if any activity (Kennedy, 1961; Hubscher, 1962; Arienti et al., 1970). Only the protozoan exchange enzyme may be localized in mitochondria (Dennis and Kennedy, 1970). In plants, microsomes showed highest activity (Vandor and Richardson, 1968).

pH optima for reactions that are most probably utilizing

similar exchange enzymes have been reported to be pH 7.4 (Hübscher et al, 1959), pH 9.0 (Dils and Hübscher, 1961), pH 8.3 (Hubscher, 1962), pH 7.7 (Crone, 1967b), pH 8.5 (Vandor and Richardson, 1968), pH 7.7 (Dennis and Kennedy, 1970) and pH 8.5 (Porcellati et al., 1971). On the other hand, the reported optimum for phospholipase D is in the range of pH 4.8 to 6.5 (Kates, 1970).

The exchange reaction is inhibited by magnesium (Dils and Hubscher, 1959). Inhibition by magnesium but stimulation by calcium is interesting in that it is the reverse situation of that found for the enzyme phosphorylcholine-cytidyl transferase (Morré et al., 1970).

A consensus seems to have developed concerning the mechanism of the exchange reaction, that is, the exchange reaction is mediated by a single enzyme system. This idea has been put foward by Borkenhagen et al. (1961), Crone (1967b), Vandor and Richardson (1968), Dennis and Kennedy (1970) and Porcellati et al. (1971). Only Artom (1961) and Hübscher (1962) have suggested separate enzymes but Crone (1967b) has stated why the differences obtained by these two workers to support separate enzymes are probably unreal. Competitive inhibition between substrates seems to be the main reason in support of a single enzyme system.

3. Decarboxylation of phosphatidylserine

In animal and bacterial systems the decarboxylation of PS has been throughly studied. In plant systems only indirect

evidence for its occurence is available.

In animals the decarboxylation of PS has been demonstrated by feeding phosphatidylserine-1-^{14}C to in vitro preparations and noting the production of labelled $^{14}CO_2$ (Borkenhagen et al., 1961; Dennis and Kennedy, 1970), and by the incorporation of DL-serine-1-^{14}C into the lipid in the presence and absence of hydroxylamine, which inhibits the decarboxylase (Dennis and Kennedy, 1970). In both of the above cases the decarboxylase was localized in the mitochondrial fraction. Crone (1967a) used specific activity data from pulse-chase experiments to give indirect evidence that PE is formed from PS and that free serine is not significantly decarboxylated. Recently Bieber et al. (1970) showed that PS formed by rat liver microsomes was translocated to the inner mitochondrial membrane where it was decarboxylated (see Getz, 1970). There were indications that electron transport and oxidative phosphorylation had little effect on the activity of the enzyme.

In bacteria the decarboxylation of PS has been demonstrated by Kanfer and Kennedy (1964) using an enzyme preparation derived from E. coli. The enzyme preparation, containing PS decarboxylase, was relatively free of L-serine-CMP phosphatidyltransferase activity. PS-1-^{14}C decarboxylation was determined by measuring $^{14}CO_2$ release. The enzyme was active in buffer containing 0.02 M EDTA at pH 6.9. Free L-serine was not attacked by the enzyme. Subsequent research on this pathway was done by Patterson and Lennarz (1971). They compared the decarboxylation of PS that was either endogenously generated on bacterial

ghosts from ^{14}C-serine and CDP-diglyceride or exogenously supplied to bacterial ghosts as a sonic dispersion. Endogenous PS was decarboxylated in the presence of buffer only, while exogenous PS required several "physical state modifiers" such as salt and detergent. The decarboxylation of exogenous PS had optimum activity at about pH 7.0 and showed a very sharp optimum of detergent (Cutscum) concentration.

In the review of Mudd (1967) it is stated that "The details of PC, PE and PS synthesis in plants are completely unknown". Indirect evidence that decarboxylation of PS may take place in plants was obtained by Sastry and Kates (1965) and Gorchein et al. (1968). Willemot and Boll (1967) fed L-serine-U-^{14}C, -3-^{14}C, -1-^{14}C and ethanolamine-^{14}C to excised tomato roots. By comparing the activities appearing in PC, PS, PE and CO_2 with the various labelled precursors supplied, evidence was found for the decarboxylation of PS and the subsequent incorporation of label in PE and PC. Direct evidence that decarboxylation of serine takes place only while in the lipid form was not obtained. Further indirect evidence was obtained by Vandor and Richardson (1968) using pea microsomal suspensions. They found that serine-U-^{14}C produced $^{14}CO_2$ whereas ethanolamine-1,2-^{14}C and serine-3-^{14}C did not.

4. Methylation of phosphatidylethanolamine to form phosphatidylcholine

The pathway from PE to PC involving stepwise methylation by S-adenosylmethionine has been shown to be operative in

animals (Kennedy, 1961) and microorganisms (Kates, 1966). In plants no direct evidence has been obtained for this pathway although there are results which suggest that such a pathway does exist (Sastry and Kates, 1965; Willemot and Boll, 1967; Gorchein et al., 1968).

It is well documented, from work using animal systems, that S-adenosylmethionine is the methyl donor in the pathway from PE to PC (Hill and Lands, 1970). The fact that S-adenosylmethionine is present in plants (Bonner and Varner, 1965) lends support to postulating a PE methylation pathway in plants.

5. Transphosphatidylase action of phospholipase D

Dils and Hübscher (1959) suggested that their observed calcium stimulated incorporation of choline and serine into rat liver particles could be explained by exchange reactions catalyzed by a phospholipase D.

Ferrari and Benson (1961) were the first to use the word "transphosphatidylation" for a phospholipid base exchange reaction but no mention was made of phospholipase D. They postulated that the reactions of PG and inositol led to the formation of PI and glycerol. They also suggested that PC and PE formation may take place via transphosphatidylation with either PI or PG. The mechanisms they postulated were based solely on kinetic data obtained from feeding $^{14}CO_2$ to Chlorella pyrenoidosa. There was no mention as to the nature of the enzyme(s) involved. Douce et al. (1966) and Bartels and Van Deenen (1966) reported the formation of phosphatidylmethanol. The first

report of transphosphatidylation by phospholipase D was by A.A. Benson, S. Freer and S.F. Yang in 1965 during the 9th International Conference on the Biochemistry of Lipids, Noordwijk ann Zee. Yang et al.(1967) extended their findings and proposed a mechanism of action. The work of Dawson and Hemington (1967), describing the transferase action of the enzyme, appeared almost simultaneously with the aforementioned work of Yang et al. (1967).

The mechanism of action proposed by Yang et al. (1967) for phospholipase D action is as follows:

PC + enzyme $\rightleftharpoons$ phosphatidyl-enzyme + choline

phosphatidyl-enzyme + water $\longrightarrow$ PA + enzyme

phosphatidyl-enzyme + alcohol $\longrightarrow$ phosphatidyl-alcohol + enzyme

This mechanism is based on the fact that PA could not be substituted for PC, indicating that PA was not involved in the reaction, and hence the reaction that produces PA from PC is not reversible; this distinguishes transphosphatidylation from a possible reverse action of phospholipase D. In addition, the reaction is inhibited by the sulfhydryl-binding reagent p-chloromercuribenzoate (but not by iodoacetamide, N-ethylmaleimide or di-isopropylfluorophosphate). These results suggest the involvement of a sulfhydryl group at the active site of the enzyme (Kates, 1970).

The phosphatidohydrolase action of phospholipase D shows a rather broad substrate specificity (Kates, 1970). The substrate specificity of the enzyme for transphosphatidylation has not been so widely investigated. The mechanism of

transphosphatidylation proposed by Yang et al. (1967) can be interpreted to mean that any substrate that can undergo hydrolysis by phospholipase D should also act as a substrate for transphosphatidylation. Yang et al. (1967) feel that transphosphatidylation reactions are implicated in the incorporation of serine and ethanolamine in animal tissues (Borkenhagen et al., 1961). It follows from this that PE and PS probably are also substrates. However, phospholipase D has not been found in animal tissues (Dawson, 1966). PC (Dawson, 1967; Yang et al., 1967) and PG (Stanacev and Stuhne-Sekalec, 1970) have been shown to be substrates. There is evidence that PE may also be a substrate (Douce et al., 1966).

It has been suggested that acceptors of the phosphatidyl unit must contain a primary alcoholic group but that not all primary alcohols will act as acceptors (Dawson, 1967). Alcohols that will act as acceptors have been noted by Yang et al. (1967), Dawson (1967) and Lennarz et al. (1967). The suggestion of Dawson (1967) that the primary alcohol probably should be water soluble was contradicted by the finding of Stanacev and Stuhne-Sekalec (1970) who showed that PG can act as an acceptor of the phosphatidyl group in the formation of cardiolipin. Dawson (1967) found no evidence that PE, PI or long-chain mono or diglycerides could act as acceptors of the phosphatidyl group.

The optimum pH for phospholipase D action (obtained mainly from work on its hydrolytic action) has been found to range from pH 4.8 to 6.5 depending on the incubation system used (Kates, 1970). Recently Antia et al. (1970) found pH 7.0 as

optimum for an algal phospholipase D. Phospholipase D hydrolysis of PI in Schizosaccharomyces pombe has an optimum of pH 8.0 (White and Hawthorne, 1970). Soŭcek et al. (1971) reported an optimum of pH 8.0 for a bacterial enzyme similar in action to phospholipase D, however, this enzyme may be completely different from plant phospholipase D. The algal enzyme of Antia et al. (1970) also differed from that of other workers' findings in that it was not activated by ethyl ether; this is similar to the situation found by Tookey and Balls (1956) for a cotton seed phospholipase D. Long et al. (1967) showed inhibition in the presence of ether. Condrea et al. (1964) have found that lipoprotein-bound PC is readily cleaved in the absence of ether or calcium whereas free PC is not. The varied responses of phospholipase D depend on the composition of the incubation medium and the presentation of the substrates. This has been discussed by Kates (1970) and Lennarz (1970).

The subcellular location of phospholipase D is not clear (Kates, 1970; Clermont and Douce, 1970). Clermont and Douce (1970) studied the localization of phospholipase D and found that mitochondria and plastids isolated on saccharose gradients, from various plant tissues, lacked activity. Impure fractions undoubtedely accounted for the results of earlier workers. It was concluded that the majority of activity was in the soluble fraction; either in the cytoplasm or the vacuoles.

The role of calcium in phospholipase D reactions, and possibly other lipid reactions, is not yet settled. Ansell and Hawthorne (1964) suggest that calcium may activate the reaction

by removing excess phosphatidic acid which would give too high a negative ζ potential. However, if incorporation occurs by transphosphatidylation, which does not result in PA formation, then the above suggestion must be incorrect (Yang et al., 1967). Davidson and Long (1958) noted that calcium concentration was independent of enzyme concentration but roughly proportional to the amount of ovalecithin used as substrate. Crone (1967b) believes that there may be affinities of the amino-alcohol with calcium as well as the absolute concentration of the substrate (in the exchange reaction). Yang et al. (1967) also feel that calcium ion activates the lipid substrate since both hydrolysis and transphosphatidylation were activated by calcium ions.

Quarles and Dawson (1969) have studied the distribution of phospholipase D in various plants and noted changes in activity with development.

II. GENERAL MATERIALS AND METHODS

A. PLANT MATERIAL

Seeds of Avena sativa L. cv. Brighton (1966-69 harvests), a hulless variety, were obtained from W.D. MacNaughton, Box 3191, Station C, Ottawa. They were soaked in tap water for about 4 hr (Morré and Key, 1967), rinsed and sown onto well-soaked Terralite Brand Vermiculite (W.D. Perron Co., Chomedy, P.Q.) in rubber trays. A thin layer of soaked vermiculite (less than 1 cm) over the seeds was found to produce more uniform growth of coleoptiles probably by affecting time of germination. It was found that these seeds germinated poorly if used within the six month period following harvest. This may represent a post-harvest dormancy period. The seedlings were grown at 25°C, 90% humidity, in total darkness in growth chambers (Model E-8, Controlled Environments Co., Winnipeg). The seedlings were irradiated with red light, while inside the growth chamber, from the 48th to the 56th hr after sowing (Morré and Key, 1967; Liau, 1968) to inhibit growth of the mesocotyl. This timing and duration of red light treatment was found to be optimum in minimizing growth of the mesocotyl with little decrease in growth of the coleoptile. The red light source used here consisted of 5 Grolux fluorescent lamps (F20T12 - Gro, Sylvania) filtered through two layers of Cinemoid filter, Cinemoid Red No. 14 and Cinemoid Amber No. 5A (Strand Electric Co., Toronto). This filter combination theoretically transmits only the 660 nm peak of the Grolux lamps eliminating the shorter

wavelengths that the lamps also produce. The intensity of the light at the distance the plants were grown (70 cm; measured from the lamps) was 2 X 10^3 ergs/cm^2-sec, measured with a light energy meter (Model 65, YSI-Kettering Radiometer, Yellow Springs, Ohio, U.S.A.).

Coleoptiles were generally harvested on the 5th day (about 25 - 30 mm long), discarding those in which the first leaf had emerged (Avery and Burkholder (1936) showed that after leaf break-through elongation ceases at the base but localized elongation regions still remain at the tip and persist for two or three days.). Coleoptiles were selected for uniformity and first leaves were removed. During harvest coleoptiles were floated in ice-cold homogenizing medium (usually 0.4 M sucrose, 0.05 M Tricine) until a sufficient number had been collected. When fresh weight determinations were required it was found that blotting with filter paper was sufficient to give a percent water content equal to that of coleoptiles collected on moist filter paper; apparently water is not drawn into the empty coleoptile cylinders to any great extent. All harvesting was carried out under a dim green light. All subsequent operations were performed at 0 - 4°C.

B. ISOLATION OF MICROSOMAL MATERIAL

Coleoptiles, 150, were ground in 0.4 M Sucrose, 0.05 M Tricine buffer at pH 8.0 through two layers of fine nylon mesh using a mortar and pestle. The final volume of the homogenate was brought to 180 ml. This brei was then centrifuged

(Servall Refrigerated-Automatic Centrifuge; SS-34 head) at 37,000g (max) for 20 min followed by ultracentrifugation of the supernatant (Spinco L2-50; type 40 head) at 105,000g (avg) for 1 hr. Ultracentrifugation was in 13.5 ml (12.5 ml capacity) Polyallomer tubes. Each pellet produced represented microsomal material from 10.4 coleoptiles. The supernatant was discarded and the tubes drained by inverting them onto tissue paper for a few minutes. The above conditions were used for all experiments, unless otherwise noted. If less than 150 coleoptiles were used the final volume of the homogenate was adjusted keeping the ratio of 150 coleoptiles per 180 ml of medium constant.

To prevent loss of microsomal material, where possible, all further operations including the initial extraction steps were carried out in the same Polyallomer tubes used for ultracentrifugation.

C. FEEDING OF LABELLED PRECURSORS

Crude labelling of whole coleoptile lipids, for the purpose of partial identification by radioautography, was done with the following radioactive materials: ^{35}S as Na_2SO_4 fed at 10.0 µCi/ml, ^{32}P-orthophosphate as Na_3PO_4 fed at 2.0 µCi/ml (Kates, 1967, p. 10) and galactose-1-^{14}C (1.0 mCi/mM) fed at 0.2 uCi/ml in water. The feeding of L-serine-U-^{14}C to whole coleoptiles was similar to the incubation used by Willemot (1964) for cultured tomato roots; 0.2 µCi/ml of L-serine-U-^{14}C (2.84 mCi/mM) adjusted to pH 7.0 with NaOH or HCl. Ten ml of incubation medium were used per gram fresh weight of

coleoptiles. Incubation of whole coleoptiles was done in total darkness (see Sect III.A.2.).

In experiments using microsomal pellets the incubation medium was usually 1.0 ml of 0.05 M Tricine, 0.005 M $CaCl_2$ at pH 8.0, unless otherwise noted. Where required, additives to the incubation medium were made and then all were titrated to pH 8.0. The incubation medium was pipetted onto the pellets in the Polyallomer tubes. The pellets were then resuspended using an extremely close fitting, electrically driven teflon pestle (833 rpm for about 10 sec). The labelled substrate, usually 25 µl, plus lipid solvents where required, were then added to the Polyallomer tubes containing the resuspended material. The mixture was then centrifuged (International Clinical Centrifuge Model Cl) for about 1 min to place the total reaction mixture in the bottom of each tube.

All incubations were carried out at 25°C for 1 hr, unless otherwise specified, in a Gallenkamp Shaking Incubator (stroke 3.0 cm; speed 56 oscillations per min).

D. LIPID EXTRACTION OF WHOLE COLEOPTILES

Lipid extraction was performed by the method of Bligh and Dyer (1959). Whole coleoptiles were blotted dry, weighed and placed in a 40 ml Tenbroeck glass homogenizer (Kontes Glass Co., Vineland, N.J., U.S.A.). Taking into account coleoptile water content (section III.A.5.(a)), sufficient chloroform, methanol and additional water, as necessary, were added to bring the ratio of C:M:W to 1:2:0.8 (v:v:v). Five strokes of the

homogenizer were sufficient to break up the material. The third extraction of the resulting brei, separated by centrifugation, failed to remove any further measureable amount of phosphorus. All extracts were combined and sufficient chloroform and water were added to bring the ratio of C:M:W to 1:1:0.9 (v:v:v). The upper layer was discarded. This washing of the lipid extract was judged satisfactory as a minimum of water soluble material remained at the origin during the subsequent chromatography on silicic acid impregnated paper in D:A:W, 8:5:1 (v:v:v).

This procedure was found to extract a consistent amount of lipid phosphorus. This was determined by extracting 5 separate groups of coleoptiles (about 20 coleoptiles per group) and making three measurements of the phosphorus content of each extract (group). No extract differed from the average value for the 5 extracts by more than 2.5% when expressed on a µg phosphorus per gram fresh weight basis.

E. LIPID EXTRACTION OF MICROSOMES

Lipid extraction of resuspended microsomes was carried out according to the method of Bligh and Dyer (1959). Water was added to the reaction mixture, in the Polyallomer tube, to bring the total volume to 2.0 ml. The tube was then emptied into a 50 ml, conical, glass-stoppered centrifuge tube containing 2.5 ml of chloroform:methanol, 1:2 (v:v; chloroform was always redistilled). Two additional 2.5 ml washes of chloroform:methanol, 1:2, were then used to rinse out the

Polyallomer tube (It was noted that cellulose nitrate ultracentrifuge tubes could not be used with this procedure because some cellulose nitrate dissolved, even with brief methanol rinses, and the material ran with the solvent front during subsequent chromatography in diiosbutylketone-acetic acid-water.). The combined washes produced a single phase, after brief mixing on a Vortex Genie, with a ratio of C:M:W of 1:2:0.9 (v:v:v). The tubes were stoppered, refrigerated for 1-2 hr and then centrifuged (International Model UV) for about 10 min at about 800g (avg) to give two distinct phases. For most experiments the upper phase and white interfacial fluff of protein were removed as completely as possible, using a water aspirator, and discarded. Where the upper phase was needed for further analysis it was removed with a pasteur pipette. The chloroform phase was dried down using a stream of nitrogen with the aid of a water bath at 37°C. When completely dry, the extract was immediately taken up in a few drops of chloroform and spotted for chromatography.

F. CHROMATOGRAPHY ON SILICIC ACID IMPREGNATED PAPER

Chromatography was carried out on Whatman 3mm paper impregnated with silicic acid as described by Marinetti (1962) and Kates (1967). In later experiments the commercially available SG-81 Silica Gel Loaded Paper (H. Reeve Angel and Co., 9 Bridewell Place, Clifton, N.J., U.S.A. or Mandel Scientific Co. Ltd., Montréal, P.Q.) was used. Papers were run for about 16-20 hr (SG-81 papers developed faster) in diisobutylketone

(redistilled):acetic acid:water, 8:5:1 (v:v:v) containing 0.05% butylated hydroxytoluene.

Kates (personal communication) and Willemot (1964) noted that the solvent D:A:W, 8:5:1 (v:v:v) improved with usage. A similar situation was found during this work: fresh solvent gave greater mobility to all components often crowding them towards the solvent front; as the solvent aged a better separation was achieved until finally old solvent caused crowding of components towards the origin. The solvent composition was occasionally varied to produce the best separation. This was done by making small changes in the relative amount of water whenever new solvent was being made up. The optimal composition was not found; however, a small improvement was sometimes noted with D:A:W:, 8:5:0.9 (v:v:v). Varying diisobutylketone or acetic acid concentration individually was not tried.

Chromatograms were dried for 45 min and then passed through a solution of 0.0012% Rhodamine 6G in water (Kates, 1967). The lipid spots were revealed under UV light and outlined. Chromatograms were allowed to dry, after which the spots were cut out for counting. Most of this work involved the assay of radioactivity in PC, PS and PE. The resolution of these three phospholipids on silicic acid impregnated paper varied with each run. Where possible the individual phosphatides were separately counted, otherwise, the three phosphatides, PC+PS+PE, were cut out and counted together. In most of the work to be presented either method of counting gives almost identical results (see Sect III.B.5.).

