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THE PERTURBATION OF PHOSPHOLIPID METABOLISM IN ESCHERICHIA COLI.

The City University of New York, Ph.D., 1972
Biochemistry

THE PERTURBATION OF PHOSPHOLIPID METABOLISM IN *ESCHERICHIA COLI*

by

WILLIAM DAVID NUNN

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

1972

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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To Geraldyne, Adrienne, and my family

Acknowledgments

I wish to express my sincere gratitude to Dr. Burton E. Tropp for his role as educator and advisor to me.

Special thanks are due Charles Shopsis, Linda Meade, and David Klein for their helpful advice and interest.

To Susan Emry, I express my deepest gratitude for her technical assistance and suggestions in preparing the manuscript.

Abstract

THE PERTURBATION OF PHOSPHOLIPID METABOLISM

IN ESCHERICHIA COLI

by

WILLIAM DAVID NUNN

Adviser: Associate Professor Burton E. Tropp

The incorporation of labeled precursors into the DNA, RNA, protein, and phospholipids of Escherichia coli cultured in the presence of phenethyl alcohol (PEA) was determined. PEA inhibits the uptake of labeled uracil to the same extent in cells exhibiting relaxed and stringent control of RNA synthesis. This indicates that PEA does not primarily affect amino acid synthesis or activation. Uptake of labeled acetate into the phospholipid fraction is more sensitive to inhibition by low concentrations of PEA than is the uptake of labeled precursors into the macromolecules. Thymine starvation or the addition of nalidixic acid has no effect on acetate incorporation. Chloramphenicol is a much less effective inhibitor of acetate incorporation than is PEA. The distribution of labeled acetate incorporated into phospholipids is markedly affected by the presence of PEA. The uptake of acetate into phosphatidylethanolamine and phosphatidylglycerol is inhibited while the uptake of acetate into the cardiolipin fraction is unaffected.

The phosphonic acid analogue of glycerol-3-phosphate,

3,4-dihydroxybutyl-1-phosphonate, was shown to be a competitive inhibitor of L-glycerol-3-phosphate:CMP phosphatidyltransferase. The three-carbon phosphonic acid analogue, 2,3-dihydroxypropyl-1-phosphonate, does not act as effectively as a competitive inhibitor of this enzyme. Phenethyl alcohol had no effect on this enzyme's activity. Neither the phosphonic acid analogues nor PEA had any effect on the enzymatic activity of L-serine:CMP phosphatidyltransferase.

Table of Contents

CHAPTER 1

INTRODUCTION

Biological membranes are composed mainly of lipid and protein. In Escherichia coli (E. coli), about 30 percent of the dry mass of membrane is lipid (1). The lipids consist mainly of phospholipids (2), which are believed to play important roles in various processes carried out by the membrane (3-8). Although synthetic pathways of bacterial phospholipids are known (9-11), the functions of the different phospholipid classes in regulatory metabolism of cellular processes are largely unknown. Studies involving perturbation of phospholipid metabolism have contributed somewhat to our knowledge of the relationship of various lipids to such membrane functions as active transport, cellular structure, macromolecular biosynthesis, energy metabolism, and cell

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2. J. H. Law, Bacteriological Proceedings, 129 (1961).
3. C. F. Fox, Proc. Nat. Acad. Sci. U.S.A. 63, 850 (1969).
4. L. Rothfield and A. Finkelstein, Ann. Rev. Biochem. 37, 463 (1968).
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6. P. S. Sastry and L. E. Hokin, J. Biol. Chem. 241, 3354 (1966).
7. J. Starka and J. Morova, J. Gen. Microbiol. 60, 251 (1970).
8. D. C. White and A. N. Tucker, J. Bacteriol. 97, 199 (1969).
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division. Some of the approaches undertaken to delineate the role of membrane phospholipids in cellular processes have been studies involving lipid mutants, metabolic inhibitors, amino acid starvation, and changes in cultural conditions.