Elution of lipids from silicic acid impregnated paper was performed according to Kates (1967, p. 15). Spots or bands were stapled to a strip of chromatography paper and eluted by descending chromatography with C:M:W:, 75:25:2 (v:v:v) followed by methanol.

G. SILICIC ACID COLUMN CHROMATOGRAPHY

Preliminary studies showed that chromatography on silicic acid impregnated paper was improved considerably by prefractionation of the lipid extract on a column of silicic acid. The procedure used was a modification of that used by Sastry and Kates (1964).

Column chromatography was performed using a glass column of 1.0 cm I.D. fitted with a sintered glass filter. The column was packed with Mallinckrodt silicic acid 100 mesh powder, previously soaked in chloroform for cleaning and removal of fines, to a column volume of 3 ml. The lipid extract was placed on top of the column using about 1 ml of chloroform. Six fractions were eluted from the column as follows:

Fraction	Solvent	Column Volumes	ml of solvent
I	C (100%)	11	33.0
II	C:M, 5:1	2	6.0
III	C:M, 5:1	3.5	10.5
IV	C:M, 1:1	2	6.0
V	C:M, 1:1	4	12.0
VI	M (100%)	2	6.0

Column chromatography was used only in the work involving identification of phospholipids (Sect III.A.1.). For all other work fractionation on a silicic acid column was not used before fractionation on silicic acid impregnated paper. The improvement in resolution would not justify the time involved or the possible losses in lipid material that could occur.

H. LIPID HYDROLYSIS AND IDENTIFICATION OF PRODUCTS

The method of hydrolysis used was essentially that of Willemot and Boll (1967). A total lipid extract, or lipid eluted off silicic acid impregnated paper, was dried down in a stream of nitrogen and refluxed for 105 min in 3 ml 3 N HCl at 100°C on a steam bath. The samples were then partitioned against diethyl ether (sometimes saving the ether extract for counting). The aqueous phase was chromatographed on Whatman #1 paper in two dimensions: first direction for 12 hr in butanol:acetic acid:water, 5:3:1 (v:v:v) and second direction for 13 hr in butanol:95% ethanol:water, 21:17:12 (v:v:v). Hydrolysis products were identified by staining reactions and by position of standards and unknowns relative to the position of the dye Tropaeoline-ooo.

The above chromatographic procedure was also used to assay for water soluble components in the Bligh and Dyer upper phase.

I. MEASUREMENT OF RADIOACTIVITY

The preliminary studies on the incorporation of labelled

lipid precursors by coleoptiles involved counting aliquots of aqueous media or lipid extracts dried onto aluminum planchettes. Counting was done, using a Nuclear Chicago Model 470 system, under "Q" gas. The efficiency of counting ^{14}Carbon was 24.6%.

Spots cut from chromatograms (Bartley and Abraham, 1965) were placed in 20 ml scintillation vials which were then filled with 15 ml of 0.4% Omnifluor (98% PPO and 2% Bis-MSB, New England Nuclear Corporation, Boston, U.S.A.) in toluene. The vials were counted in a Beckman CPM-100 Scintillation Counter at a gain setting of 350. Channels were selected using Beckman Isosets. The efficiency was about 75% for ^{14}Carbon counted in the $^{3}H+^{14}C$ channel with a background of 32 cpm. The validity of this counting technique was confirmed by tests showing that: a) no labelled lipids dissolved in the scintillation fluid from the silicic acid impregnated paper, b) there was no detectable quenching due to the Rhodamine 6G stain used to locate lipids on the chromatograms, and c) the counting efficiency was not affected by the size of the spot on which the labelled lipids appeared or by placing additional chromatography paper in the vial.

In experiments involving the counting of $^{14}CO_2$ the labelled substrate was preincubated over 1N NaOH for 1 hr to remove dissolved CO_2 (see Kanfer and Kennedy, 1964). Carbon dioxide was collected in polypropylene center wells of Reaction Flasks (Kontes Glass Co., Vineland, N.J., U.S.A.) containing 0.2 ml of Packard Hydroxide of Hyamine and a piece of filter paper. The center wells were suspended from rubber stoppers

(Kontes Glass Co.) which fitted tightly on the 13.5 ml Polyallomer ultracentrifuge tubes used for incubation as noted above (Sect II.C.). At the end of the incubation period 1% TCA was added to the incubation medium, through the rubber stopper, bringing the volume to 2.0 ml. The reaction mixture was shaken for an additional 30 min to attain equilibrium. The center well and contents were then dropped into 15 ml of 0.4% Omnifluor in toluene and counted in a Packard TRI-CARB Spectrometer at an efficiency of about 72%. Refrigerated counting was an absolute necessity when using Hydroxide of Hyamine, or any organic quaternary ammonium base, because it decreased the very high background due to photoactivation and presumably, subsequent phosphorescence.

Duncombe and Rising (1969) have shown that organic CO_2 trapping agents can cause gross interference of metabolic processes. To test this possibility Hydroxide of Hyamine was injected into center wells of individual tubes both at the end as well as at the beginning of the incubation period. The results revealed no significant differences in the levels of $^{14}CO_2$ collected.

For double label experiments a gain of 300 produced about 0.5% spillover of tritium counts into the ^{14}Carbon channel. The efficiency of ^{14}Carbon counted on paper in the ^{14}C channel (Isoset) was 57.4% with a background of 15 cpm. The efficiency of tritium counted on paper was 14.7% with a background of 15 cpm. CPM due to tritium were calculated from the number of counts in the ^{3}H channel minus the proportion of ^{14}C counts

that fell into the ^{3}H channel. This method of counting, using a solid support (Whatmann 3mm) along with double isotope discrimination settings, has recently been criticized by Bransome and Grower (1970). The relatively large size of phospholipid molecules, and possibly their surface active properties, may keep them from penetrating far into the Whatmann 3mm matrix where their activity could be considerably quenched.

J. LIPID PHOSPHORUS DETERMINATION

Phosphorus was determined by the method of Rouser et al. (1966). Lipids dissolved in chloroform were pipetted into 30 ml Kjeldahl digestion flasks and dried down in an oven at about 80°C. Nine-tenths ml of 72% perchloric acid was added to each flask and heated to give very gentle refluxing for 20 min (Rhee and Dagon, 1967). Flasks were cooled and the walls washed down with 7.0 ml water. One ml of 2.5% ammonium molybdate solution followed by 1.0 ml 10% ascorbic acid solution was added to the flask. The total contents of each flask were then transferred to test tubes, for convenience, and heated for 5 min in a boiling water bath. Twenty min after heating, the optical density was read at 820 nm on a Unicam SP 800B against a suitable blank. The range of this determination was 0.5-6.0 ug phosphorus per 9.9 ml reaction mixture. A standard curve was run with each group of determinations using K_3PO_4, K_2HPO_4 or KH_2PO_4. Replicated determinations on known quantities of phosphorus rarely differed by more than 3%.

K. RNA DETERMINATION

Protein and nucleic acid were determined by modifying the method used by Davies (1968). Protein and nucleic acid were precipitated by taking an aliquot of suspended material, adding an equal volume of 10% (w/v) trichloroacetic acid (TCA) and centrifuging for at least 10 min in a clinical centrifuge (top speed) at $4^{o}C$. The supernatant was poured off and the pellet was washed with cold 5% TCA, centrifuged, washed with cold acetone and centrifuged. The supernatants were discarded. Precipitated nucleic acid was extracted from the pellet at $70^{o}C$ during two-one hr periods each time with 2.0 ml of 0.5 N perchloric acid. These two extracts were combined and RNA was estimated by measuring $OD_{260}-OD_{280}$ values on a Unicam SP 800B. Standardcurves were run using yeast RNA. The range of determination was approximately 0.020-0.180 mg RNA per 4.0 ml extraction volume.

L. PROTEIN DETERMINATION

Protein was determined by the method of Lowry et al. (1961) on TCA precipitates produced as above (Sect II.K.). Protein was dissolved in 0.5 ml of 1 N NaOH. The determination was performed by adding 5.0 ml of solution D, waiting 10 min and adding 0.5 ml of solution E with rapid mixing. After 20 min the O.D. was read at 500 nm on a Unicam SP 800B against an appropriate blank. A standard curve, based on known amounts of crystalline bovine serum albumin (BSA), was run along with each set of determinations. The range of determinations was

0.005-0.060 mg of BSA per 6.0 ml reaction mixture.

M. LIST OF COMMERCIAL SOURCES FROM WHICH CHEMICALS WERE OBTAINED

Buffers	
N-Tris(hyroxymethyl)methyl-glycine (Tricine)	Calbiochem Los Angeles, Calif., U.S.A.
N,N-Bis(2-hydroxyethyl)-glycine (Bicine)	"
Inhibitors	
N-ethylmaleimide	Mann Research Labs., Inc. New York, N.Y., U.S.A.
p-chloromercuribenzoate	"
Lipids	
L-α-lecithin(dipalmitoyl, synthetic)	General Biochemicals, Inc. Chagrin Falls, Ohio, U.S.A.
Phosphatidylinositol	"
Phosphatidylethanolamine	Nutritional Biochemicals Co. Cleveland, Ohio, U.S.A.
Phosphatidylserine	"
Nucleotides	
Adenosine-5'-Triphosphate, disodium, $\cdot 4H_2O$, M.A.	Mann Research Laboratories Inc., N.Y., N.Y., U.S.A.
Cytidine-5'-Triphosphate, trilithium, $\cdot 6H_2O$, M.A.	"
Radioactive Chemicals	
All	New England Nuclear Corp. Boston, Mass., U.S.A.

Standards for Assays

Albumin, bovine serum, A grade	Calbiochem Los Angeles, Calif., U.S.A.
Ribosenucleic acid (yeast) soluble, A grade	"

III. RESULTS

A. PRELIMINARY EXPERIMENTS

1. Identification of oat coleoptile lipids on silicic acid impregnated paper

A major portion of this work involves the radioactive counting of specific lipid spots that appear on silicic acid impregnated paper after chromatography. It was thus important to determine what type of lipid each spot represented paying careful attention to the relative location of PC, PS and PE. The following techniques were used for identification of lipid spots:

a) lipid staining reactions with Rhodamine 6G (Marinetti, 1962; modified), ninhydrin for amino-containing lipids (Marinetti, 1962), modified Dragendorff's reagent for choline (Bregoff et al., 1953) and periodate-schiff reagent for vicinal glycol groups (Kates, 1967),

b) co-chromatography with commercially available lipid standards (see Sect II.M.),

c) phosphorus and sulfur containing lipids located by radioautography of chromatograms; lipids were heavily labelled with $Na_3{}^{32}PO_4$ or $Na_2{}^{35}SO_4$ (Kates, 1967),

d) prefractionation of the lipid extract into acetone soluble and acetone insoluble material (Haverkate and Van Deenen, 1965) or on a column of

silicic acid prior to chromatography on silicic acid impregnated paper and

e) spots eluted from silicic acid impregnated paper subjected to acid hydrolysis and the resulting water bases chromatographed along with standards.

The following oat coleoptile microsomal polar lipids were identified: PC, PE, PS and PA. PI, monogalactosyldiglyceride and digalactosyldiglyceride may also be present. The polar lipid composition of whole coleoptiles was essentially the same as that of the microsomal fraction except that sulfolipid (believed to be sulfoquinovosyl diglyceride) was present. The galactolipids appeared, visually, to be in higher concentration, relative to the other lipid components, than in the microsomes. Phosphatidylglycerol may also be present in the microsomal fraction.

2. Feeding of labelled lipid precursors to coleoptiles

The incorporation of various radioactive lipid precursors by coleoptiles was briefly studied. Some of this work involved the same experiments used to identify lipid components (Sect III.A.1.).

L-serine-U-^{14}C was fed to whole coleoptiles and its appearance in various lipids was detected by radioautography of the silicic acid impregnated paper chromatogram. Activity was first detected in neutral lipid after 1 hr of incubation whereas activity in phospholipid appeared after 2 hr. It was not determined which parts of the phospholipid molecules were labelled.

When feeding L-serine-U-^{14}C, galactose-1-^{14}C, $Na_2{}^{35}SO_4$ or $Na_2{}^{32}PO_4$ it was noted that changes in the amount of incorporation over a 24 hr period differed for each precursor. L-serine-U-^{14}C showed little further increase in incorporation into lipid after the first 12 hr of incubation. Galactose-1-^{14}C and $Na_3{}^{35}PO_4$ incorporation increased steadily from 0-24 hr. The activity from $Na_2{}^{35}SO_4$ appeared more rapidly during the second 12 hr of incubation, than during the first 12 hr in lipid. It was noted that for each precursor the loss of activity from the incubation medium with time always correlated qualitatively with its appearance in the lipid extract.

Incorporation of $Na_3{}^{32}PO_4$ into lipids under a 60 watt incandescent lamp was about 25% greater than a dark incubated control. Thus all feeding to coleoptiles was done in total darkness.

The lipid phosphorus content (ug per gm fresh weight) of coleoptiles decreased rapidly with incubation, about 20% being lost during the first 4 hr of incubation. The rate of loss decreased with further incubation up to 24 hr (fresh weight measured at time zero only).

3. Replication of microsomal preparations

The first quantitative work undertaken here on the incorporation of L-serine-U-^{14}C into microsomal phospholipids revealed great differences between replicates.

To test the possibility that the pellets were of different sizes lipid phosphorus was measured in each pellet after

ultracentrifugation. No pellet differed by more than 6% from the average lipid phosphorus content of all pellets determined. Thus the pellets in each tube were sufficiently similar to indicate that the variability in L-serine-U-^{14}C incorporation was due to some other factor.

It was found that good replicates of incorporation could be obtained if pellets were resuspended in the Polyallomer ultracentrifuge tubes using an extremely close fitting teflon pestle. The pestle was spun at 833 rpm for 10 sec during which time two passes into the microsomal pellet and incubation (resuspending) medium were made.

The necessity for this method of resuspension is emphasized by the fact that: a)Inadequate resuspension produced poor replications, b)incubation of whole pellets (not resuspended) resulted in good replications but low levels of incorporation and c)resuspension by the above method gave good replications and good incorporation levels, therefore apparently producing a uniformly suspended pellet.

4. Hydrolytic action during extraction procedures

To determine whether or not precautions should be taken to prevent the possible stimulation of phospholipase D action during extraction procedures (Kates, 1957), the following experiment was performed.

Microsomal pellets, incubated for 1 hr at 25°C were extracted by the method of Bligh and Dyer (1959), half of them receiving a 100°C, 5 min heat treatment (Bartels and Van Deenen, 1966) just prior to extraction. The extracts were

chromatographed, PA was eluted and its phosphorus content determined. PA decreased from 0.30 to 0.23 µg lipid phosphorus per pellet or 6 to 4.6% of total pellet lipid phosphorus as a consequence of the heat treatment. Because of such small changes in PA concentration it was considered unnecessary to use a routine enzyme killing procedure prior to extraction. However, it is possible that during extraction phospholipase D action resulted in the formation of phosphatidylmethanol (Yang et al., 1967). In this respect it was noted that during the above experiment the concentration of PC, PS and PE, on a visual basis, did not change, nor was there formation of a new spot (see Douce et al., 1966) when comparing the lipids from heat treated and untreated pellets.

It was noted that in the presence of ethyl ether (0.5 ml added to a 1.0 ml reaction mixture) microsomal pellets incubated for 1 hr (ether dried off before extraction; no heat treatment) showed an increase in PA concentration to 0.75 µg lipid phosphorus per pellet from 0.30 µg (of the non-ether containing control). This may indicate that ether stimulates PA formation probably via increased phospholipase D activity.

5. Development of the oat coleoptile

Some aspects of the development of the oat coleoptile were investigated by progressively harvesting coleoptiles that had attained a length of 10, 20, 30, 40 or 50 mm and performing the necessary assays. After 30 mm there is a high percentage of coleoptiles in which the first leaf had emerged. These coleoptiles were never selected as the effect of emergence on

coleoptile development was not known.

(a). Developmental changes in the whole coleoptile

Changes in fresh and dry weight were studied and the results are presented in Figure 1. The change in percent water, also shown, is taken into account during the extraction of lipids by the method of Bligh and Dyer (1959).

Figure 2 shows the change in lipid phosphorus per gm fresh weight and per coleoptile. The measurement of lipid phosphorus was considered to be a measurement of phospholipid content. Throughout development total coleoptile phospholipid decreased from 3.9% of dry weight at 10mm to 1.9% at 50 mm (calculated by assuming an average molecular weight of 750 for phospholipids.). Ferrari and Benson (1961) noted that Chlorella phospholipids were 5.5% of dry weight. It was noted (data not presented) that the concentration of PC, PS and PE, as percent of total lipid phosphorus, remained relatively constant throughout development at about 31, 4 and 17%, respectively. In another study it was found that coleoptiles approximately 25 mm long had about 30% more phospholipid on a gm fresh weight basis in the tip (upper 2 mm) than in the remainder of the coleoptile. Whether this relationship changed with further development was not studied.

Changes in protein and RNA are presented in Figure 3. The decrease in RNA content as the coleoptile approaches 50 mm in length should be noted.

Figure 1. Changes in fresh weight, dry weight and percent water of the whole coleoptile with increasing length of the coleoptile

At any one length three determinations were made using 20 coleoptiles per determination. The lines join the means of the three determinations.

○ DRY WT / COLEOPTILE (mg)
● FRESH WT / COLEOPTILE (mg x 10⁻¹)
4.0
3.0
2.0
1.0
0
10
20
30
40
50
% WATER
95
94
93
92
0
10
20
30
40
50
COLEOPTILE LENGTH (mm)

Figure 2. Changes in lipid phosphorus content of the whole coleoptile with increasing length of the coleoptile.

The results are expressed on a gram fresh weight and a per coleoptile basis. At any one length 3 determinations were made (2 at 30 mm) using 20 coleoptiles for each determination. Each determination is itself the average of 3 phosphorus determinations on aliquots of any one extract. The lines join the means of the determinations.

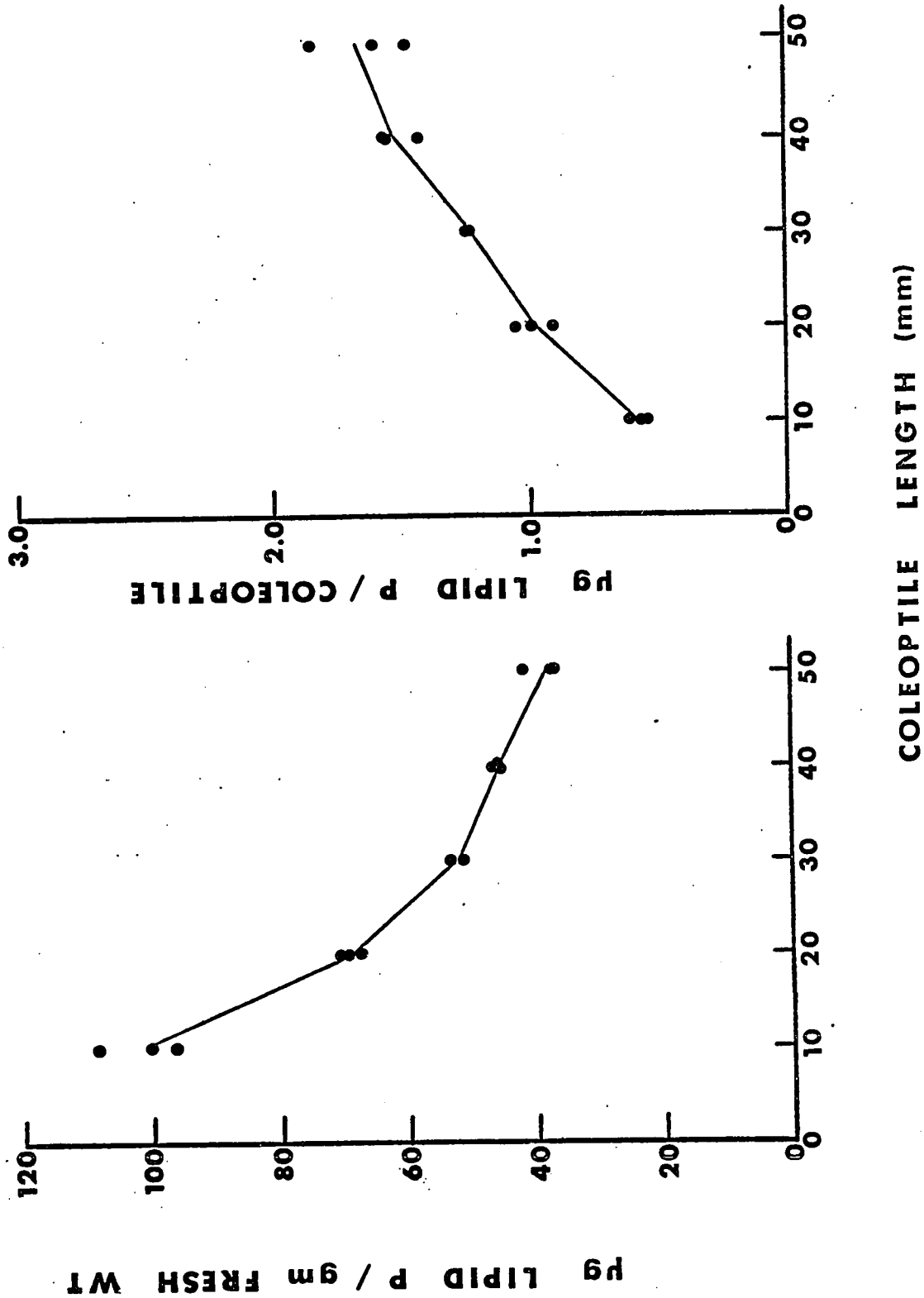
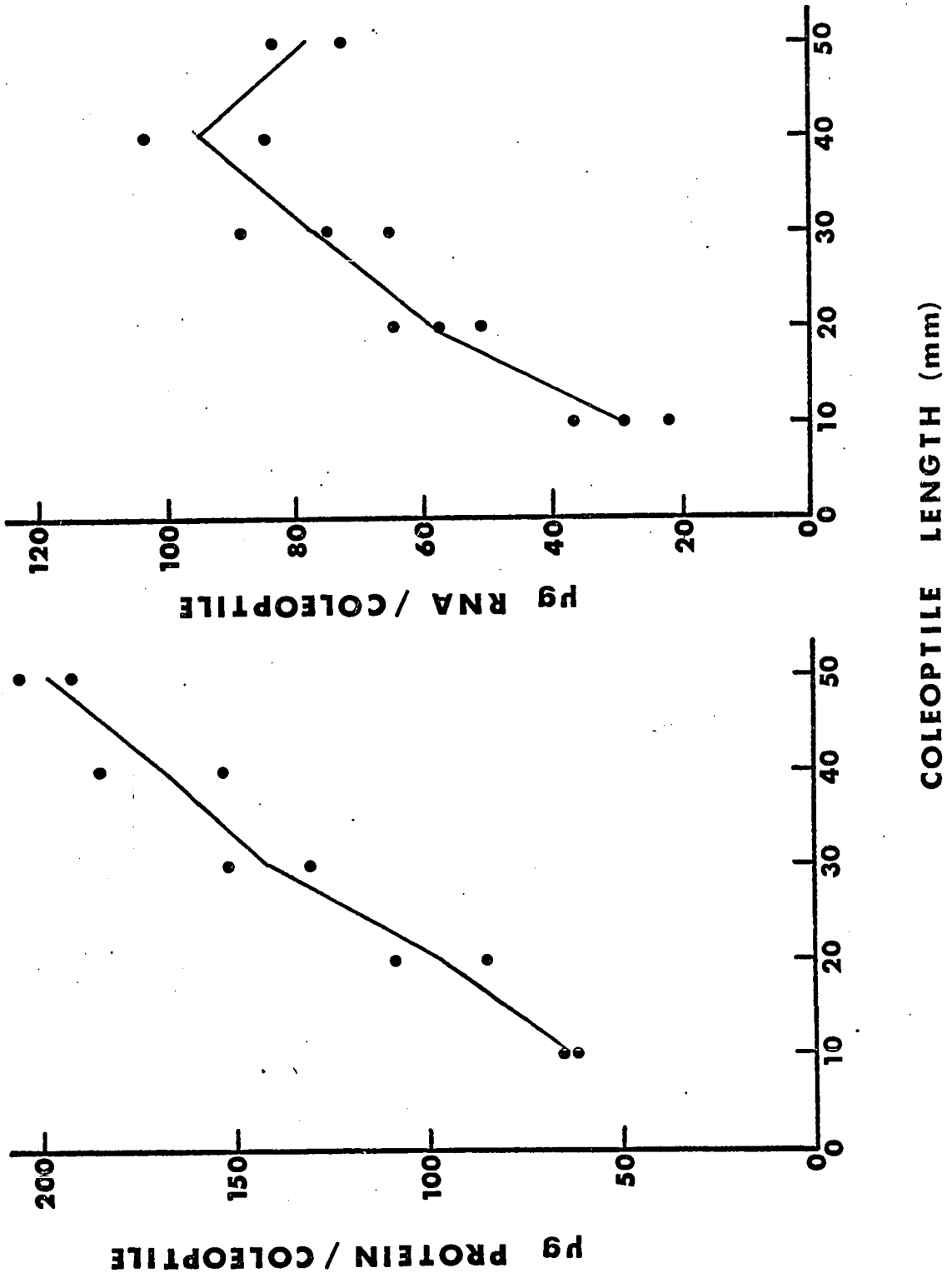

µg LIPID P / gm FRESH WT
120
100
80
60
40
20
0
µg LIPID P / COLEOPTILE
3.0
2.0
1.0
0
10
20
30
40
50
COLEOPTILE LENGTH (mm)

Figure 3. Changes in protein and RNA content of the whole coleoptile with increasing length of the coleoptile.

Two or three determinations were made at any one length. Thirty coleoptiles at each length were homogenized in 30 ml of 0.2 M borate-boric acid buffer, pH 8.0. TCA precipitates were made on 2 ml aliquots of the homogenates. RNA was determined on perchloric acid soluble material from trichloroacetic acid precipitates. Protein was determined on aliquots of a trichloroacetic acid precipitate dissolved in 0.5 ml of 1N NaOH. The lines join the means of the determinations.


µg RNA / COLEOPTILE
0
20
40
60
80
100
120
µg PROTEIN / COLEOPTILE
0
50
100
150
200
COLEOPTILE LENGTH (mm)
0
10
20
30
40
50

(b). Developmental changes in the microsomal fraction

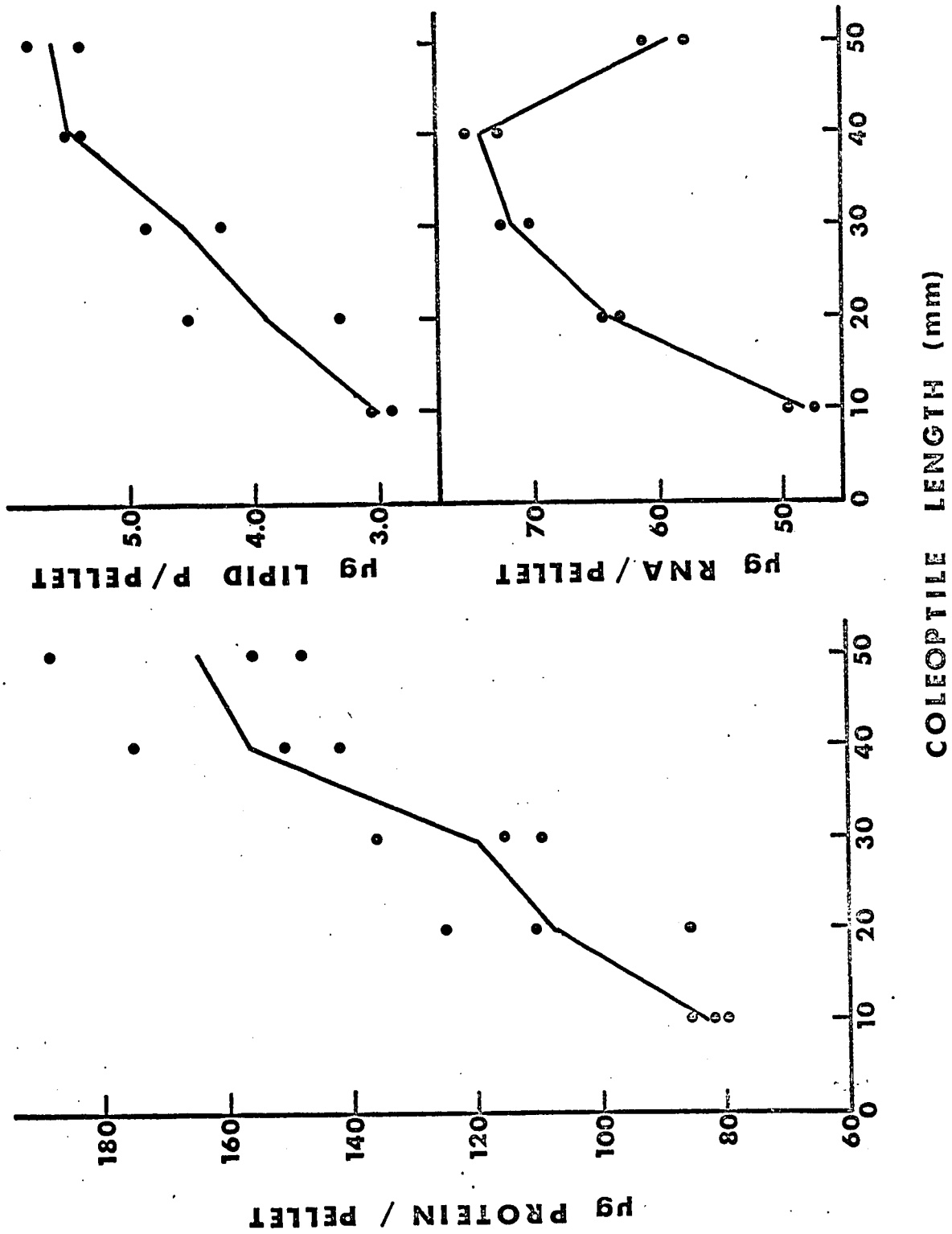
The results of one study on the development of the microsomal fraction is presented in Figure 4. The decrease in RNA content between 40 and 50 mm is somewhat more pronounced in the microsomal fraction than in the whole coleoptile (Fig 3). Microsomal protein and phospholipid phosphorus increased throughout the growth of the coleoptile. If RNA to protein ratios are plotted against increasing coleoptile length from which microsomal pellets were derived (plot not presented), both the magnitude of the ratios and its decrease with age are in close agreement with the data obtained from pea root developmental studies (Loening, 1961).

Masuda et al. (1966) found that ribosomes do not increase in the growing coleoptile and decrease only when the coleoptile is fully grown. It can be assumed that the RNA assayed here was largely ribosomal. No explanation can be offered for the increase with development noted here since the procedure for formation of the microsomal fraction used here was almost identical to that used by Masuda et al. (1966).

Setterfield (1961) has shown an increase of vesicular material in microsomal pellets with late coleoptile development. Setterfield (1961) and Masuda et al. (1966) have shown a corresponding increase in membrane-bound ribosomes as coleoptiles become fully elongated. It was hoped that the determination of phospholipid in expanding coleoptile microsomes would detect this late increase in membrane material. The data show however, a steady increase throughout development.

Figure 4. Changes in protein, RNA and lipid phosphorus content of the microsomal pellet with increasing length of the coleoptile.

Coleoptiles were homogenized in 0.2 M borate-boric acid buffer, pH 8.0, and microsomal pellets were produced. One pellet contains microsomal material obtained from 10.4 coleoptiles. Trichloroacetic acid precipitates were formed from 5 pellets. Three of these pellets were used for protein determination and two for RNA determination. Lipid phosphorus was determined on two separate pellets. The lines join the means of the determinations.


µg PROTEIN / PELLET
µg LIPID P/ PELLET
µg RNA / PELLET
COLEOPTILE LENGTH (mm)

An attempt was made to separate free and membrane-bound ribosomes on discontinuous sucrose gradients. The object of this study, based on the findings of Setterfield (1961), was to eventually correlate membrane composition and lipid biosynthetic activity with increasing numbers of membrane-bound ribosomes. The technique of Blobel and Potter (1967) was used for fractionation of the microsomes. However, the common technical problem of gelling of the preparations on the addition of sodium deoxycholate was never solved.

B. INCORPORATION OF PHOSPHOLIPID PRECURSORS INTO MICROSOMAL PHOSPHOLIPIDS

1. Buffers and pH

The incorporation of L-serine-U-^{14}C and ethanolamine-1,2-^{14}C into microsomal phospholipids as a function of pH is presented in Figures 5 and 6, respectively. Optimum incorporation occurred at pH 8.0 (serine) and pH 7.6 (ethanolamine).

In screening for a proper buffer L-serine-U-^{14}C was supplied to microsomes in either 0.1 M phosphate, 0.05 M Tris (Tris(hydroxymethyl)aminomethane)-HCl, 0.2 M boric acid-borax (0.2 M in terms of borate), 0.05 M Tricine (N-tris(hydroxymethyl)methylglycine)-NaOH, 0.05 M Tricine-KOH or 0.05 M Bicine (N,N-Bis(2-hydroxyethyl)glycine)-NaOH. The optimum pH for incorporation of L-serine-U-^{14}C into microsomal phospholipids was about 8.0 in all buffers tested; pH optima were not determined for Tricine-KOH and Bicine-NaOH.

At pH 8.0 incorporation of L-serine-U-^{14}C was about 20%

Figure 5. The incorporation of L-serine-U-^{14}C into microsomal phospholipids as a function of pH.

The incorporation of L-serine-U-^{14}C (0.5 μCi; 123 mCi/mM, 50 μl) into the microsomal phospholipids PC+PS+PE was determined at various pHs buffered by 0.05 M Tricine-NaOH. Each three points at each pH tested represent two determinations and the mean.

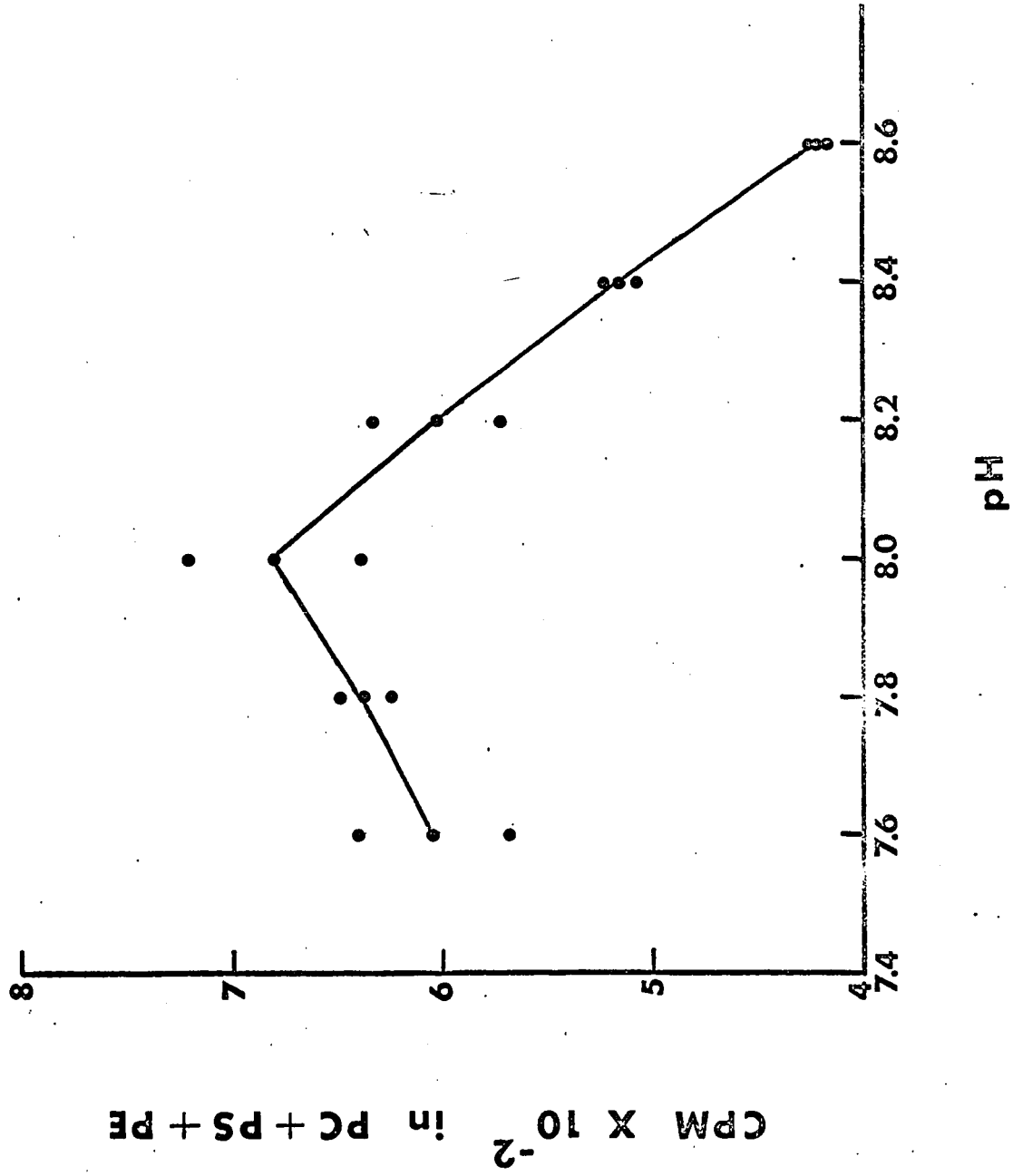
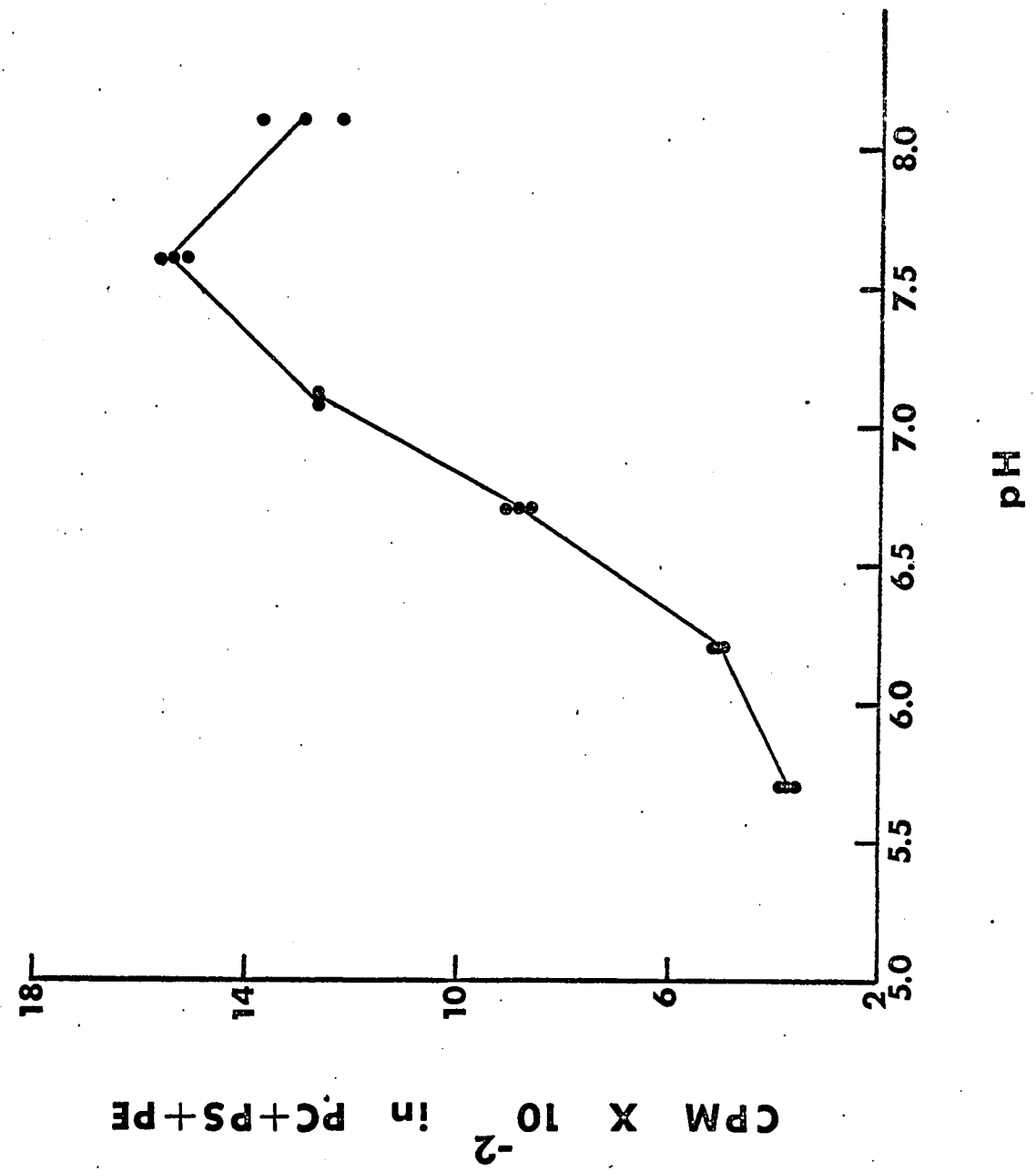

CPM X 10-2 in PC + PS + PE
8
7
6
5
4
7.4
7.6
7.8
8.0
8.2
8.4
8.6
pH

Figure 6. The incorporation of ethanolamine-1,2-^{14}C into microsomal phospholipids as a function of pH.