The isolation of E. coli auxotrophs defective in the biosynthesis of unsaturated fatty acids was first accomplished by Silbert and Vagelos (12) and subsequently by other laboratories (13, 14). These mutants are capable of synthesizing saturated fatty acids but have lost their capacity to make unsaturated fatty acids (12). Cronan, Birge, and Vagelos (15) have demonstrated the existence of two genes (cistrons) involved in unsaturated fatty acid biosynthesis. One of these, designated fab A, has been characterized (12) as deficient in β-hydroxydecanoyl thioester dehydrase which is the enzyme responsible for the formation of cis-Δ^3-decanoyl acyl carrier protein (ACP), the first intermediate in the biosynthesis of unsaturated fatty acids of E. coli (16). The other cistron has an enzymatic defect which has not been identified yet (15). In addition, to natural constituents, these mutants can be supplemented with a broad spectrum of cis- and trans-unsaturated fatty acids not normally present in E. coli. Studies

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12. D. F. Silbert and P. R. Vagelos, Proc. Nat. Acad. Sci. U.S.A. 58, 1579 (1967).

13. U. Henning, G. Dennert, K. Rehn, and G. Deppe, J. Bacteriol. 98, 784 (1969).

14. P. Overath, E-M. Raufuss, W. Stoffel, and W. Ecker, Biochem. Biophys. Res. Commun. 29, 28 (1967).

15. J. E. Cronan, Jr., C. H. Birge, and P. R. Vagelos, J. Bacteriol. 100, 601 (1969).

16. K. Bloch, P. Baronowsky, H. Goldfine, W. J. Lennarz, R. Light, A. T. Norris, and G. Schauerbrandt, Fed. Proc. 20, 921 (1961).

of structural and functional changes in biological membranes have been made possible by varying the unsaturated fatty acyl moieties of the phospholipids of these mutants. Silbert et al. observed that the supplementation of unsaturated fatty acid auxotrophs with exogenous fatty acids of unusual geometry causes altered growth (12). If these auxotrophs are supplemented with fatty acids which support growth poorly, their growth rate is altered and the cells tend either to lyse or grow as nonseptated filaments (12). When these mutants are starved of an appropriate unsaturated fatty acid, the cell continues to grow and divide for a generation before lysis and cell death occur (12). Henning, Dennert, Rehn, and Deppe observed that the removal of fatty acids from unsaturated fatty acid auxotrophs resulted in the inhibition of DNA, RNA, and to a slight extent protein synthesis (13). In addition, they observed that the deprival of unsaturated fatty acids from these mutants resulted in a shift in the proportion of phospholipids in the membrane such that there was a decrease in phosphatidylglycerol and an increase in cardiolipin (13). Fox utilized the unsaturated fatty acid auxotrophs to examine the function of phospholipids in the synthesis of the β-galactoside transport system (3). His experiments demonstrated that unsaturated fatty acids are required for the induction of the β-galactoside transport system (3). On the other hand, results of experiments by Overath, Hill, and Lamnek (17) are in direct conflict with those of Fox. These workers found that a functional β-galactoside transport system could be induced after

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17. P. Overath, F. F. Hill, and I. Lamnek, Nature New Biology, in press.

removal of the required unsaturated fatty acids from an unsaturated fatty acid auxotroph (17). In view of the conflicting data, additional studies should be performed to resolve the differences. Esfahani, Ioneda, and Wakil have reported that E. coli have a control mechanism which minimizes differences in the physical properties of their phospholipids (18). They showed that an unsaturated fatty acid auxotroph incorporated exogenous fatty acids of various chain lengths in such a way that the ratio of unsaturated to saturated fatty acids incorporated into phospholipids increases with increasing chain length or decreasing number of double bonds (18).

A second type of lipid mutant which is unable to synthesize or catabolize glycerol was isolated by Mindich (19). These mutants of Bacillus subtilis require glycerol for net synthesis of phospholipid, and almost all of the labeled glycerol added to cultures of these mutants can be recovered in the lipid extract (19). During glycerol starvation, the rate of fatty acid synthesis slows to 25 percent of that observed during exponential growth with glycerol. Although there was no net synthesis of phospholipid, the lipid composition of the membrane was altered dramatically during glycerol starvation (20). This alteration in phospholipid composition was manifested by a decrease in phosphatidyl glycerol and an accumulation of cardiolipin (20). When the glycerol starved mutants were supplemented with glycerol, the relative proportion of phosphatidylglycerol and cardiolipin were restored to