The incorporation of ethanolamine-1,2-^{14}C (1.0 μCi, 1.5 mCi/mM) into the microsomal phospholipids PC+PS+PE was determined at various pHs buffered by 0.05 M Tricine-NaOH. Each three points at each pH tested represent two determinations and the mean..


CPM X 10^-2 in PC+PS+PE
18
14
10
6
2
5.0
5.5
6.0
6.5
7.0
7.5
8.0
pH

higher using 0.05 M Tricine-NaOH buffer as compared with 0.2M borate. It was also noted that borate altered the pH of the homogenizing medium presumably because of its ability to complex with sucrose (see Good et al.,1966).

Incorporation using 0.05 M Tricine-NaOH at pH 8.0 was at least 100% higher than with 0.05 M Bicine-NaOH.

On theoretical grounds phosphate buffer was considered undesirable because pH 8.0 is outside its effective buffering range; however, no changes in pH of a phosphate buffered reaction mixture were found during a 1 hr incubation period. It has subsequently been found (Antia et al., 1970) that phosphate buffer inhibits 50-70% of phospholipase D action as compared with activity in the zwitterionic buffers of Good et al. (1966).

There were indications that Tris-HCl may inhibit serine and ethanolamine incorporation. Tris buffer has been found to be a competitive inhibitor of the calcium stimulated incorporation of ethanolamine into phosphatides of the larval fat body of Musca domestica (Crone, 1966). Good et al. (1966) state that Tris is "a primary aliphatic amine of considerable reactivity and consequently it is often inhibitory". The main reason for rejecting Tris-HCl was its ability to form Schiff bases with amino acids (Matsuo, 1967), a property which I wanted to avoid because of an interest in the decarboxylation of PS and the role of PALP.

From the above results and considerations it was concluded that Tricine-NaOH is the most suitable buffer for the study of

the incorporation of L-serine-U-^{14}C into phospholipids of oat coleoptile microsomes. For further studies pH 8.0 was chosen for both serine and ethanolamine incorporation. The difficulties involved in finding such small differences in pH optima indicated that they may not be truly different. Using the same pH, and hence the same buffer for both substrates, would eliminate a possible source of error.

Buffering by 0.05 M Tricine-NaOH at pH 8.0 was considered sufficient. Occasional pH measurements at the end of incubations were always the same as the incubation medium pH before addition to the microsomal pellet.

2. Calcium chloride dependent incorporation

The effects of varied $CaCl_2$ concentration on the incorporation of L-serine-U-^{14}C and ethanolamine-1,2-^{14}C into microsomal phospholipids are shown in Figures 7 and 8. Optimum $CaCl_2$ concentration was about 0.006 M for both bases. Higher or lower levels of $CaCl_2$ resulted in lowered incorporation. All experiments, except where otherwise noted, were carried out at a $CaCl_2$ concentration of 5.0 µmoles per 1.025 ml total incubation volume. This choice followed from early experiments using borate buffer where the optimum concentration was found to be about 0.005 M.

The $CaCl_2$ requirement for incorporation of L-serine-U-^{14}C is illustrated by the results of an early experiment (using 0.2 M borate buffer) presented in Table 1. EDTA, 0.002 M, almost completely inhibited incorporation, presumably by binding all endogenous $CaCl_2$. The over titration of EDTA with

Figure 7. The incorporation of L-serine-U-^{14}C into microsomal phospholipids as a function of $CaCl_2$ concentration.

Microsomal pellets were fed L-serine-U-^{14}C (0.25 µCi; 123 mCi/mM). Incorporation into the microsomal phospholipids PC+PS+PE was determined. Each three points at each concentration tested represent two determinations and the mean. The results are representative of those found in three similar experiments.

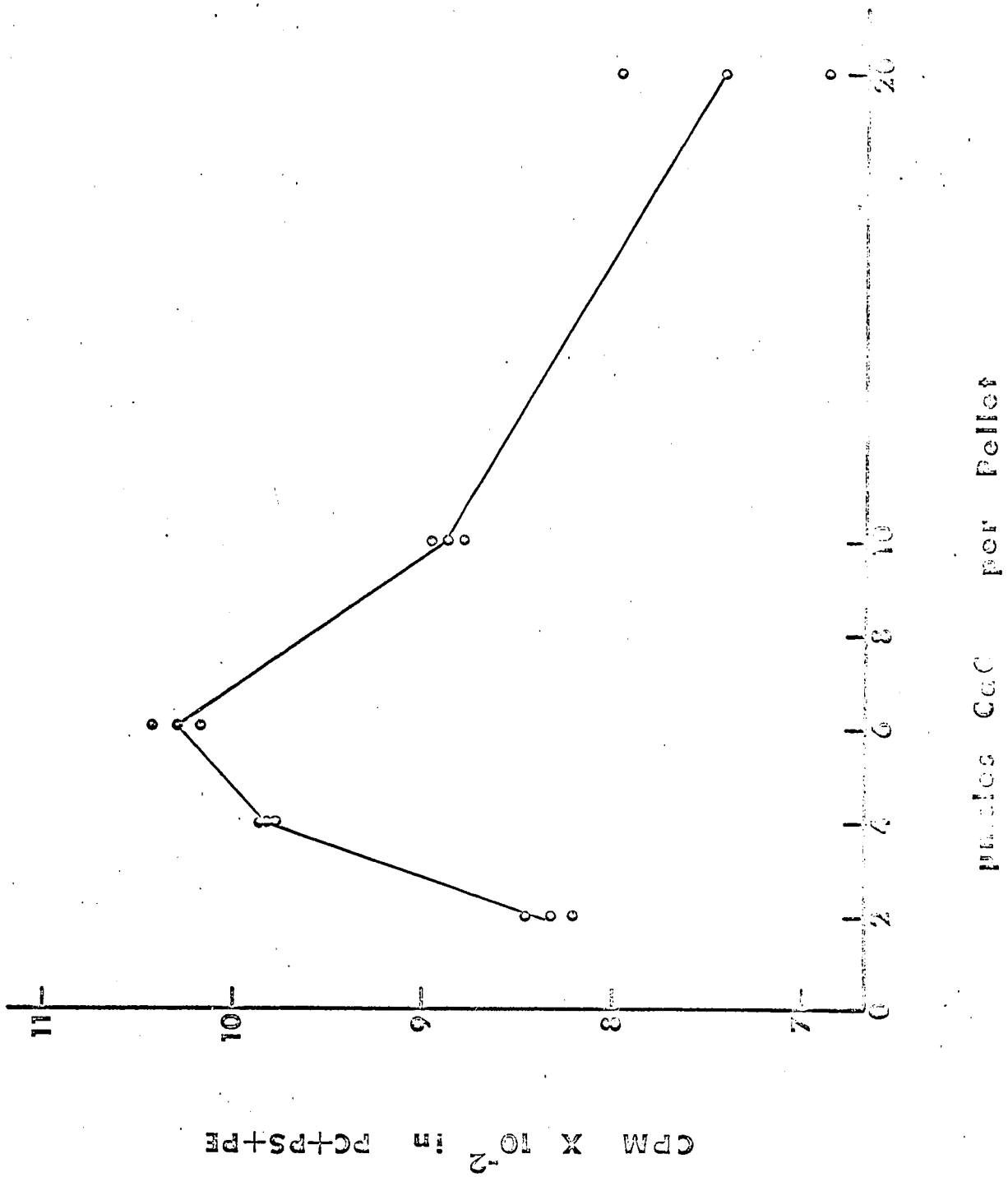

CPM X 10⁻² in PC+PS+PE
11
10
9
8
7
0
2
4
6
8
10
20
Micrograms CaCl per Pellet

Figure 8. The incorporation of ethanolamine-1,2-^{14}C into microsomal phospholipids as a function of $CaCl_2$ concentration.

Microsomal pellets were fed ethanolamine-1,2-^{14}C (0.5 µCi; 1.85 mCi/mM). Incorporation into microsomal PE was determined. Each three points at each concentration tested represent two determinations and the mean. This experiment was not repeated.

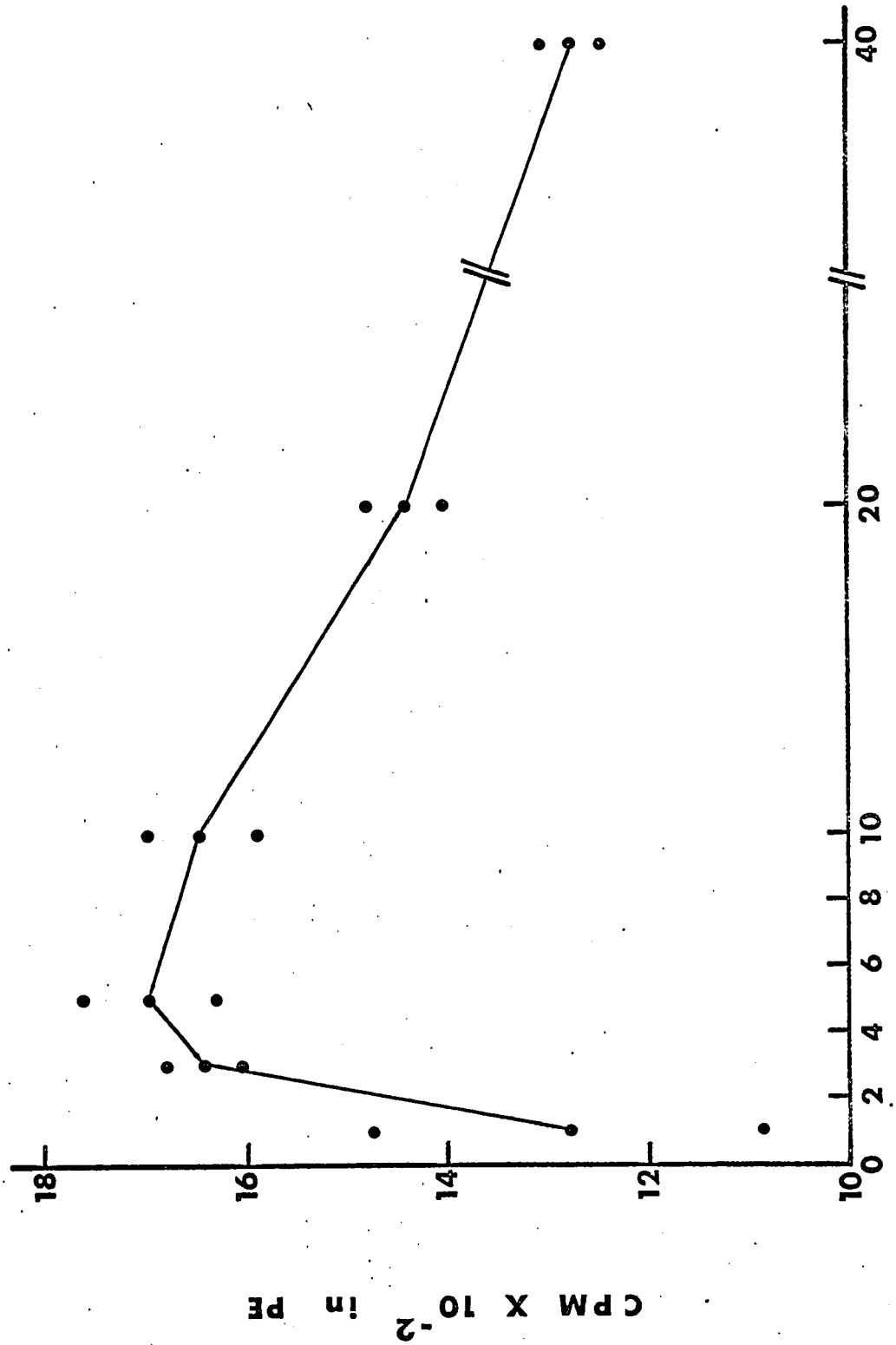

CPM X 10^{-2} in PE
18
16
14
12
10
0
2
4
6
8
10
20
40
μmoles $CaCl_2$ per Pellet

Table 1. The effect of EDTA and $CaCl_2$ on the incorporation of L-serine-U-^{14}C

Six microsomal pellets were resuspended in 0.2 M boric acid-borax buffer (0.2 M in terms of borate) at pH 8.0. Each pellet was fed L-serine-U-^{14}C (0.25 µCi; 122 mCi/mM) and the additions noted.

Pellet	Addition per pellet	cpm in PC+PS+PE	percent of pellets #1 and #2
1	5.0 µmoles $CaCl_2$	868	100
2	"	831	
3	2.0 µmoles EDTA	12	1.8
4	"	17	
5	2.0 µmoles EDTA + 7.0 µmoles $CaCl_2$	765	92
6	" "	795	

$CaCl_2$ restored incorporation to expected levels. When serine or ethanolamine were incubated in 0.05 M Tricine buffer (pH 8.0), 0.002 M EDTA also had the same inhibitory effect.

The optimum $CaCl_2$ concentration for choline-^{14}C-chloride incorporation was never tested. It was noted that at pH 8.0 choline incorporation, in the presence of 0.005 M $CaCl_2$ increased only 11% over a control lacking exogenously added $CaCl_2$. Also radically different from serine and ethanolamine incorporation was the effect of 0.005 M EDTA on choline incorporation; it caused a 33% decrease in incorporation. Higher levels of $CaCl_2$ or EDTA were not tested.

Oat coleoptile microsomes showed the same optimum $CaCl_2$ concentration for serine and ethanolamine incorporation even though they were fed at very different concentrations. This suggests that optimal Ca^{++} concentration is a function of lipid substrate or enzyme concentration. Other workers believe that optimum Ca^{++} concentration is directly related to the concentration of lipid substrate (see Sect I.B.5.). Davidson and Long (1958) found that for optimum phospholipase D action the ratio was 15 µmoles Ca^{++} ions/µmole ovalecithin. Results obtained here give a ratio of 6.0 µmoles Ca^{++} ions/4.3 µg lipid phosphorus (lipid phosphorus content of one microsomal pellet) or about 40 µmoles Ca^{++} ions/µmole phospholipid. The fact that these values are of the same magnitude may indicate similarities between phospholipase D action and the incorporation of serine and ethanolamine studied here. However, it may only be a reflection of total phospholipid binding of cations since

the ratio calculated here is based on total microsomal phospholipid and not lecithin alone. The ratio may possibly be altered by the classes of phospholipids present or by organic solvents (Seeman et al., 1971); Davidson and Long (1958) included ether in their reaction mixture.

3. <u>The effect of serine or ethanolamine concentration on their incorporation into microsomal phospholipids</u>

The effect of L-serine-U-^{14}C concentration its incorporation into the microsomal phospholipids PC+PS+PE is shown in Figure 9. The K_m for L-serine is about 0.2 mM.

The effect of ethanolamine-1,2-^{14}C concentration on its incorporation into the microsomal phospholipid PE is shown in Figure 10. The K_m for ethanolamine is about 0.4 mM.

A rectilinear relationship was found between microsomal concentration and amount of incorporation of L-serine-U-^{14}C. Heat treated microsomes, 5 min at 100°C, did not incorporate L-serine-U-^{14}C.

All work presented here was done in the presence of limiting substrate. Rapid counting of samples to about 1% error (2σ) required feeding of labelled substrates at relatively high specific activity. The feeding of high specific activity material at enzyme saturation levels was prohibited by its high cost. Most of the experiments presented in this thesis cost approximately $2 each in labelled serine or ethanolamine. To obtain a similar amount of activity incorporated, at enzyme saturation levels, the cost would be a minimum of $50 per experiment. None of the conclusions reached in this work require that enzyme

Figure 9. The effect of L-serine-U-^{14}C concentration on its incorporation into the microsomal phospholipid PC+PS+PE.

L-serine-U-^{14}C was fed at a specific activity of 137 mCi/mM. Each point represents one determination. s = mM X 10^{-1} L-serine-U-^{14}C fed; v = µmoles X 10^{-4} L-serine-U-^{14}C incorporated into PC+PS+PE per hour. The line through the points was fitted using standard regression techniques which gave the following results:

Intercept = 0.214 X 10^{-4} µmoles^{-1} hr

Slope = 0.403 X 10^{3} µmoles^{-1} hr·mM

± 0.010 X 10^{3} (Standard error of the regression coefficient)

The regression coefficient was significant at the 0.01 level.

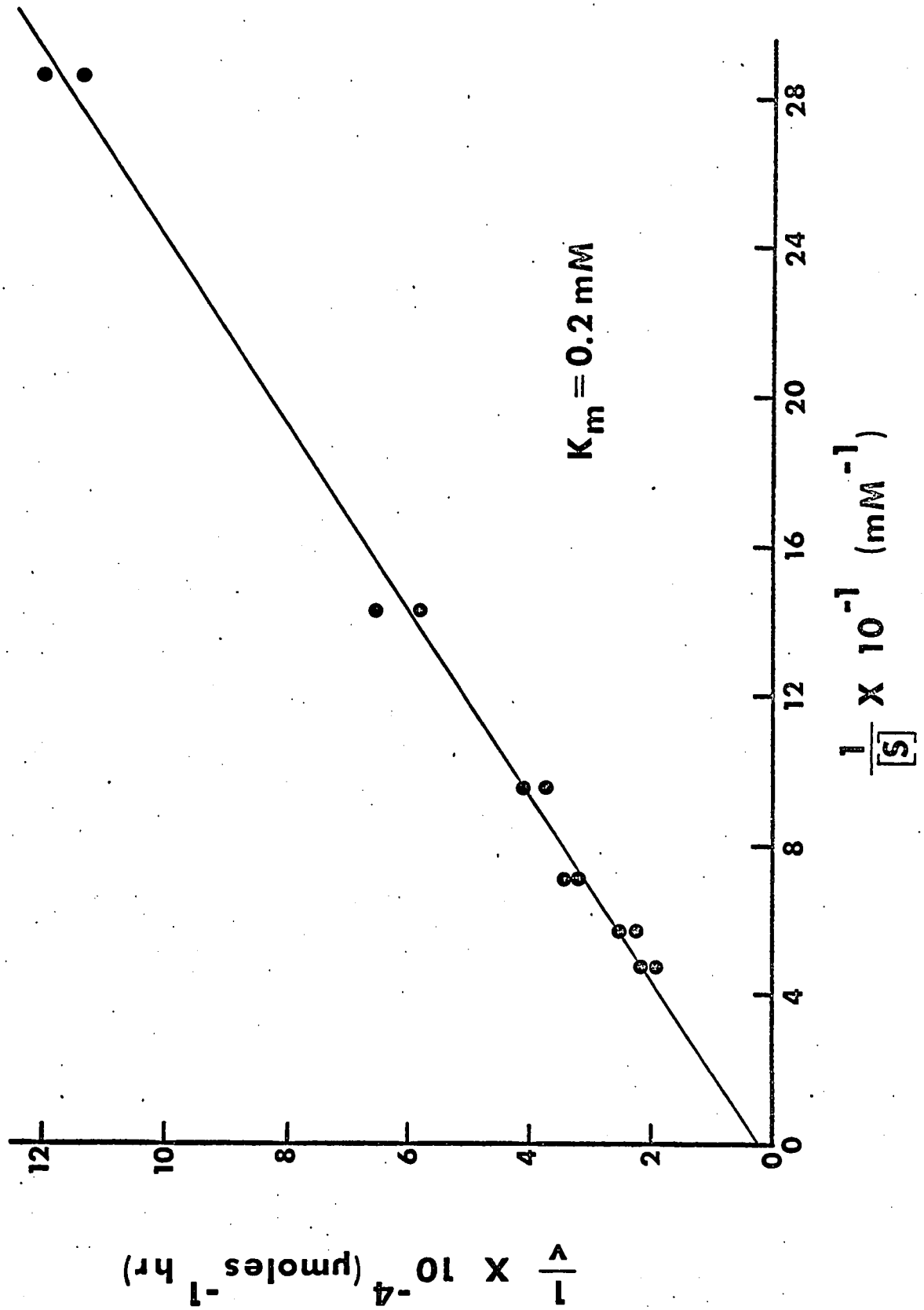

$\frac{1}{v}$ X 10^{-4} (μmoles^{-1} hr)
0
2
4
6
8
10
12
$K_m = 0.2$ mM
0
4
8
12
16
20
24
28
$\frac{1}{[S]}$ X 10^{-1} (mM^{-1})

Figure 10. The effect of ethanolamine-1,2-^{14}C concentration on its incorporation into the microsomal phospholipid PE.

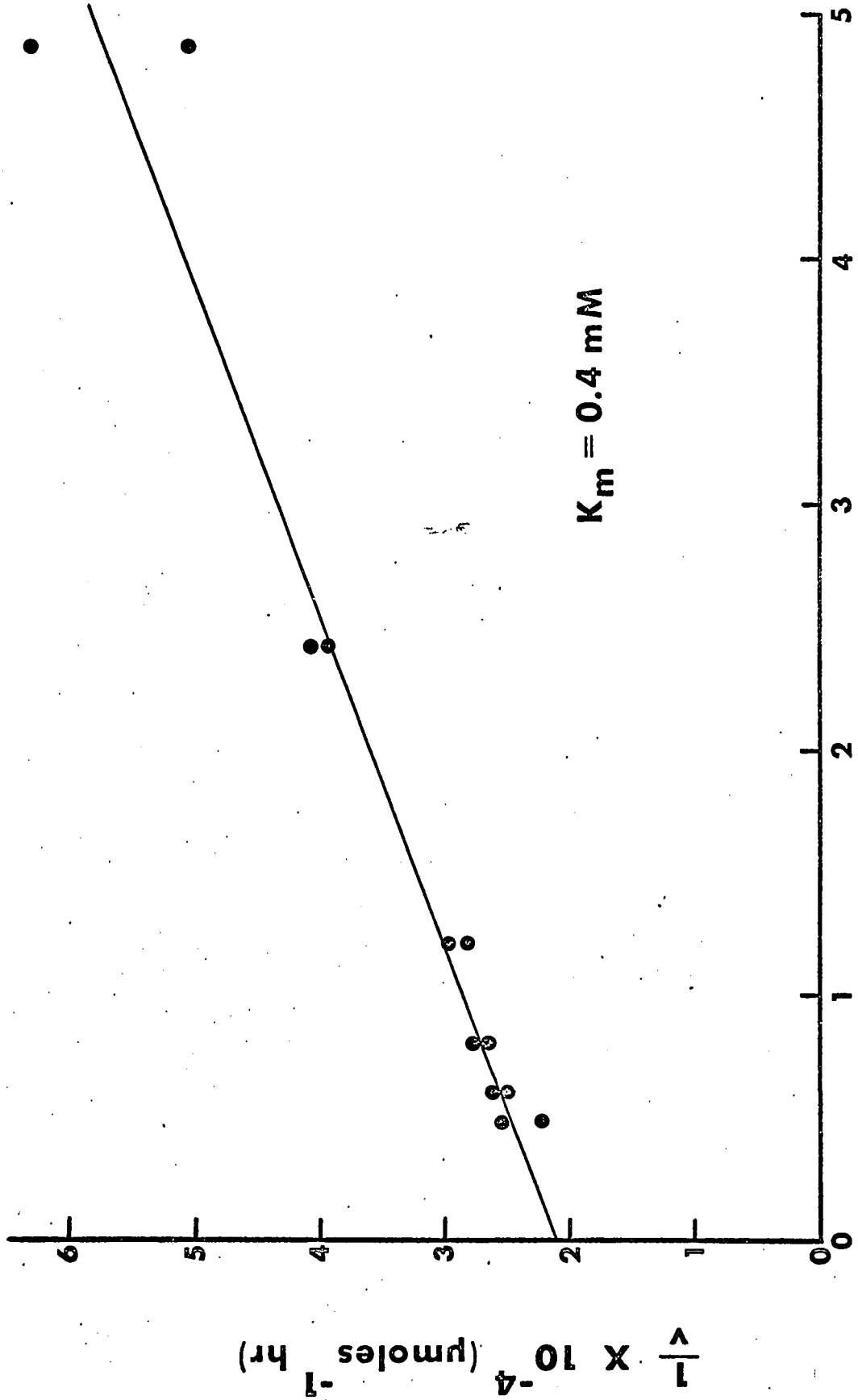
Ethanolamine-1,2-^{14}C was fed at a specific activity of 1.85 mCi/mM. Each point represents one determination. s = mM ethanolamine-1,2-^{14}C fed; v = μmoles X 10^4 ethanolamine-1,2-^{14}C incorporated into PE per hour. The line through the points was fitted using standard regression techniques which gave the following results:

Intercept = 2.09 X 10^4 μmoles^{-1} hr

Slope = 0.747 X 10^4 μmoles^{-1} hr·mM

± 0.055 X 10^4 (Standard error of the regression coefficient).

The regression coefficient was significant at the 0.01 level.


$\frac{1}{v}$ x 10^{-4} (μmoles^{-1} hr)
$\frac{1}{[S]}$ (mM^{-1})
$K_m = 0.4$ mM
0
1
2
3
4
5
6

saturating quantities of substrate be used.

4. The rate of incorporation of L-serine-U-^{14}C

The amount of incorporation versus time of L-serine-U-^{14}C into microsomal phospholipids was studied to determine the time course of the reaction. When L-serine-U-^{14}C was supplied at a fractional concentration of that required for enzyme saturation (Fig 11) the period approximating initial velocity was about 30 min. However, a one hr incubation period was used for most of the work reported here. The conclusions arrived at should not be affected by the greater deviation from constant velocity resulting from this longer incubation period. At near enzyme saturation levels (6 μmoles L-serine per incubation) the period approximating initial constant velocity is about 7 min (Fig 12).

The time course for ethanolamine incorporation was not investigated.

5. Products of enzyme action

When feeding L-serine-U-^{14}C at least 90% of all labelled lipid formed was found in the PS area of the chromatogram. To prove that this activity was in PS, and furthermore to prove that it was largely in the serine moiety of PS, further fractionation of the lipid was performed after eluting the PC, PS and PE areas of the chromatogram. The eluant was hydrolyzed and the resulting hydrolysis products were partitioned against ethyl ether. This ether extract contained negligible amounts of activity. The hydrolysis products were then chromatographed

Figure 11. The incorporation of L-serine-U-^{14}C (fed at subsaturating levels) into the microsomal phospholipids PC+PS+PE as a function of time.

L-serine-U-^{14}C (0.20 μCi; 137 mCi/mM) was fed to resuspended microsomal pellets at a concentration well below enzyme saturating levels. The three points given at each time interval are two determinations and the mean.

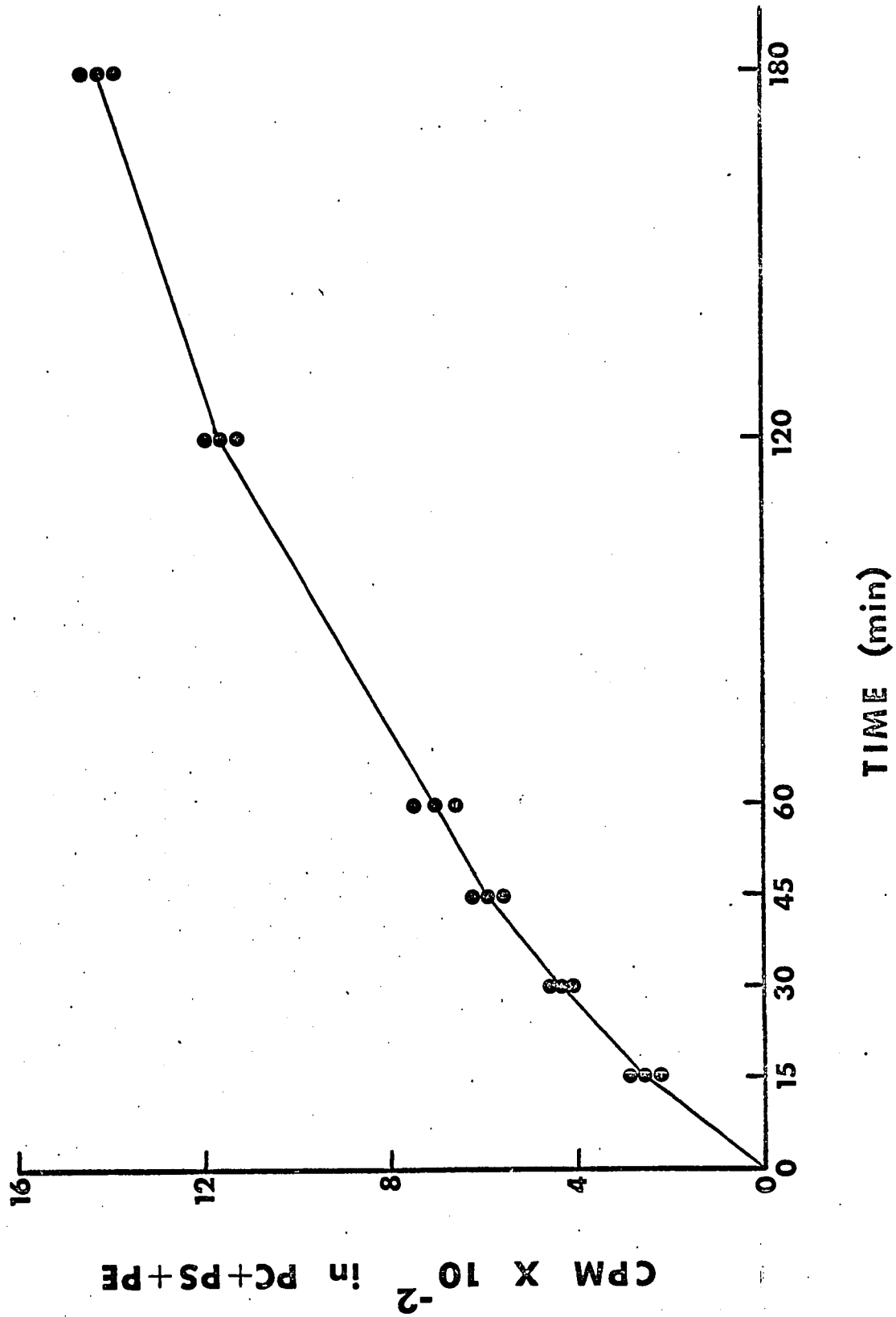
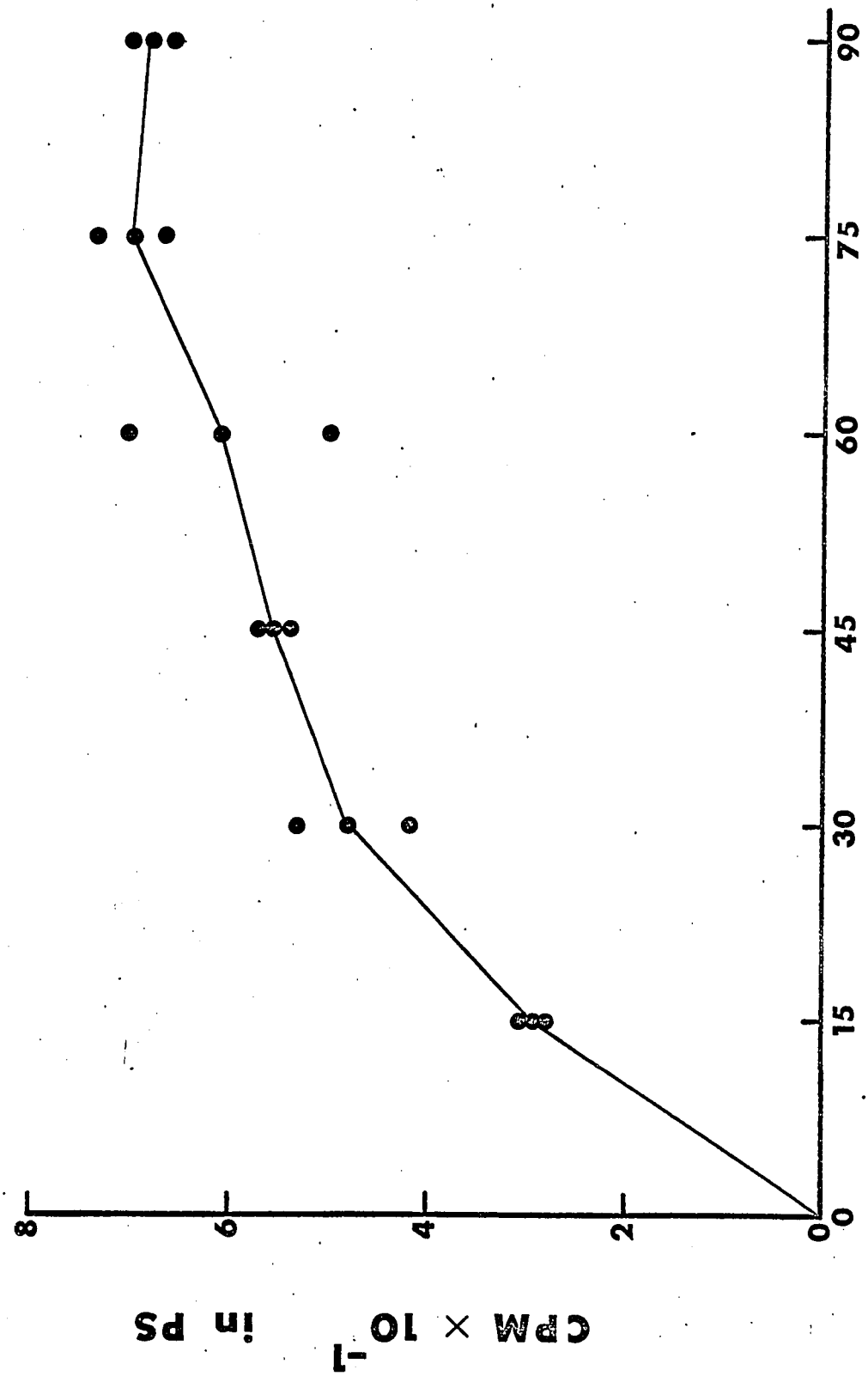

CPM X 10-2 in PC+PS+PE
0
4
8
12
16
0
15
30
45
60
120
180
TIME (min)

Figure 12. The incorporation of L-serine-U-^{14}C (fed at enzyme saturating levels) into the microsomal phospholipid PS as a function of time.

L-serine-U-^{14}C (0.40 μCi; 0.67 mCi/mM) was fed to resuspended microsomal pellets at a concentration approaching enzyme saturation. The three points given at each time interval are two determinations and the mean.


CPM × 10⁻¹ in PS
0
2
4
6
8
0
15
30
45
60
75
90
TIME (min)

on Whatmann #1 paper for 12 hr in butanol:acetic acid:water, 5:3:1 (v:v:v), as described in Section II.H. . Material that co-chromatographed with an L-serine standard contained 93% of the total activity of the hydrolysate; about 6% of the activity was found in the ethanolamine and choline areas.

A similar situation was noted for the incorporation of ethanolamine-1,2-^{14}C; about 87% of the total activity in PC+ PS+PE was found in PE. Choline-1,2-^{14}C incorporation, however, resulted in an activity distribution of about 60% in PC, 20% in PS and 20% in PE. These results were obtained after lipid fractionation only.

As noted in Section II.F. when chromatography on silicic acid impregnated paper resulted in incomplete separation of PC, PS and PE the three phospholipid spots were counted together. The fact that feeding of serine or ethanolamine results in incorporation largely into their corresponding phospholipids means that counting of individual phospholipids or the group should produce similar results.

6. Pulse-chase experiments with L-serine-U-^{14}C

A pulse-chase experiment was performed to help elucidate the pathway of incorporation of serine into microsomal phospholipids. L-serine-U-^{14}C, fed at high specific activity, was chased by addition of a larger amount of cold L-serine sufficient to decrease specific activity to negligible levels. The experiment was then repeated modifying the chase by replacing $CaCl_2$ with EDTA. EDTA prevented further incorporation; in other words EDTA prevented the chase. It was reasoned that if an

exchange reaction producing labelled PS followed by its decarboxylation to form labelled PE takes place, as proposed by Borkenhagen et al.(1961), then a chase with EDTA should prevent the loss of this labelled PE.

In the pulse-chase experiment without EDTA, Figure 13, after the start of the chase there was an initial rise in PS activity. In the pulse-"chase" experiment with EDTA, Figure 14, there was also possibly an initial rise in PS activity (plotted as PS+PC activity due to poor chromatographic separation) but no subsequent fall; activity remained quite constant for up to 335 min. In Figure 13 the incorporation of ^{14}C into PE and PC was very low. However, one may note the rise and fall of PE and PC activity following the beginning of the fall of PS activity. After 60 min of chase PE activity became zero and remained close to zero for up to 180 min. This is an interesting point indicating that PE and PC may be participating in an exchange reaction. Figure 14, on the other hand, shows that in the presence of EDTA PE activity increased slowly throughout the experiment. This will be dealt with in the discussion.

7. Competitive inhibition of serine incorporation by ethanolamine

Ethanolamine is believed to compete with L-serine-U-^{14}C for incorporation into PE and PS, respectively (see Section I.B.2.). A double reciprocal plot of the Lineweaver-Burke type was prepared from data of L-serine-U-^{14}C incorporation into PC+PS+PE in the absence and presence of ethanolamine. This is

Figure 13. Pulse-chase experiment using L-serine-U-^{14}C and $CaCl_2$.

Microsomal pellets were incubated at 30°C with L-serine-U-^{14}C (0.5 µCi/pellet; 137 mCi/mM) for 30 min. The chase was started by the addition of 50 ul of a solution containing 6.0 umoles cold L-serine. Reactions were stopped after various time intervals by the addition of 0.9 ml 1% TCA followed by immediate extraction. Time zero represents the beginning of the chase period. The three points at each time interval are two determinations and the mean.

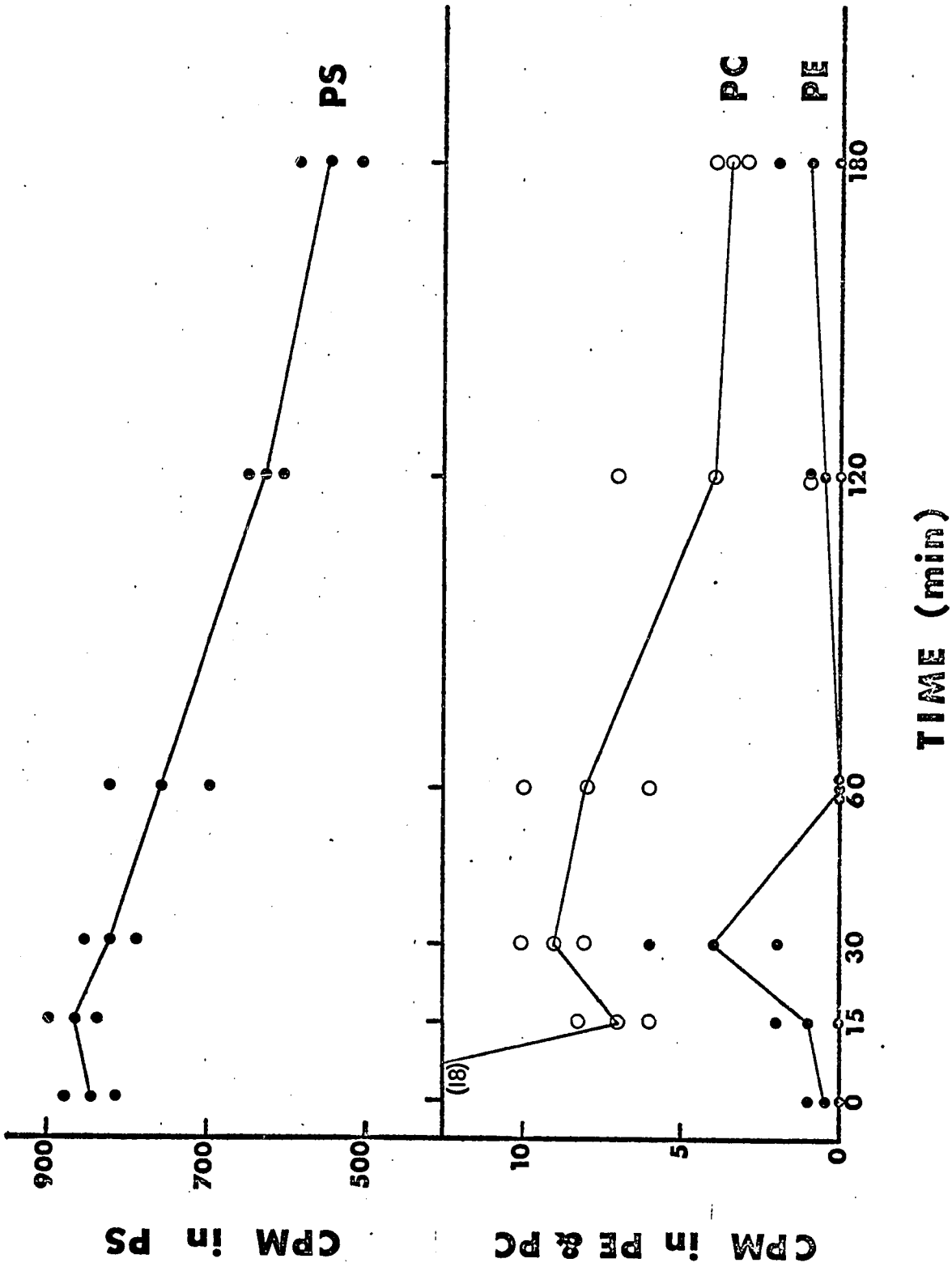
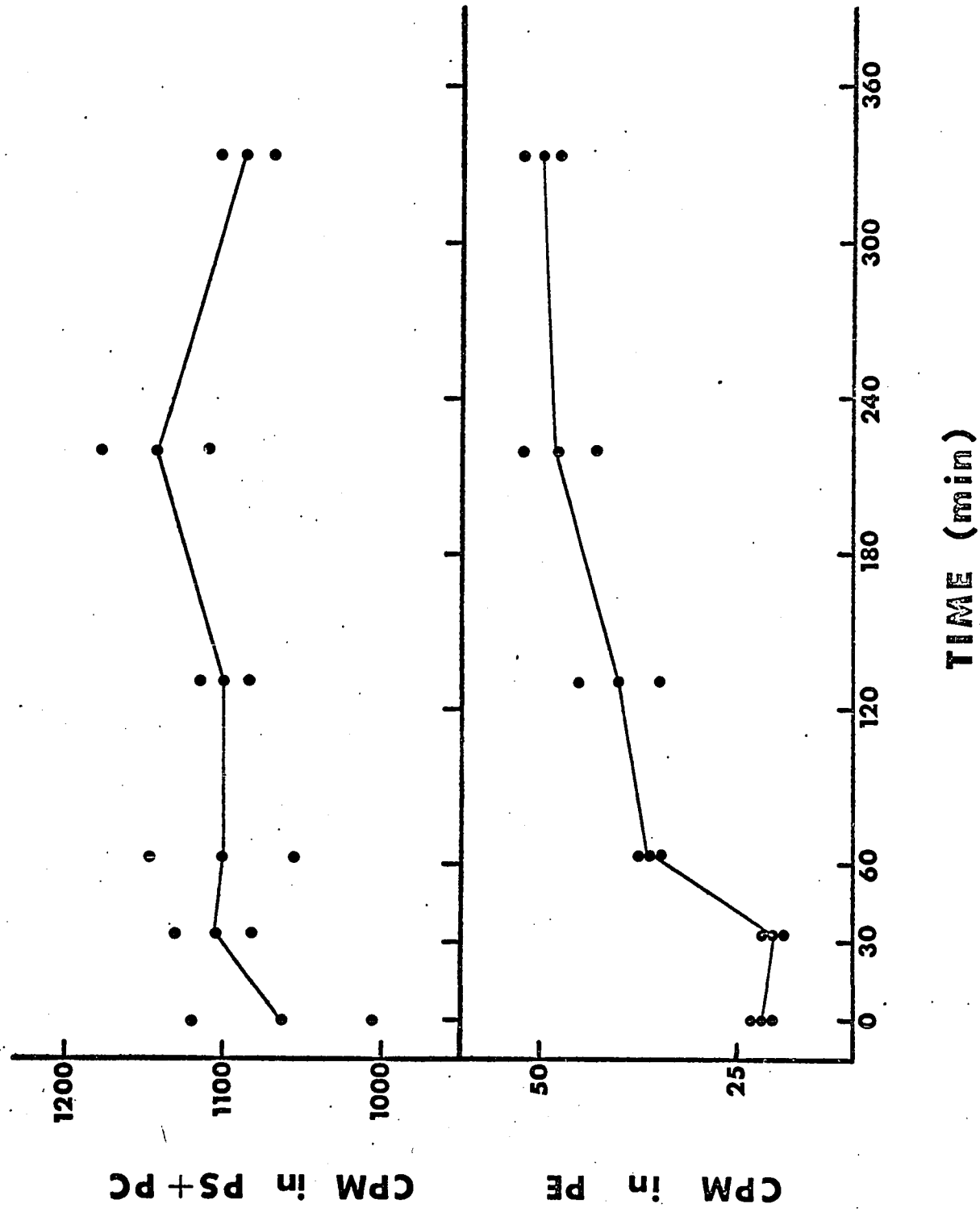