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18. M. Esfahani, T. Ioneda, and S. Wakil, J. Biol. Chem. 246, 50 (1971).
19. L. Mindich, J. Mol. Biol. 49, 415 (1970).
20. T. T. Lillich and D. C. White, J. Bacteriol. 107, 790 (1971).

the predeprivation membrane composition (20). When these mutants are deprived of glycerol, they undergo a slow increase in cell density and remain viable for at least 4 hours (19). In other experiments, Mindich showed that, during the period of slow increase in cell mass in the absence of net lipid synthesis, the mutant maintained a slow synthesis of DNA until a doubling of the total DNA occurred. These cells were able to initiate a new round of DNA synthesis (19). In the absence of glycerol, these mutants support bacteriophage growth and induced enzyme formation. Ribosomal RNA synthesis is drastically inhibited when these mutants are deprived of glycerol (19). Uptake of labeled leucine into the cell membrane continues at the same rate as total protein synthesis in the deprived cultures, resulting in membranes with increased density (19). These results complement those of Hsu and Fox (21), who observed that DNA and protein synthesis continue for a short period of time even though phospholipid synthesis had ceased immediately after removal of glycerol from E. coli glycerol auxotrophs. In addition, these authors observed that the blocking of lipid synthesis by glycerol deprivation resulted in a decrease in the inducibility of the β-galactoside transport system (21). In contrast to these results, Willecke and Mindich, using glycerol auxotrophs of B. subtilis, reported that the citrate transport system could be induced in the absence of glycerol (22). In support of the data of Willecke et al., Mindich found that net lipid synthesis is not required for the induction of lactose permease in

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21. C. Hsu and C. F. Fox, J. Bacteriol. 103, 410 (1970).

22. K. Willecke and L. Mindich, J. Bacteriol. 106, 514 (1971).

Staphylococcus aureus (23). Therefore, it seems that the dependence on phospholipid synthesis for the formation of an active transport system is not a general phenomena.

Perturbations of the asymmetric fatty acid acylation of sn-glycerol-3-phosphate which normally results in the formation of phosphatidic acid can produce dramatic disturbances in cellular processes. Therefore, enzyme mechanisms which confer positional specificity of fatty acids, such that unsaturated fatty acids are preferentially esterified at position 2 and saturated fatty acids at position 1 of the glycerol molecule (24), are of central importance to the cell. The isolation of temperature sensitive mutants of E. coli which were incapable of completing the acylation sequence has contributed immensely to the understanding of these mechanisms. The first acyltransferase mutants were found by Cronan, Ray, and Vagelos (25) and were isolated by a selection technique based on killing of non-mutant cells with tritiated sn-glycerol-3-phosphate. Reversion and enzymatic (25) analysis of temperature resistant revertants have indicated that the phenotype of this mutant is due to a mutation which has been mapped at a single locus, called plsa (26). This mutant contains normal monoacyl-

---

23. L. Mindich, Proc. Nat. Acad. Sci. U.S.A. 68, 420 (1971).

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26. J. E. Cronan, Jr., and G. N. Godson, Molecular and General Genetics, in press.

transferase activity and thermolabile sn-glycerol-3-phosphate acyl-transferase activity (25). Hechemy and Goldfine (27) have isolated temperature sensitive mutants which are thermolabile for monoacyl-sn-glycerol-3-phosphate activity but normal for sn-glycerol-3-phosphate acyltransferase activity. The existence of these two types of mutants establish that at least two different enzymes are required for the conversion of sn-glycerol-3-phosphate to phosphatidic acid. The enzymatic reactions for each of these enzymes are listed below:

[1] sn-glycerol-3-phosphate + acyl CoA →
monoacyl-sn-glycerol-3-phosphate + CoA

[2] monoacyl-sn-glycerol-3-phosphate + acyl CoA →
phosphatidic acid + CoA

When the temperature sensitive acyltransferase mutants are shifted to nonpermissive temperatures, there is an immediate and profound decrease in phospholipid synthesis followed by an abrupt halt in cell growth (28) and the loss of viability. Cell death is probably due to the irreversibility of the enzyme inactivation (25). Preliminary studies involving these mutants at the nonpermissive temperature indicate that there is some inhibition of DNA, RNA, and protein synthesis (27). Further kinetic studies of cellular processes in these mutants should be performed for the purpose of determining whether or not there is a temporal relationship between this form of perturbation of phospholipid synthesis and some specific macromolecular synthesizing process.