PS
PC
PE
CPM in PS
CPM in PE & PC
TIME (min)
900
700
500
10
5
0
0
15
30
60
120
180
(18)

Figure 14. Pulse-"chase" experiment using L-serine-U-^{14}C and EDTA.

Microsomal pellets were incubated at 30°C with 50 ul of L-serine-U-^{14}C (1.0 μCi/pellet; 137 mCi/mM) for 15 min. After the pulse the "chase" was started by the addition of 50 ul of a solution containing 12.0 umoles L-serine and 6.0 umoles EDTA. Reactions were stopped after various time intervals by the addition of 0.9 ml 1% TCA followed by immediate extraction. Time zero represents the beginning of the "chase" period. The three points at each time interval are two determinations and the mean.


CPM in PS+PC
1200
1100
1000
CPM in PE
50
25
0
30
60
120
180
240
300
360
TIME (min)

shown in Figure 15. The inhibition appears to be competitive.

The K_i for ethanolamine is about 6 mM. The error in this value must be large since relatively few points were available for plotting Figure 15. However, it seems likely that a K_i for ethanolamine of about 6 mM is significantly greater than the K_m for ethanolamine which is equal to 0.4 mM (Sect III.B.3.). On another occasion a K_i for ethanolamine equal to about 11 mM was calculated (data not presented).

The converse experiment, varying the concentration of ethanolamine-1,2-^{14}C in the absence and presence of L-serine, was not performed. However, it was found that ethanolamine-1,2-^{14}C incorporation was inhibited 62% by the presence of an equimolar quantity of L-serine.

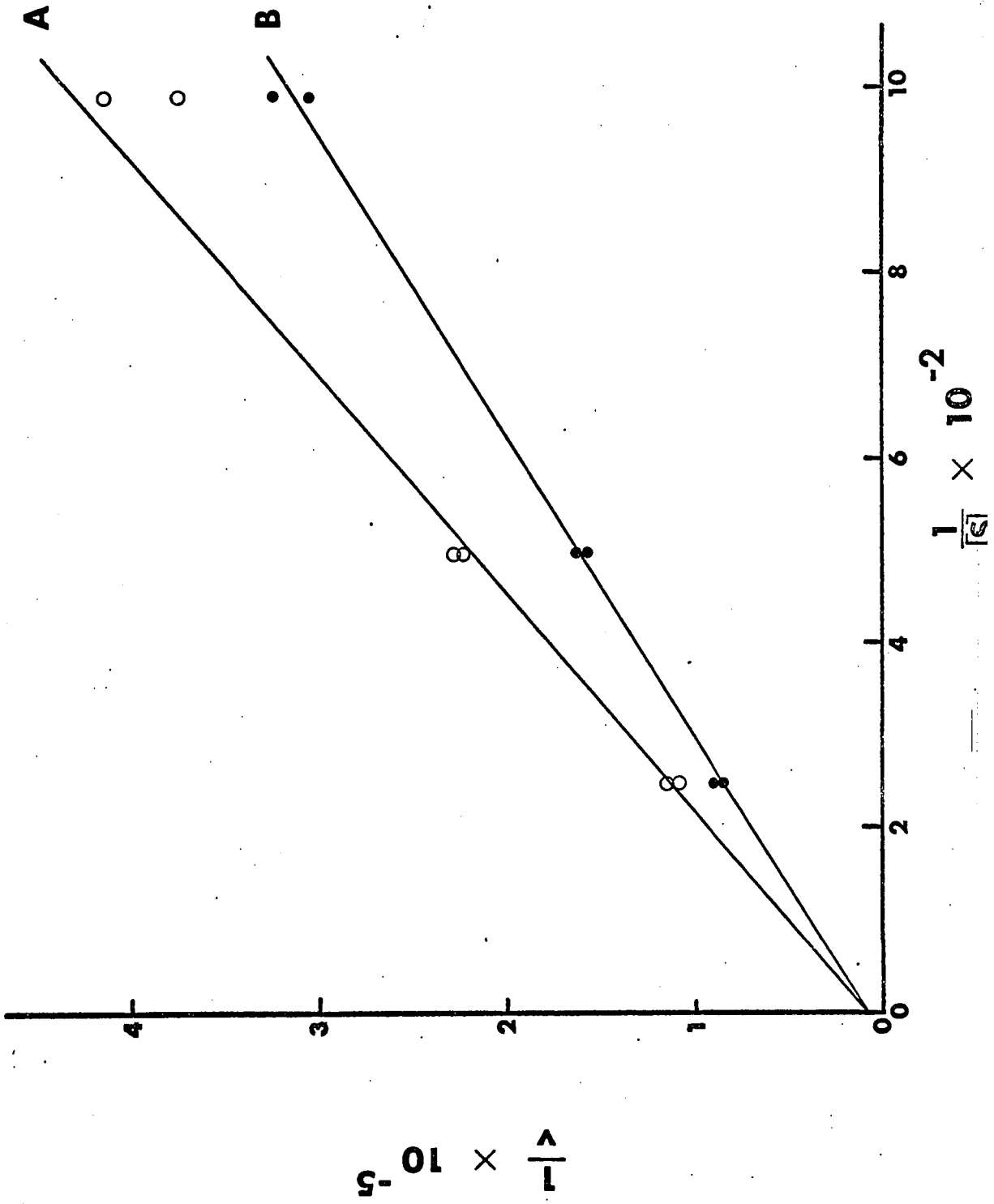
8. <u>The effect of D-serine on the incorporation of L-serine-U-^{14}C into microsomal phospholipids</u>

Microsomal pellets were incubated with L-serine-U-^{14}C (0.25 µCi/pellet; 118 mCi/mM). The addition of D-serine at molarities of 20, 200 and 2000 times that of the L-serine-U-^{14}C fed, caused 6, 12 and 59% inhibition, respectively, as compared with controls.

Such relatively high concentrations of D-serine, in comparison with the amount of L-serine fed, suggest a mechanism of inhibition different from that between L-serine and ethanolamine. For comparison, an equimolar amount of ethanolamine inhibits L-serine-U-^{14}C incorporation by about 30%. Contaminating amounts of L-serine and/or possibly racemization in the aqueous medium (Dawson et al., 1969, p.54) are the most likely

Figure 15. Competitive inhibition of L-serine-U-^{14}C incorporation by ethanolamine.

Double reciprocal plot of L-serine-U-^{14}C incorporation into microsomal phospholipids in the presence (line A) and absence (line B) of ethanolamine. Each of 12 microsomal pellets were fed L-serine-U-^{14}C (118 mCi/mM) at three different concentrations (4 pellets per concentration). Ethanolamine was fed to 6 pellets at 0.002118 µmoles per pellet. Total incubation volume was 1.05 ml. s = mM X 10^{-2} L-serine-U-^{14}C fed; v = µmoles X 10^{-5} serine incorporated into PC+PS+PE per hr. Each point represents one determination. For line B the line drawn through the points was fitted using standard regression techniques. For line A the line was drawn by eye giving greater weight to the points obtained at higher concentrations. Similar results were obtained in another experiment using half the concentration of ethanolamine.


A
B
0
1
2
3
4
0
2
4
6
8
10
$\frac{1}{v} \times 10^{-5}$
$\frac{1}{[S]} \times 10^{-2}$

explanations.

9. Effect of feeding L-serine on the quantity of phosphatidylserine, phosphatidylethanolamine and free ethanolamine.

A double label experiment was designed to see what effects the incorporation of serine had on the quantities of PS, PE and free ethanolamine in a resuspended microsomal pellet. The experiment should test the mechanism of serine incorporation proposed by Borkenhagen et al. (1961). Providing this is the only reaction occurring the molar ratio of PS formed to PE lost should be 1:1.

Ethanolamine-1,2-^{14}C was fed to each of twelve resuspended microsomal pellets. After a one hr incubation all tubes were brought to 0°C and a sufficient quantity and concentration of Tricine-Sucrose solution was added so as to recreate the initial homogenizing medium (except for the presence of a small amount of $CaCl_2$) and also to dilute out the remaining free ethanolamine. The pellets were reformed by ultracentrifugation after which the supernatant, containing free ethanolamine, was discarded. The pellets were again resuspended in 0.05 M Tricine, 0.005 M $CaCl_2$ at pH 8.0. Six of the pellets received no further additions; three were extracted immediately and three after 15 min of incubation. The other six received an enzyme saturating quantity of L-serine-^{3}H(G) and were extracted after either 15 min or 60 min of incubation. The results are presented in Table 2. Radioactivity was counted in PC, PS, PE, the origin of the chromatogram and the upper aqueous phase of the

Table 2. The effect of feeding L-serine on the quantity of PS, PE and free ethanolamine.

The data in this table are taken from two separate experiments. Ethanolamine-1,2-^{14}C (0.5 μCi/pellet; 1.85 mCi/mM) was fed to all tubes during the first incubation of 1 hr. L-serine-^{3}H(G) (12.5 μCi; 2.08 mCi/mM) was fed to tubes in treatment III and IV during the second incubation for the times indicated. Each determination is the mean obtained from three pellets.

		Expt 1		Expt 2
Treatment Number	Second Incubation Time (min)	CPM in PE (^{14}C channel)	CPM in PS (^{3}H channel)	CPM in ethanolamine (^{14}C channel)
I	0	1111	1	120
II	15	972	1	
III	15	993	149	
IV	60	895	220	129

	μmoles	μmoles PE lost: μmoles PS formed
Serine-^{3}H(G) incorporated into PS after 60 min	3.23×10^{-4}	
PE lost (Specific activity of PE = 0.036 mCi/mM)	47×10^{-4}	14.6 : 1
PE lost (Specific activity of PE = 1.85 mCi/mM)	0.92×10^{-4}	0.28: 1

Bligh and Dyer extract. The last two counts were combined. On one occasion (Table 2, Expt 2) the water soluble fraction was chromatographed to resolve ethanolamine from serine.

The results indicate that after 15 min of incubation, incorporation of L-serine-^{3}H(G) into PS does not cause a decrease in PE-^{14}C activity when compared with its control(Treatment II). A possible explanation for not finding a decrease in PE-^{14}C activity is that in the absence of L-serine PE participates in other exchange reactions, for example, exchange with residual ethanolamine or hydrolysis to produce PA. Phospholipase D action is known to act in this way (Yang et al., 1967). If this is so then one can assume that after 60 min the decrease in activity in PE is, at least in part, related to the increase in activity in PS.

Following the reasoning given above the ratio µmoles PE lost : µmoles PS formed was calculated. The results are either 14.6:1 or 0.28:1 depending on the specific activity of PE that is participating in the exchange reaction. If one considers that total PE was available for the exchange reaction then the specific activity was 0.036 mCi/mmole (calculated from 1111 cpm in 0.75 µgm PE-phosphorus or 0.0242 µmoles PE). On the other hand if PE available for the exchange reaction was only that formed during the first incubation with ethanolamine-1,2-^{14}C, then the specific activity was the same as the ethanolamine fed, i.e., 1.85 mCi/mmole . The two ratios imply the existence of separate pools of PE. The idea of separate pools of PE has been suggested by Willemot and Boll (1967) and Dennis

and Kennedy (1970). Dennis and Kennedy feel that only a small pool of PE may be available for the exchange reaction in Tetrahymena pyriformis.

During Expt 2 (Table 2) an increase in free ethanolamine was noted. It is of doubtful significance. These results were obtained by fractionation of the water solubles (upper phase and origin of chromatogram) by chromatography as described in Section II.H. The activity in the ethanolamine spot was then measured. However, during Expt 1 the total water solubles were counted. Large amounts of activity from L-serine-^{3}H(G) in the ^{14}C channel (0.5% spillover) obscured small differences in cpm due to ^{14}C-ethanolamine.

During the course of the present work a similar experiment was done by Dennis and Kennedy (1970). Their results indicate that the reaction scheme proposed by Borkenhagen et al. (1961) operates in Tetrahymena pyriformis.

10. The relationship between serine and ethanolamine incorporation and the transphosphatidylase activity of phospholipase D.

There exists the possibility that incorporation of serine and ethanolamine into oat coleoptile microsomal phospholipids is accomplished via transphosphatidylation by phospholipase D as described by Yang et al. (1967). The existence of phospholipase D action in oat coleoptile microsomal preparations was suggested by the results on calcium dependency and energy independence reported above. In order to obtain further evidence on this point the incorporating system described in previous

sections was subjected to many of the same tests to which Yang et al. (1967) subjected phospholipase D.

(a). Sulfhydryl-group inhibitors

The levels of incorporation of L-serine-U-^{14}C and ethanolamine-1,2-^{14}C into PS and PE, respectively, were tested in the presence of 0.1 mM p-chloromercuribenzoate (PCMB) and 1.0 mM N-ethylmaleimide (NEM). The results are presented in Table 3. Inhibition by PCMB and not by NEM, and in the case of ethanolamine the slight stimulation of incorporation by NEM, are fully in agreement with the results of Yang et al. (1967) for the hydrolysis of PC by phospholipase D ("phosphatidohydrolase" activity). The reversal of inhibition by glutathione was not tested.

(b). Heat coagulation

The purification of phospholipase D from cabbage-leaf by the method of Davidson and Long (1958) and later used by Yang et al. (1967) involved coagulation of the 13,000g supernatant by heating for 5 min at 55°C followed by an additional centrifugation (13,000g, 5 min) to remove the "bulky precipitate" that formed. This treatment slightly increased the total number of enzyme units which most probably indicates that no degradation of the enzyme took place and possibly that an inhibitor was removed. On the other hand Kates (1954) noted that plastid-associated phospholipase D is thermolabile.

An experiment was performed to see what effects heating had on the incorporation of serine and ethanolamine into micro-

Table 3. The effect of two sulfhydryl-group inhibitors on the incorporation of L-serine-U-^{14}C and ethanolamine-1,2-^{14}C.

Incorporation of serine and ethanolamine was determined by measuring incorporation into their respective microsomal phospholipids. L-serine-U-^{14}C (0.5 µCi/pellet; 137 mCi/mM) or ethanolamine-1,2-^{14}C (1.0 µCi/pellet; 1.5 mCi/mM) was fed to microsomal pellets in the absence and presence of 0.1 mM PCMB or 1.0 mM NEM. Each value is the mean of two replicates. This experiment was not repeated.

Labelled Material	Inhibitor	CPM incorporated into respective phospholipids (PS or PE)	Percent of Control
L-serine-U-^{14}C	none	2013	100
"	0.1 mM PCMB	155	7.6
"	1.0 mM NEM	1832	91
Ethanolamine-1,2-^{14}C	none	1385	100
"	0.1 mM PCMB	534	39
"	1.0 mM NEM	1580	114

somal phospholipids. The 37,000g supernatant was heated at 55°C for 5 min followed by rapid cooling in an ice bath. An additional centrifugation (37,000g; 20 min) was performed and from the resulting supernatant microsomal pellets were formed by ultracentrifugation. The heat treated pellets showed a 70% inhibition of L-serine-U-^{14}C incorporation and a 33% inhibition of ethanolamine-1,2-^{14}C incorporation. This experiment is substantially different from the heat treatment used by Davidson and Long(1958)so that no direct comparisons can safely be made. However, it is clear that this method of heat treatment lowers the ability of oat coleoptile microsomal pellets to incorporate serine and ethanolamine.

There was the possibility that the above results were due to increased sedimentation of microsomal material during the second 37,000g centrifugation. To test this possibility a similar experiment was performed by following the heat treatment directly with ultracentrifugation to form microsomal pellets. The heat treated pellets showed a 93% inhibition of L-serine-U-^{14}C incorporation and a 74% inhibition of ethanolamine-1,2-^{14}C incorporation. The results indicate that in the system studied here heat treatment causes a loss of incorporating ability most probably by enzyme denaturation.

Direct heat treatment of microsomal pellets was not tried.

(c). Ethyl ether

Two separate experiments were performed to test the effect of ethyl ether on the incorporation of serine and ethanolamine in microsomal phospholipids (Table 4). The second experiment

Table 4. The effect of ethyl ether on the incorporation of serine and ethanolamine into microsomal phospholipids.

L-serine-U-^{14}C (0.5 µCi/pellet; 137 mCi/mM) or ethanolamine-1,2-^{14}C (1.0 µCi/pellet; 1.5 mCi/mM) was fed to microsomal pellets either in the presence or absence of 0.5 ml ethyl ether. Experiment 2 is identical to Expt 1 except that the 37,000g supernatant used to produce the microsomal pellets was heat treated and recentrifuged at 37,000g (see Sect III.B.10.(b).). The reaction was stopped by the addition of 1 ml of 1% TCA. The ether was dried off under a stream of N_2 before performing lipid extraction. Each treatment is the mean of three determinations.

Substrate	Addition	CPM in PC+PS+PE	Percent of control
Expt 1			
L-serine-U-^{14}C	none	1297	100
"	ether	620	47.8
Ethanolamine-1,2-^{14}C	none	945	100
"	ether	1377	145.7
Expt 2			
L-serine-U-^{14}C	none	463	100
"	ether	66	14.3
Ethanolamine-1,2-^{14}C	none	460	100
"	ether	789	171

differed from the first in that the 37,000g supernatant used to produce microsomal pellets was heat treated and then recentrifuged at 37,000g as described in the preceding section. Prior to use the ethyl ether was washed three times with an equal volume of water. This procedure removes the ethanol contaminant which could act as a transphosphatidylation acceptor (Yang et al., 1967).

Both with and without heat pretreatment ether inhibited incorporation of L-serine-U-^{14}C but stimulated ethanolamine-1,2-^{14}C incorporation. Heat pretreatment accentuated these effects of ether.

(d). Various alcohols

Yang et al. (1967) noted that ethanol, methanol and glycerol were excellent acceptors for transphosphatidylation by phospholipase D. The incorporation of these alcohols into oat coleoptile microsomal lipids was not tested here. However, it was anticipated that if these alcohols are being incorporated by the same enzyme as that incorporating serine and ethanolamine, the inhibition of one alcohol by another should be detectable. The competitive inhibition of serine by ethanolamine, shown above (Sect III.B.7.), suggests this relationship. With this in mind ethanol, methanol, glycerol and choline were tested to see their effects on the incorporation of L-serine-U-^{14}C and/or ethanolamine-1,2-^{14}C into microsomal phospholipids.

Data from a number of experiments are compiled in Table 5. In Expt 1 the unlabelled alcohols were fed at a concentration of 4.6% by volume. At this concentration the transphosphatidyl-

Table 5. The effect of various alcohols on the incorporation of serine and ethanolamine into microsomal phospholipids.

L-serine-U-^{14}C (0.00399 µmoles; 125 mCi/mM) or ethanolamine-1,2-^{14}C (0.663 µmoles; 1.5 mCi/mmole) was fed in reaction mixtures of total volume 1.075 ml (Expts 1 and 2) or 1.025 ml (Expt 3).

Labelled substrate	Unlabelled alcohol (µmoles fed)	CPM in PC+PS+PE (replicates)	Percent of Control
Expt 1			
L-serine-U-^{14}C	none	1996 (2)	100
"	Methanol (1230)	1614 (2)	81
"	Ethanol (860)	2019 (2)	101
"	Glycerol (680)	1846 (2)	92
Ethanolamine-1,2-^{14}C	none	1424 (2)	100
"	Methanol (1230)	1326 (2)	93
Expt 2			
Ethanolamine-1,2-^{14}C	none	1600 (2)	100
"	Ethanol (170)	1432 (2)	90
Expt 3			
L-serine-U-^{14}C	none	2102 (2)	100
"	Choline (0.00798)	2053 (2)	98
"	Choline (1.32)	2063 (1)	98
Ethanolamine-1,2-^{14}C	none	1422 (2)	100
"	Choline (1.32)	1603 (1)	113

ase action of phospholipase D is greater than its phosphatidohydrolase action (Yang et al., 1967). In Expt 2 ethanol was fed at a concentration of about 1%. In Expt 3 choline chloride was fed with L-serine-U-^{14}C at concentrations of 2 and 330 times the molarity of the L-serine-U-^{14}C fed. For the ethanolamine-1,2-^{14}C incubation (Expt 3) choline chloride was fed at only one concentration, twice the molarity of the labelled substrate. Choline chloride did not inhibit serine or ethanolamine incorporation and even slightly stimulated ethanolamine incorporation. Similar results were obtained by Porcellati et al. (1961) using chick brain microsomes. Lack of inhibition by choline led to the testing of the calcium chloride requirement for choline-^{14}C-chloride incorporation (see Sect III.B.2.); a requirement considered very characteristic of the system incorporating serine and ethanolamine.

Yang et al. (1967) noted that at a concentration of 4% both glycerol and ethanolamine were inhibitory to overall phospholipase activity; ethanol was inhibitory at a concentration of over 10%. Thus inhibition by 4.6% glycerol but no inhibition by 4.6% ethanol (Expt 1, L-serine-U-^{14}C) are similar to the findings of Yang et al. (1967). However, this similarity is not considered sufficient evidence for phospholipase D action but is more likely a general phenomenon of interaction between organic solvent and lipid reaction.

The low levels of inhibition found here are not what would be expected from the results of Yang et al. (1967) if a similar system is operating in oat coleoptile microsomes, i.e.,

the results are not suggestive of competition between substrate and other alcohols for incorporation.

11. A comparison of the rates of incorporation of serine and ethanolamine into microsomal phospholipids under various conditions.

Data from various experiments, in which both serine and ethanolamine incorporation were studied is presented in Table 6. The ratio (column S/E) of the rate of serine incorporated per umole serine fed to the rate of ethanolamine incorporated per umole ethanolamine fed is presented. From this ratio a comparison of serine and ethanolamine incorporation under various conditions can be made. The conditions to which the microsomal pellets were subjected are given in the legend of Table 6.

The most valid comparisons can be made only within any one experiment where variability in microsomal pellets was at a minimum. This is the case within Expts 7,8,9 and 10. These experiments showed that addition of ethyl ether (Expt 7), heat treatment (Expt 8), ageing of pellets at $4^{o}C$ (Expt 9) or addition of sulfhydryl-group inhibitors (Expt 10) decreased the ratio of S/E.

In Expt 6 microsomal pellets were aged at $-20^{o}C$. Even though this experiment lacks a value for an untreated pellet it can be compared with the untreated pellets in other Expts. In all experiments the S/E values for untreated pellets ranges from 1.74 to 4.45 with an average of 2.74. The value for the treated pellets in Expt 6 is about 0.28 which is some 6 times

Table 6. Ratios of the rate of serine incorporated per umole serine fed to the rate of ethanolamine incorporated per umole ethanolamine fed.

Microsomal pellets were fed either L-serine-U-^{14}C (S) or ethanolamine-1,2-^{14}C (E). Activity incorporated into microsomal PC+PS+PE during a 1 hr incubation was determined and used to calculate umoles incorporated per hr. Two microsomal pellets (3 in Expt 7) were used for each determination. Only the average incorporation is recorded. Microsomal pellets used in any one experiment were identical in that they were produced by ultracentrifugation, at one time, from the same post-mitochondrial supernatant (37,000g supernatant). Microsomal pellets used in one experiment were incubated at the same time except in Expts 6 and 9 where time was a factor. Treatments were as follows: a) Pellets were held for 6 days at about -20°C, b) pellets were held for 36 days at -20°C, c) 0.5 ml ethyl ether was layered onto the reaction mixture, d) 37,000g supernatant was heated for 5 min at 55°C followed by an additional 37,000 g centrifugation, e) pellets were held at 4°C for 55 min before incubation, f) pellets were held at 4°C for 205 min before incubation, g) reaction mixture contained 0.1 mM PCMB and h) reaction mixture contained 1.0 mM NEM. Data listed under Expts 7, 8 and 10 are also dealt with in Section III.B.10. of this thesis.

Expt	Treatment	substrate fed (μmoles)	μmoles X 10^{-4} incorporated per hr	μmoles incorporated X 10^{-3} / μmole fed·hr	$\frac{S}{E}$ *
1	-	S(0.00399)	0.132	3.30	
	-	E(0.665)	7.44	1.12	2.95
2	-	S(0.00399)	0.125	3.14	
	-	E(0.665)	7.45	1.12	2.80
3	-	S(0.00399)	0.119	2.98	
	-	E(0.665)	7.66	1.15	2.59
4	-	S(0.00732)	0.159	2.17	
	-	E(0.665)	5.73	0.861	2.52
5	-	S(0.00366)	0.089	2.46	
	-	E(0.665)	7.25	1.09	2.25
6	a	S(0.00366)	0.015	0.287	
	a	E(0.665)	7.18	1.08	0.28
	b	S(0.00366)	0.0085	0.233	
	b	E(0.665)	5.83	0.877	0.27
7	-	S(0.00366)	0.074	2.03	
	-	E(0.665)	4.94	0.743	2.73
	c	S(0.00366)	0.035	0.970	
	c	E(0.665)	7.20	1.08	0.89
8	-	S(0.00366)	0.089	2.42	
	-	E(0.665)	3.62	0.544	4.45
	d	S(0.00366)	0.027	0.724	
	d	E(0.665)	2.41	0.362	2.0
9	-	S(0.00399)	0.161	4.03	
	-	E(0.539)	12.5	2.32	1.74
	e	S(0.00399)	0.130	3.25	
	e	E(0.539)	11.5	2.13	1.53
	f	S(0.00399)	0.127	3.18	
	f	E(0.539)	12.8	2.37	1.34
10	-	S(0.00366)	0.119	3.24	
	-	E(0.665)	8.07	1.21	2.68
	g	S(0.00366)	0.0099	0.272	
	g	E(0.665)	3.35	0.503	0.54
	h	S(0.00366)	0.109	2.98	
	h	E(0.665)	9.31	1.40	2.13

* $\frac{S}{E} = \frac{\text{μmoles serine incorporated X } 10^{-3} \text{ / μmole fed per hr}}{\text{μmoles ethanolamine incorporated X } 10^{-3} \text{ / μmole fed per hr}}$

lower than the lowest value for the untreated pellets. It is unlikely that such a difference is due to variability between experiments. Hence it is concluded that ageing at -20°C also causes a decrease in the S/E ratio.

The data presented here suggest that the mechanism incorporating serine and the mechanism incorporating ethanolamine do not respond in the same way to various treatments.

C. DECARBOXYLATION OF PHOSPHATIDYLSERINE

When feeding L-serine-U-^{14}C to whole coleoptiles, activity appeared rapidly in PC (Sect III.A.2.). In the microsomal fraction there are relatively high levels of endogenous PE and PC and low levels of endogenous PS. This could be due to the presence of an active PS decarboxylase. The low levels of CO_2 evolution when feeding L-serine-U-^{14}C to microsomal pellets (Table 7) were considered to be due to the in vitro system used here, i.e., incubation in 0.05 M Tricine, 0.005 M $CaCl_2$ at pH 8.0. Attempts were therefore made to enhance in vitro decarboxylation and at the same time to detect loss of activity in PS and appearance in PE.

Pyridoxal phosphate at concentrations ranging from 0.02 mM to 5 mM had no effect on $^{14}CO_2$ generation from L-serine-U-^{14}C. Protein preparations of either a total coleoptile homogenate, a 37,000g precipitate or the 105,000g supernatant added to the microsomal pellets also had no effect. Toluene (0.1 ml per incubation) did not stimulate decarboxylation but it did inhibit the incorporation of L-serine-U-^{14}C into PS. Attempts

to push decarboxylation by saturating the enzyme system incorporating serine into PS had no effect on the amount of CO_2 released.

1. Comparison of $^{14}CO_2$ release from L-serine-U-^{14}C and DL-serine-1-^{14}C

If decarboxylation of serine is occurring, feeding serine-1-^{14}C should theoretically produce three times the amount of $^{14}CO_2$ as is produced when feeding an equal quantity and activity of serine-U-^{14}C.

Table 7 presents the results of such an experiment comparing $^{14}CO_2$ generation from DL-serine-1-^{14}C and L-serine-U-^{14}C in the presence and absence of both microsomal pellets and toluene. The ratio of labelled CO_2 generated from DL-serine-1-^{14} to L-serine-U-^{14}C was found to be 6.7 in the absence of toluene and 8.5 in the presence of toluene. The theoretical ratio is 7.5. It is calculated by taking into account activities fed, assuming utilization of only the L-isomer and decarboxylation of the number one carbon.

2. Feeding of ^{14}C-labelled phosphatidylserine

^{14}C-labelled PS was biosynthesized by feeding oat coleoptile microsomal pellets L-serine-U-^{14}C. The labelled lipid was extracted by the usual Bligh and Dyer method and purified by chromatography on silicic acid impregnated paper followed by elution.

Resuspended pellets were fed ^{14}C-labelled PS by introducing it into the reaction mixture, during two separate experi-

Table 7. Comparison of $^{14}CO_2$ release from L-serine-U-^{14}C and DL-serine-1-^{14}C.

Either 10 ul L-serine-U-^{14}C (0.2 µCi/pellet; 137 mCi/mM) or 25 ul DL-serine-1-^{14}C (1.0 µCi/pellet; 7.55 mCi/mM) was used. Toluene, 0.1 ml, was added as noted. Incubation was at 30°C for 1 hr. The reaction was stopped by the addition of 1 ml of 1% TCA and shaken for an additional 15 min. All labelled materials were held over 1.0 N NaOH for 1 hr before use.

Labelled Material	Microsomal Pellet	Toluene	CO_2 collected (cpm)	Net CO_2 generated (cpm)	Ratio*
L-serine-U-^{14}C	yes	no	27.2		
"	yes	no	26.2	5.9	6.7[a]
"	yes	yes	22.9	2.3	8.5[b]
DL-serine-1-^{14}C	yes	no	163.2		
"	yes	no	155.0	39.5	
"	yes	yes	123.5	19.6	
L-serine-U-^{14}C	no	no	20.6		
"	no	no	20.9		
"	no	yes	20.6		
DL-serine-1-^{14}C	no	no	120.8		
"	no	no	118.4		
"	no	yes	103.9		

* Ratio CO_2 generated from DL-serine-1-^{14}C to L-serine-U-^{14}C.
Theoretical ratio = 7.5 = 5.0 (ratio of activities fed) X
0.5 (utilization of L-isomer only)
X 3.0 (activity in carbon #1)

[a] without toluene [b] with toluene

ments, in either 50 ul chloroform or 50 ul aliquots of a sonicated water dispersion. Within the limitations of the experiments no differences were noted between the two methodsof feeding.

The data for the feeding of ^{14}C-labelled PS, as a sonicated water dispersion, to microsomal pellets are presented in Table 8. The results show low and variable levels of $^{14}CO_2$ collected. Thus $^{14}CO_2$ release by microsomes cannot be considered significantly greater than the microsome-lacking control (Treatment IV). Lipid chromatography of the incubated microsomes after feeding the labelled lipid showed the greatest decrease in PS activity and the greatest increase in PE activity in the microsome-lacking treatment. This may indicate that microsomes protect PS against non-enzymatic degradation.

The activity found in PC and PE indicate that the labelled PS fed had a minimum purity of 80%.

The detection of decarboxylation of PS by the above method may only require the feeding of much higher specific activity ^{14}C-labelled PS. Presentation of the substrate to the enzyme may also be the problem. Difficulties in feeding a lipid substrate have been noted by Patterson and Lennarz (1971) for PS and by Dennis and Kennedy (1970) for PE.

D. ENERGY-DEPENDENT INCORPORATION OF SERINE, ETHANOLAMINE AND CHOLINE

The possibility that energy-dependent pathways exist for the incorporation of serine, ethanolamine and choline into the microsomal phospholipids of the oat coleoptile was examined.

Table 8. Feeding of ^{14}C-labelled PS to microsomal pellets.

^{14}C-labelled PS (600 cpm/50 µl per pellet; 4.7 X 10^{-3} mCi/mM) was fed as a sonicated water dispersion to resuspended microsomal pellets (no microsomal pellets in Treatment IV). Pellets were resuspended in 1.0 ml 0.05 M Tricine, 0.005 M EDTA at pH 8.0. The reaction was stopped by the addition of 0.95 ml 1% TCA. Additions: a = 0.005 M PALP; b = 0.005 M PALP, 0.1 ml toluene; c = 0.005 M PALP, 0.1 ml toluene (no microsomes).

Treatment Number	Additions	CPM in CO_2	Net CPM in CO_2	CPM in PC	CPM in PS	CPM in PE
I	none	32.2 21.6	5.2	33 39	512 506	56 57
II	a	22.2 22.8	0.8	55 94	551 445	62 46
III	b	22.5 21.6	0.4	32 37	495 547	54 50
IV	c	20.4 23.0		52 120	399 307	72 50

Pellets resuspended in 0.05 M Tricine, 0.005 M EDTA showed no increase in incorporation of L-serine-U-^{14}C, ethanolamine-1,2-^{14}C or choline-1,2-^{14}C chloride in the presence of ATP or CTP. When feeding was carried out in 0.05 M Tricine, 0.01 M KCl, 0.005 M $MgCl_2$, 0.01 M KH_2PO_4 and 0.002 M EDTA at pH 8.0 (modified method of Bygrave and Bucher (1968)), the addition of 0.001 M ATP plus 0.001 M CTP caused increased incorporation of serine, ethanolamine and choline into PC+PS+PE equal to 154, 14 and 29% of controls, respectively (Table 9, Expt 1). However, the levels of incorporation for serine and ethanolamine with the nucleotides were no more than 10% that of the calcium stimulated reaction (for incorporation of choline in the presence of $CaCl_2$ or EDTA see Section III.B.2.). There was some indication that for serine incorporation ATP gave more stimultaion than did CTP (Table 9, Expt 2).

The findings of Morré et al. (1970) indicate that higher levels of incorporation may require the presence of other cell fractions.

E. DEVELOPMENTAL CHANGES IN LIPID BIOSYNTHETIC SYSTEMS. INCORPORATION OF L-SERINE-U-^{14}C AND ETHANOLAMINE-1,2-^{14}C INTO PHOSPHOLIPIDS OF MICROSOMES PREPARED FROM COLEOPTILES OF INCREASING LENGTH.

L-serine-U-^{14}C and ethanolamine-1,2-^{14}C were fed to microsomal pellets prepared from coleoptiles of different lengths. Every attempt was made to standardize the procedure from one harvest to another.

The results (Fig 16) are similar for both serine and ethan-

Table 9. The effect of ATP and CTP on the incorporation of serine, ethanolamine and choline into microsomal phospholipids.

Microsomal pellets produced by ultracentrifugation in 0.05 M Tricine, 0.4 M Sucrose, pH 8.0 were incubated in 0.05 M Tricine, 0.01 M KCl, 0.005 M $MgCl_2$, 0.01 M KH_2PO_4 and 0.002 M EDTA at pH 8.0 with the addition of either 0.001 M ATP and/or 0.001 M CTP. Incubations were at 37°C for 30 min with 25 ul of either L-serine-U-^{14}C (0.5 µCi/pellet, 137 mCi/mM), ethanolamine-1,2-^{14}C (1.0 µCi/pellet; 1.5 mCi/mM) or choline-1,2-^{14}C (1.0 µCi/pellet; 5.0 mCi/mM). Each value is the average determined using two microsomal pellets.

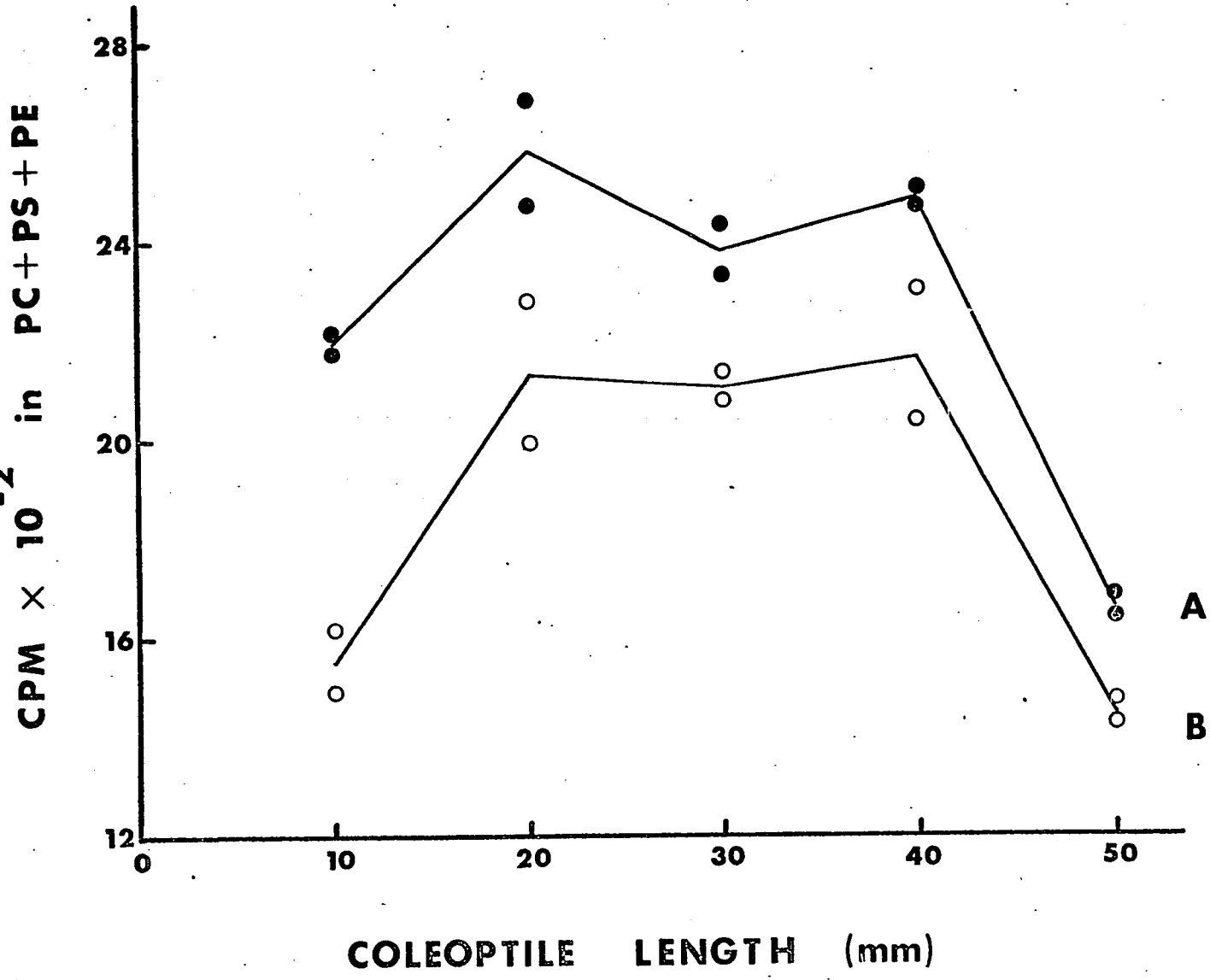
Labelled Substrate	Additions	PC	PS	PE	Total CPM	Percent Increase
Expt 1:						
L-serine-U-^{14}C	--	14	9	14	37	
"	ATP + CTP	38	28	28	94	154
Ethanolamine-1,2-^{14}C	--	40	21	76	137	
"	ATP + CTP	50	25	81	156	14
Choline-1,2-^{14}C	--	382	76	105	563	
"	ATP + CTP	514	93	118	725	29
Expt 2:						
L-serine-U-^{14}C	--	5	5	7	17	
"	ATP	6	11	7	24	41
"	CTP	3	8	5	16	-6
"	ATP + CTP	6	13	6	25	47

Figure 16. Changes in the rate of incorporation of L-serine-U-^{14}C and ethanolamine-1,2-^{14}C into the phospholipids of microsomal pellets which were prepared from coleoptiles of increasing length.