---

27. K. Hechemy and H. Goldfine, Biochem. Biophys. Res. Commun. 42, 245 (1971).

28. J. E. Cronan, Jr., personal communication.

By using a variation of the tritiated suicide technique, Cronan has isolated 20 temperature sensitive mutants which form all three E. coli phospholipids--cardiolipin, phosphatidylglycerol, and phosphatidylethanolamine--at 25°C but form only the first two lipids at 37°C (28). However, studies correlating specific dependence of a cellular process to the presence of this phospholipid must await further characterization of these mutants.

Recently, Lusk and Kennedy (29) isolated a mutant of E. coli which is inhibited by low concentrations of NaCl. This mutant has a normal metal content, but it cannot tolerate levels of sodium to which the wild type is indifferent (29). The addition of NaCl to cultures of these mutants results in a decrease in the synthesis of phosphatidylethanolamine and an increase in the synthesis of cardiolipin while apparently causing no effect on DNA, RNA, protein, and total lipid synthesis (29). The enzyme(s) directly responsible for these changes in phospholipid synthesis is not known.

Several laboratories have studied phospholipid metabolism under conditions in which various metabolic processes had been inhibited. From such studies, Ballesta and Schaechter (30) concluded that phospholipid synthesis in E. coli can be dissociated from macromolecular biosynthesis. They demonstrated that the inhibition of protein synthesis by chlortetracycline, of DNA synthesis by mitomycin, or the multiple effects of levallorphan (31) did not result in short-term inhibition

---

29. J. E. Lusk and E. P. Kennedy, J. Bacteriol. 109, 1034 (1972).

30. J. P. G. Ballesta and M. Schaechter, J. Bacteriol. 107, 251 (1971).

31. F. F. Gale, Mol. Pharmacol. 6, 134 (1970).

of phospholipid synthesis (30). Sokowa, Nakuo, and Kaziro obtained similar results when they used chloramphenicol to inhibit protein synthesis (32). However, no studies involving the distribution of label into the three major phospholipids were performed with chloramphenicol treated bacteria.

Treatment of E. coli with the morphine analogue, levorphanol, resulted in a decrease in the uptake of labeled inorganic phosphorus (^{32}P) into the total lipid fraction (33). This drug had been reported to inhibit bacterial growth (34) and ribosomal RNA synthesis (35). In addition, it was demonstrated that levorphanol caused the accelerated efflux of ATP, GTP, putrescine, amino acids, and potassium ion from the cells into the medium (36, 37). Since these results suggested that levorphanol affected the membrane, Wurster, Elsbach, Rand, and Simon investigated the effects of this drug on the synthesis and distribution of the major phospholipids in E. coli (33). They not only found that lipid synthesis was inhibited but that the labeling of membrane phospholipids was strikingly altered (33). After 60 minutes incubation with levorphanol the relative amounts of individual phospholipids were

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33. N. Wurster, P. Elsbach, J. Rand, and E. J. Simon, Biochim. Biophys. Acta 248, 252 (1971).

34. E. J. Simon, Science 144, 543 (1966).

35. E. J. Simon and D. Van Praag, Proc. Nat. Acad. Sci. U.S.A. 51, 877 (1964).

36. R. Greene and B. Magasnik, Mol. Pharmacol. 3, 453 (1967).

37. E. J. Simon, L. Schapira, and N. Wurster, Mol. Pharmacol. 6, 577 (1970).

45 percent phosphatidylethanolamine, 10 percent phosphatidylglycerol, and 45 percent cardiolipin, as compared with 67 percent phosphatidylethanolamine, 25 percent phosphatidylglycerol, and 7 percent cardiolipin in untreated bacteria (33). This altered lipid composition could be reversed to normal when the drug was removed from the medium (33). It is still not clear, though, whether or not the effect of levorphanol on lipid synthesis is primary or secondary.