L-serine-U-^{14}C (0.5 µCi/pellet; 125 mCi/mM) or ethanolamine-1,2-^{14}C (1.0 µCi/pellet; 1.85 mCi/mM) was fed to microsomal pellets prepared from coleoptiles of different length. Incorporation into PC+PS+PE was determined. Four pellets were used at each length; two for serine and two for ethanolamine. The lines join the means of the determinations. This experiment was not repeated.

A = L-serine-U-^{14}C

B = Ethanolamine-1,2-^{14}C


28
24
20
16
12
0
10
20
30
40
50
CPM × 10^-2 in PC+PS+PE
A
B
COLEOPTILE LENGTH (mm)

olamine showing a rise and fall in the amount of labelled substrate incorporated. For both serine and ethanolamine the average result for 30 mm coleoptile microsomes is about 10% lower than a smooth curve drawn through the other points. Since pellets are produced on different days this lowered incorporation suggests some difference in the production of microsomes or the incubation procedure that would affect their incorporating ability. However, the possibility that a true dip exists cannot be excluded.

Microsomal protein per pellet was also assayed at each developmental stage during this experiment (data not presented). A steady rise similar to that shown in Figure 4 was found indicating that changes in incorporating ability were not due to variation in size of the microsomal pellets produced on different dates.

The ratio S/E was calculated (see Section III.B.11.) at each developmental stage. The changes in this ratio with increasing length of the coleoptile are as follows:

length (mm)	S/E
10	2.82
20	2.17
30	2.25
40	2.29
50	2.45

A rapid fall followed by a steady increase should be noted. Such a pattern would suggest real changes with development. Varying amounts of denaturation during each assay, causing changes in

the S/E ratio, should present a more random picture.

IV. DISCUSSION

The aim of this work was to investigate and characterize the pathways leading to PC, PS and PE in the oat coleoptile microsomal fraction. It was then anticipated that subsequent assay of the then characterized pathways, during the course of development of the coleoptile, would be used to reveal the development of membranes, or at least membrane components, in the oat coleoptile and give clues as to mechanisms of membrane biogenesis.

The discussion is organized in three parts. The first part concerns the pathways incorporating serine, ethanolamine and choline into phospholipids. The decarboxylation of PS to form PE will then be discussed. Finally the preliminary results concerning the developmental changes in the whole coleoptile and coleoptile microsomal fraction will be considered.

A. THE INCORPORATION OF SERINE, ETHANOLAMINE AND CHOLINE INTO OAT COLEOPTILE MICROSOMAL PHOSPHOLIPIDS.

The results of studies on the incorporation of serine, ethanolamine and choline into microsomal phospholipids has led to the following conclusions concerning their mechanisms of incorporation:

L-serine, ethanolamine and choline are incorporated into their respective phospholipids by calcium stimulated reactions. Three specific enzyme sites exist for this incorporation, one for each of these phospholipid bases. The enzyme(s) mediating this incorporation may be unspecific for their lipid substrates

(PS, PE or PC). Incorporation is not via transphosphatidylation by phospholipase D.

Choline is also incorporated into PC by a calcium independent mechanism.

The existence of energy dependent pathways for the incorporation of serine, ethanolamine and choline into oat coleoptile microsomal phospholipids is uncertain.

These views are based upon the following points:

1) $CaCl_2$ is required for the incorporation of serine, ethanolamine and at least one reaction incorporating choline (Sect III.B.2.).

2) The following evidence indicates that serine, ethanolamine and choline are incorporated at three separate sites:

a) The mechanisms incorporating serine and ethanolamine respond very differently to ageing, heat treatment, ether and two sulfhydryl-group inhibitors (Sect III.B.11.). The indication is that distinct enzymic sites exist for serine and ethanolamine incorporation.

b) The K_i for ethanolamine is considerably higher than its K_m (Sect III.B.3. and Sect III.B.7.). This indicates that the site at which ethanolamine inhibits serine incorporation differs from the site at which ethanolamine is incorporated itself.

c) Choline does not inhibit L-serine-U-^{14}C or ethanolamine-1,2-^{14}C incorporation (Sect III.B.10.(d).). Inhibition would be expected if choline was incorporated at the same site as serine or ethanolamine. The slight stimulation of ethanol-

amine-1,2-^{14}C incorporation in the presence of equimolar choline is not easily explained and may be an artefact.

d) Ethanolamine was found to competitively inhibit the incorporation of L-serine-U-^{14}C into PS (Sect III.B.7.). Inhibition of ethanolamine-1,2-^{14}C by serine was found; the results of other workers indicate that this inhibition was probably also competitive. Inhibition at one site and incorporation at another would be expected because of similarities in the structure of serine and ethanolamine. The relatively different structure of choline would lessen the possibility of it being an inhibitor at a serine or ethanolamine site.

e) There are indications that the optimum pH for serine and ethanolamine incorporation are different (Sect III.B.1.)

3) The following evidence leads me to speculate that the enzyme or enzymes mediating the incorporation of serine and, by extension, ethanolamine and choline are not specific for a particular phospholipid substrate (this assumes that there is only one site for serine incorporation):

a) The pulse chase experiments (Sect III.B.6.) show that activity in PS, PC and PE decreased in the presence of unlabelled L-serine and $CaCl_2$. In the presence of L-serine and EDTA, PS and PE activity did not fall. The presence of a phospholipase D, known to require $CaCl_2$, may be the explanation of the above. However, phospholipase D type action was shown to be very low in microsomes under conditions which should cause stimulation of its activity (Sect III.A.4.). It may be that PC, PS and PE are participating in exchange reactions which result

in the incorporation of L-serine to form PS with the loss of activity in PC, PS and PE. If this is correct then the enzyme incorporating L-serine may be unspecific for phospholipid substrates.

b) The experiment (Sect III.B.9.) testing the proposed exchange mechanism of Borkenhagen et al. (1961) gave no direct evidence in favor of the reaction: S + PE$\rightleftharpoons$E + PS. However, the calculations presented are considered to show that a 1:1 ratio of PE lost to PS formed may be obtained by taking into consideration pools of PE and/or the use of other phospholipids by the exchange enzyme(s).

4) The following evidence and interpretation indicates that the incorporation of serine, ethanolamine and choline, studied here, is not due to the transphosphatidylase action of phospholipase D:

a) Various alcohols that are known acceptors for transphosphatidylation by phospholipase D (Yang et al., 1967) were tested to see if they inhibit serine and ethanolamine incorporation. The levels of inhibition found were insufficient to account for a competitive type mechanism as suggested in Section III.B.10.(d).

b) Addition of ether (Sect III.B.10.(c).) had an effect on the incorporation of serine and ethanolamine quite different from the effect it had on phospholipase D action (Yang et al., 1967). If incorporation was mediated by phospholipase D, similar responses should be expected for both substrates. In an earlier experiment (Sect III.A.4.) it was noted

that ethyl ether stimulated formation of PA, thus indicating the action of phospholipase D. If ether stimulates the hydrolytic action of phospholipase D it should also stimulate all its transphosphatidylation reactions (see Yang et al., 1967). Thus the results indicate that phospholipase D action is not involved in serine and ethanolamine incorporation. The effect of ether may be explained by the differing solubilities of serine and ethanolamine in ethyl ether.

c) The effect of heat treatment (Sect III.B.10.(b).) on the incorporation of serine and ethanolamine was quite different from its effect on phospholipase D.action. However, a valid comparison of the ability of any two enzyme systems to resist heat treatment can only be made with highly purified enzyme preparations.

d) The effects of the sulfhydryl-group inhibitors PCMB and NEM on the incorporation of serine and ethanolamine, studied here (Sect III.B.10.(a).), were identical with their effects on "phosphatidohydrolase" activity (Yang et al., 1967). The fact that the two sulfhydryl-group inhibitors have different effects when their mode of action is supposedly similar, gives support to the view that the enzyme system studied here may be phospholipase D. However, this similarity is weakened by the fact that nucleoside tetraphosphate hydrolase exhibits similar responses (see Yang et al., 1967).

e) If the incorporation of serine, ethanolamine and choline takes place at three different enzyme sites, and therefore possibly by three distinct enzymes, then this is evidence

against incorporation via transphosphatidylation by phospholipase D; which is believed to be a reaction catalyzed by a single enzyme.

5) Choline-1,2-^{14}C was found to be incorporated by a calcium independent reaction in addition to the calcium dependent reaction discussed above. This follows from the finding that choline-1,2-^{14}C incorporation differed from serine and ethanolamine incorporation in that 66% of choline incorporation, as measured here, was not inhibited by 0.005 M EDTA (Sect III.B. 2.). Results from studies of serine and ethanolamine incorporation indicate that 0.005 M EDTA binds all endogenous, free Ca^{++} (Table 1).

Three possibilities are suggested for this incorporation:

a) The incorporation is independent of divalent cations,

b) the incorporation is dependent on divalent cations which are already strongly bound onto reaction "sites" and

c) the incorporation is dependent on a divalent cation , other than Ca^{++} that is present in the microsomal fraction at a concentration greater than that which can be chelated by the available EDTA. This is an unlikely possibility considering the amount of dilution of the original homogenate.

The apparent independence of this reaction from divalent cations indicates that it is not a typical energy dependent incorporation. To my knowledge, this kind of incorporation of choline into PC has not been reported previously.

6) A brief study was done to determine whether or not energy dependent pathways exist for the incorporation of serine, ethanolamine or choline into microsomal phospholipids. The data show enhancement of incorporation by ATP plus CTP but give few clues as to the mechanism involved (Sect III.D.).

The low levels of incorporation of serine, ethanolamine and choline in the presence of ATP plus CTP, found here, can be interpreted as showing that various energy dependent enzyme systems are at very low levels in oat coleoptile microsomes. This interpretation is supported by the fact that Morré et al. (1970) have shown that choline kinase activity is predominantly in the soluble fraction.

The 29% increase in choline incorporation with ATP plus CTP corresponds to the 33% increase with 0.005 M $CaCl_2$ (Sect III.B.2.). An explanation may be that ATP and/or CTP act only by affecting calcium availability in an EDTA containing system. Such an effect involving ATP has been suggested by Artom (see Crone, 1967b) but, to my knowledge, the suggestion was not examined experimentally. It was found here that ATP caused more enhancement of serine incorporation than did CTP. The effect on choline incorporation of ATP and CTP separately was not tested. Should ATP produce greater enhancement than CTP the explanation involving calcium availability would be supported.

B. THE DECARBOXYLATION OF PHOSPHATIDYLSERINE

The results of studies on the incorporation of L-serine into PS and its subsequent fate have led to the following conclusions:

Phosphatidylserine is decarboxylated to form PE. The reaction appears to be mediated by a phosphatidylserine decarboxylase similar to that found in bacterial and animal systems. The activity of the decarboxylase is very low in oat coleoptile microsomal preparations.

The evidence and interpretations leading to these conclusions are as follows:

1) L-serine-U-^{14}C fed to whole coleoptiles was incorporated into PC, PS and PE (Sect III.A.2.) suggesting in vivo decarboxylation of PS to form PE.

2) Oat coleoptile microsomal fractions decarboxylated L-serine (Sect III.C.1.).

3) The low levels of serine decarboxylation (Sect III.C.1.) are comparable with the low levels of increase in activity in PE found during the pulse-chase experiment with EDTA (Sect III. B.6.); thus it is assumed that the two reactions are the same.

4) Pulse-chase experiments with L-serine-U-^{14}C (Sect III. B.6.) showed that activity in PE increased in the presence of EDTA; the most probable source of this activity was PS. PS decarboxylase in bacterial and animal systems operates in the presence of EDTA (Sect I.B.3.).

5) Ethanolamine incorporation into PE was completely inhibited in the presence of EDTA (Sect III.B.2.). Consideration of this and the previous point (#4) leads to rejection of the possibility that decarboxylation of free serine and subsequent incorporation of ethanolamine into PE is taking place.

6) Attempts to demonstrate the decarboxylation of PS by

feeding ^{14}C-labelled PS to microsomes was unsuccessful. The lowest levels of $^{14}CO_2$ release actually occurred in the presence of substances that are supposed to stimulate decarboxylation. For reasons given in Section III.C.2. the failure to show decarboxylation of ^{14}C-labelled PS by microsomes should not be used to reject the existence of a phosphatidylserine decarboxylase in oat coleoptile microsomal preparations.

7) Work using animal material has shown that the decarboxylase is localized in the mitochondrial fraction. The low levels of decarboxylation found here may therefore be due to the presence of mitochondrial fragments in the microsomal fraction. This implies that the ER (microsomes considered to be mainly ER fragments) of oat coleoptiles does not contain PS decarboxylase.

C. DEVELOPMENT

The preliminary data on developmental changes in the oat coleoptile raise the following points:

1) The steady increase in microsomal phospholipid per coleoptile (Fig 2) suggests the synthesis of membranous material throughout the whole period of cell expansion. However, an increase in ER and membrane-bound ribosomes has been found only in older coleoptiles (Setterfield, 1961). This may suggest the synthesis of membrane lipids without the actual formation of membranes. The rapid formation of cytoplasmic membranes in response to various stimuli (Whaley et al., 1964) may be a consequence of such preformed membrane components.

2) The data show that microsomal phospholipid content did

not increase as rapidly in coleoptiles of 40 to 50 mm in length as it did in shorter coleoptiles (Fig 4). This was unexpected because increases in ER membranes, together with increases in membrane-bound ribosomes, are reported to occur in older coleoptiles (Setterfield, 1961). An explanation may be a loss of recently formed, heavy membrane fragments sedimenting during the 37,000g centrifugation. If this is so it may mean that the results (Fig 4) which show a decrease in RNA content of preparations from older coleoptiles does not represent an actual decrease in ribosomes. The points raised here emphasize that microsomal preparations from different stages of development do not always represent the same membrane structures in the cell. This is, therefore, a complication in the use of microsomal preparations for the study of changes in the composition of membranes, and changes in phospholipid biosynthetic systems in membranes, with development

3) Considering the limitations discussed above (#2), the changes with development in the ability of the microsomal fraction to incorporate serine and ethanolamine into phospholipids (Sect III.E.) appear to be similar. However, the calculations of the ratio S/E (Sect III.E.) indicate that they may change independently with development. It would be of interest to know how such changes are related to the formation of membranes in the oat coleoptile. One must first answer the question: Do the pathways of incorporation occur in vivo and, if they do, how important are they when compared with other pathways that may be operating? No data are presented in this the-

sis that can answer this question other than the fact that whole coleoptiles incorporated serine into phospholipids.

SUMMARY

1) A study of the biosynthesis of various phospholipids in the oat coleoptile microsomal fraction was initiated. It is proposed that assays of these biosynthetic pathways during development can be used as indicators of membrane biosynthesis and give clues as to mechanisms of membrane biogenesis.

2) A review of the literature concerned with the biosynthesis of nitrogenous phospholipids in plants is presented. The following topics are covered: Energy dependent incorporation of phospholipids, the exchange reaction, decarboxylation of PS, methylation of PE to PC and transphosphatidylase action of phospholipase D. This review was completed August, 1971. A brief literature review of developmental changes in the ER and microsomal fraction of plants is also presented.

3) The various techniques used for this study are presented. In certain cases the value of the data they give is discussed.

4) Preliminary experiments have revealed the following:

a) The major phospholipids of whole coleoptiles and microsomes are PC, PS and PE,

b) L-serine-U-^{14}C, fed to whole coleoptiles, appeared most rapidly in neutral lipid as detected by radioautography,

c) thorough resuspension of microsomal pellets was necessary to obtain good replicates,

d)phospholipase D action, in the absence of ether, was not significantly stimulated by the Bligh and Dyer (1959) extraction procedure used in this work,

e) lipid phosphorus, protein and RNA content of the whole coleoptile increased during development of the coleoptile. RNA content decreased in coleoptiles of 40 - 50 mm length and

f) similar changes to (e) were found in the microsomal fraction.

5) Serine was incorporated into PS and ethanolamine into PE by oat coleoptile microsomal fractions. In the presence of 0.05 M Tricine-NaOH buffer, 0.005 M $CaCl_2$, optimum serine incorporation occurred at pH 8.0; optimum ethanolamine incorporation at pH 7.6.

6) Optimum calcium chloride concentration for serine and ethanolamine incorporation was 0.006 M. It is reasoned that optimum $CaCl_2$ concentration is related to enzyme and/or lipid substrate concentration but not to serine or ethanolamine concentration.

7) Choline appears to be incorporated by two separate reactions; one calcium dependent, the other calcium independent.

8) The K_m for L-serine was about 0.2 mM; for ethanolamine K_m= 0.4 mM.

9) L-serine and ethanolamine were incorporated, almost totally, into their respective phospholipids. Choline incorporation, however, resulted in PC having about 60% of the total activity (in PC+PS+PE).

10) Data from two pulse-chase type experiments are presented. A comparison of the two indicates:

a) PS was converted to PE and PC,

b) decarboxylation of PS to form PE took place in the

presence of EDTA and

c) PS, PC and PE may participate in exchange reactions incorporating serine into PS.

11) Ethanolamine competitively inhibited the incorporation of L-serine-U-^{14}C into PS. The K_i for ethanolamine was considerably higher than its K_m (for incorporation into PE). This indicates separate sites of action for ethanolamine; one where ethanolamine is incorporated into PE and one where ethanolamine acts as an inhibitor of serine incorporation. D-serine did not seem to readily inhibit incorporation of L-serine-U-^{14}C into PS. Choline-1,2-^{14}C did not inhibit serine incorporation and slightly enhanced ethanolamine incorporation.

12) No evidence was obtained for the participation of the exchange reaction of Borkenhagen et al. (1961) in incorporating L-serine into PS. The results may be explained by the fact that PC, PS and PE all act as lipid substrates for the exchange enzyme(s). It is also possible that only a certain pool of PE participates in the exchange reaction.

13) A series of experiments were performed which indicate that incorporation of serine and ethanolamine, as studied here, was not mediated by the transphosphatidylase action of phospholipase D.

14) A comparison of the rates of incorporation of serine and ethanolamine into microsomal phospholipids, under various conditions, indicate that separate sites of incorporation exist for serine and ethanolamine. A separate site is also indicated for choline incorporation.

15) Experiments were performed which demonstrate that low levels of PS decarboxylation exist in the microsomal fraction. Decarboxylation of PS could not be demonstrated by using exogenously supplied ^{14}C-labelled PS.

16) The existence of energy dependent pathways for the incorporation of serine, ethanolamine and choline into oat coleoptile microsomal phospholipids is uncertain. There was some indication that, for serine incorporation, ATP gave more stimulation than did CTP.

17) A study was made of the incorporation of serine and ethanolamine into phospholipids of microsomes prepared from coleoptiles of increasing length. Changes in rates of serine and ethanolamine incorporation with development are presented.

18) The discussion covers:

a) The pathways incorporating serine, ethanolamine and choline into oat coleoptile microsomal phospholipids,

b) the decarboxylation of PS and

c) the development of the whole coleoptile and coleoptile microsomal fraction.

CLAIM OF ORIGINALITY

1) Evidence is presented which has been interpreted to mean that three distinct sites exist for serine, ethanolamine and choline incorporation, by way of calcium stimulated reactions, into oat coleoptile microsomal phospholipids. The interpretation of the majority of workers, only one of whom used plant material, is that incorporation is mediated by a single enzyme or single enzyme system.

2) Choline-1,2-^{14}C has been found to be incorporated into oat coleoptile microsomal phospholipids by a pathway that is apparently independent of divalent cations. This kind of incorporation of choline into PC, to the best of my knowledge, is unique.

3) Indirect evidence has led to the conclusion that PS is decarboxylated to form PE. The results cannot be explained as decarboxylation of serine to ethanolamine and its subsequent incorporation into PE. The reaction appears to be mediated by a PS decarboxylase similar to that found in animal and bacterial systems in that it operates in the presence of EDTA.

4) Preliminary biochemical work is presented on the development of the oat coleoptile and the oat coleoptile microsomal fraction. The results of assays of phospholipid content and assays of enzyme activities mediating the biosynthesis of microsomal phospholipids, throughout development, are presented.

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