In view of the recent findings that phospholipids are found not only in the cell membrane but also in the cell wall (38, 39, 40), the studies of Starka and Morova (7), involving the effect of penicillin on phospholipid synthesis, are of interest. These workers reported that low dosages of penicillin inhibited cell division but did not affect DNA, RNA, or protein synthesis. Although penicillin had no effect on the total rate of phospholipid synthesis, it did alter the ratio of individual phospholipids from that characteristic of untreated bacteria (7). The treated cells were found to contain more cardiolipin and less phosphatidylglycerol than normal dividing cells (7). When penicillinase was added to the penicillin treated cells, cell division commenced and the normal phospholipid distribution was restored (7). On the basis of these results, Starka and Morova (7) suggested that phospholipid synthesis may play an important role in the cell division process.

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38. T. Miura and S. Mizushima, Biochim. Biophys. Acta 150, 159 (1968).

39. C. F. Fox, J. H. Law, T. Tsukagashi, and G. Wilson, Proc. Nat. Acad. Sci. U.S.A. 67, 598 (1970).

40. C. A. Schnaitman, J. Bacteriol. 104, 882 (1970).

The synthesis of the major phospholipids was also affected by various inhibitors of cellular phosphorylation such as cyanide (30, 41), forced anaerobsis (30), dinitrophenol (30), and benzo(a)pyrene. (42). These respiratory poisons caused a decrease in the rate of phospholipid synthesis. An analysis of the distribution of the label among the individual phospholipids after treatment with any of these inhibitors indicates that there is a greater net accumulation of phosphatidylglycerol than of phosphatidylethanolamine (30, 41, 42). These results have led some workers to propose that phosphatidylglycerol plays an important role in the respiration process (42).

Sokowa et al. (32) have shown that lipid synthesis is inhibited in stringent cells deprived of an amino acid. In contrast, Tropp, Meade, and Thomas reported that the rate of lipid synthesis was not affected by the presence or absence of a required amino acid in either relaxed or stringent cells (43). Recent experiments by Meade, Nunn, and Tropp (44) provide an explanation for the differences. They found that under conditions of lowered aeration, the results of Tropp et al. (43) were obtained. When the rate of aeration was constant, the results of Sokowa et al. (32) were obtained. On the basis of these findings, Meade et al. (44) have concluded that the relaxed phenotype affects

---

41. R. Peterson and C. Buller, J. Virology 3, 463 (1969).

42. G. H. Joyce and D. C. White, J. Bacteriol. 106, 403 (1971).

43. B. E. Tropp, L. C. Meade, and P. J. Thomas, J. Biol. Chem. 245, 855 (1970).

44. L. C. Meade, W. D. Nunn, and B. E. Tropp, unpublished data.

lipid synthesis only under certain conditions, and this effect was secondary to the effect on RNA synthesis.

Other studies have been undertaken to correlate phospholipid synthesis with changes in cultural conditions. Four changes in phospholipid composition occur during the transition of E. coli cultures from exponential growth to stationary growth phase (45, 46). These changes are an increase in cardiolipin, a decrease in phosphatidylglycerol, an increase in cyclopropane fatty acids, and a decrease in unsaturated fatty acids (46).

The amount of lipid in E. coli has been shown to be independent of the growth medium used (46, 47, 48). Various carbon sources had no effect on the amount or proportion of phospholipids synthesized (46). Some of the carbon sources tested were glucose, glycerol, acetate, succinate, amino acids, and fatty acids (46).

Ballesta and Schaechter (30) demonstrated that the shift down of E. coli from rich to poor medium resulted in the inhibition of phosphatidylethanolamine synthesis and the incorporation of radioactive glycerol into the distal portion of phosphatidylglycerol. When cells are shifted down from rich to poor medium, the cells stop growing but continue to

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45. J. E. Cronan, Jr., J. Bacteriol. 95, 2054 (1968).

46. J. E. Cronan, Jr., and P. R. Vagelos, Biochim. Biophys. Acta 265, 25 (1972).

47. A. P. Damaglau and E. A. Dawes, Biochem. J. 110, 775 (1968).

48. H. Kamayama, K. Higashio, and A. Gato, J. Agr. Chem. Soc. Japan 43, 273 (1969).

divide for some time (49). On the basis of their experiments, Ballesta and Schaechter (30) concluded that phosphatidylethanolamine synthesis is related to growth and cell division.

Several laboratories have observed that E. coli adjusts the fatty acid composition of its phospholipids in response to a change in the growth temperature (46, 50, 51). When there is a decrease in the growth temperature, there is a corresponding increase in the ratio of unsaturated to saturated fatty acids in the membrane (46). The lipids isolated at various temperatures have been found to have different physical properties (46). The higher the growth temperature of E. coli cultures, the greater the proportion of saturated fatty acids in the membrane (52). When the temperature of the bacterial growth culture is increased, the permeability of liposomes derived from such cells decreases (46). These changes are attributed to the change in fatty acid composition (46) as the relative proportion of phospholipids does not change at different temperatures (46). The explanation frequently given for the changes in fatty acid composition of phospholipids in response to temperature changes stresses the necessity of maintaining

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49. O. N. Kjeldgaard, O. Maaloe, and M. Schaechter, Gen. Microbiol. 19, 607 (1958).

50. A. G. Marr and J. L. Ingram, J. Bacteriol. 84, 1260 (1962).

51. C. W. M. Haest, J. Degier, and L. L. M. Van Deenan, Chem. Phys. Lipids 3, 413 (1969).

52. M. Sinensky, J. Bacteriol. 106, 449 (1971).

the proper degree of membrane fluidity (51, 53, 54, 55). Sinensky has shown that the temperature effect is mediated at the enzymatic level rather than at the genetic level (52). He noted that the acyltransferases, sn-glycerol-3-phosphate and monoacyl-sn-glycerol-3-phosphate, are the primary enzymes responsible for the temperature adaptations.

The work described in this thesis has been designed to examine the particular perturbation of phospholipid synthesis produced by phenethyl alcohol, and to determine whether or not this perturbation is an indirect consequence of some inhibitory action on one of the other cellular processes. Attempts are made to elucidate whether any of the resulting changes in cellular processes are primarily accountable to altered phospholipid synthesis. In vitro studies of two enzymes involved in phospholipid biosynthesis are performed for the specific purpose of determining whether phenethyl alcohol or a phosphonic acid analogue of glycerol-3-phosphate has any effect at these enzyme-substrate levels.

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53. K. E. Knivett and J. Cullen, Biochem. J. 96, 771 (1965).

54. J. M. Steim, Proc. Nat. Acad. Sci. U.S.A. 63, 104 (1969).

55. G. Wilson, S. Roie, and C. F. Fox, Biochem. Biophys. Res. Commun. 38, 617 (1970).

CHAPTER 2

EFFECT OF PHENETHYL ALCOHOL ON PHOSPHOLIPID SYNTHESIS

Introduction (1)

Phenethyl alcohol (PEA) is a bacteriostatic agent which inhibits the growth of many gram-negative bacteria (2, 3). Berrah and Konetzka (2) reported that PEA acts by selectively inhibiting deoxyribonucleic acid (DNA) synthesis in Escherichia coli. Soon after, Treick and Konetzka (4) observed that the addition of PEA to a culture of E. coli in exponential-growth phase inhibited DNA synthesis after an initial increase in DNA corresponding to 1.4 to 1.6 times the amount of DNA present at the time of addition. In addition, they observed that protein and ribonucleic acid (RNA) synthesis continued after the cessation of DNA synthesis. These workers noted the similarity between the effects of PEA addition and amino acid deprivation upon DNA replication. They suggested that PEA prevents the initiation of DNA synthesis (4). Subsequently, Lark and Lark (5) extended this hypothesis by proposing that two proteins were required for the initiation of DNA synthesis. The synthesis of one of the proteins was postulated to be sensitive to PEA

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1. The studies reported in this chapter have been published. W. D. Nunn and B. E. Tropp, J. Bacteriol. 109, 162 (1972).

2. G. Berrah and W. A. Konetzka, J. Bacteriol. 83, 738 (1962).

3. B. O. Lilley and J. H. Brewer, J. Amer. Pharm. Ass. Sci. Ed. 42, 6 (1953).

4. R. W. Treick and W. A. Konetzka, J. Bacteriol. 88, 1580 (1964).

5. K. G. Lark and C. Lark, J. Mol. Biol. 20, 9 (1966).

and insensitive to chloramphenicol. The other protein was postulated to be sensitive to chloramphenicol but insensitive to PEA.

However, there has been difficulty in several laboratories in finding conditions for the selective inhibition of DNA synthesis while protein and RNA synthesis continued. Rosenkranz, Carr, and Rose (6, 7) reexamined the effects of PEA on DNA, RNA, and protein synthesis and investigated the effect of PEA on the induction of alkaline phosphatase and β-galactosidase synthesis. From these studies, they concluded that PEA exerts its primary effects on RNA synthesis and on the induction of enzyme synthesis. The kinetic data of Prevost and Moses (8) tend to support the conclusion that RNA synthesis is more sensitive to PEA than is DNA synthesis, but not the conclusion concerning the induction of enzyme synthesis. Recent studies suggest that PEA does not inhibit the transcription or translation steps involved in the formation of alkaline phosphatase but does inhibit the conversion of inactive monomeric subunits into the active form of the enzyme (9).

PEA has been shown to exert profound effects on the membrane of

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6. H. S. Rosenkranz, H. S. Carr, and H. M. Rose, Biochem. Biophys. Res. Commun. 17, 196 (1964).

7. H. S. Rosenkranz, H. S. Carr, and H. M. Rose, J. Bacteriol. 89, 1354 (1965).

8. C. Prevost and V. Moses, J. Bacteriol. 91, 1446 (1966).

9. R. C. Tribhuvan, A. K. Pilgaokar, D. S. Pradhan, and A. Screenivasan, Biochem. Biophys. Res. Commun. 41, 244 (1970).

E. coli and Neurospora crassa (N. crassa) (10-12). Lester demonstrated that PEA alters the permeability of N. crassa to a variety of amino acids (10). Silver and Wendt (11) observed that PEA caused E. coli to take up large amounts of acriflavine, a compound normally impermeable to healthy growing cells. In addition, they noted that PEA caused the accelerated efflux of cellular potassium. On the basis of their findings, Silver and Wendt proposed that the primary effect of PEA is a limited breakdown of the cell membrane and that the inhibition of DNA and other cellular processes is a result of the alteration in the membrane structure.

All of these experimental results concerning the mode of action of PEA might be reconciled if PEA: (i) inhibits the synthesis of an essential protein; (ii) affects some aspect of phospholipid synthesis; or (iii) alters membrane structure. The studies described in this chapter were primarily designed to investigate the first two possibilities. This chapter also describes some of the changes that we have observed in phospholipid metabolism of E. coli cultured in the presence of PEA and other metabolic inhibitors. Phospholipid synthesis was monitored by measuring the incorporation of labeled acetate into the phospholipid fraction. PEA has a very marked effect on phospholipid metabolism.

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11. S. Silver and L. Wendt, J. Bacteriol. 93, 560 (1967).

12. T. Yura and C. Wada, Genetics 59, 177 (1967).

Materials and Methods

Chemicals. Uracil-6-^{3}H (3.1 Ci per mmole) and acetate-2-^{14}C (39 mCi per mmole) were purchased from Schwarz/Mann, Orangeburg, N.Y.; L-phenylalanine-^{3}H (20 Ci per mmole), thymine methyl-^{3}H (13.6 Ci per mmole), and L-isoleucine-^{3}H (1 mCi per 0.087 mg) were products purchased from New England Nuclear Corp., Boston, Mass. Phenethyl alcohol (PEA) was obtained from Matheson, Coleman and Bell. Nalidixic acid was a product of Schwarz/Mann, Orangeburg, N.Y. Chloramphenicol was purchased from Parke, Davis and Company, Detroit, Mich. Supelcosil silica gel 12A and the "chromatographically pure" phospholipids, bacterial phosphatidylethanolamine, cardiolipin, and phosphatidylglycerol, were obtained from Supelco, Inc., Bellefonte, Pa. The scintillation fluid used in all experiments for monitoring ^{14}C and ^{3}H was as previously described (13). All other chemicals were of reagent grade and all solvents were distilled before use.

Bacterial strains and media. Escherichia coli W3110 (pol^{+}, $endI^{+}$), a thymine requiring derivative of E. coli K-12, was generously provided by Dr. J. Cairns. E. coli PA1 (arg^{-}, rel^{-}) and E. coli PA2 (arg^{-}, rel^{+}), an isogenic pair except for the rel locus, belong to the TLB_1 family of E. coli K-12. The bacteria were kindly provided by Dr. R. Lavallé. The bacteria were grown in synthetic medium of Davis and Mingioli (14), which for E. coli W3110 were supplemented with 2 mg thymine per liter.

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13. B. E. Tropp, L. C. Meade, and P. J. Thomas, J. Biol. Chem. 245, 855 (1970).

14. B. D. Davis and E. S. Mingioli, J. Bacteriol. 60, 17 (1950).

The growth medium for PA1 and PA2 was supplemented with thiamine-HCl, 0.5 mg per liter, and L-arginine, 100 mg per liter. Glucose was added to all media to a final concentration of 0.5 percent. All cultures were incubated with adequate aeration at 37°C. Fully supplemented overnight cultures were then incubated at 37°C in a New Brunswick Metabolyte water-bath shaker, model G77, at 200 cycles per minute. Cell growth was determined at 660 nm by turbidimetric readings on a Klett-Summerson colorimeter. All experiments were initiated when the turbidity reached 40 to 50 Klett units.

Medium changes. In those experiments in which the addition of PEA was the only change in the culture medium, the amounts added varied from 0.0 to 0.20 percent v/v (refer to the figures). Otherwise, to change growth media or remove supplements, cells were chilled and then harvested at 4°C in the Sorvall RC-2 centrifuge in an SS-34 rotor at 10,000 r.p.m. for 5 minutes. The cells were then washed twice with unsupplemented synthetic medium. One-half the volume of the initial aliquot was used in each washing. The cells were then resuspended in the original volume of synthetic medium and supplements were added to give the desired experimental conditions. The following inhibitors of biosynthetic processes were added to the culture medium as indicated: nalidixic acid (10 μg per ml); valine (250 μg per ml); and chloramphenicol (25 μg per ml).

Assays for macromolecular synthesis. To detect changes in the rate of cellular macromolecular synthesis, radioactive precursors and PEA were added simultaneously. Incorporation of label into protein was determined by a slight modification of the procedure of Byfield

and Scherbaum (13, 15). The culture medium for this procedure contained L-isoleucine (15 μg per ml) and L-isoleucine-^{3}H (0.15 μCi per ml) or L-phenylalanine (10 μg per ml) and L-phenylalanine-^{3}H (0.20 μCi per ml) as supplements. Aliquots containing 0.1-ml samples of the culture were spotted on Whatman No. 3MM filter paper squares which were immediately immersed in 5% trichloroacetic acid (TCA) and treated as previously described (13). The dry squares were counted in toluene scintillator fluid using the Beckman model LS-200 scintillation counter. RNA and DNA synthesis were followed by measuring the incorporation of labeled uracil and thymine by the filter square method described in the protein assay. The culture medium supplements for the RNA assay were as previously described (13). Thymine (2 μg per ml) and thymine-methyl-^{3}H (1.5 μCi per ml) were added to the culture medium used for measuring DNA synthesis.

Assay for lipid synthesis. The synthesis of lipids was followed by measuring the incorporation of labeled acetate. The culture medium was supplemented with potassium acetate (100 μg per ml) and acetate-2-^{14}C (0.02 μCi per ml). The PEA and untreated cultures were incubated at 37°C for 90 minutes, during which period duplicate samples were removed at various time intervals for the determination of radioactivity in the lipid fraction. Zero time was always designated as the time of PEA addition. Great care was taken to insure that the rate of shaking was not varied during any of the incubations, since such variation can complicate the interpretation of experiments on lipid metabolism (16).

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15. J. Byfield and O. Scherbaum, Anal. Biochem. 17, 434 (1966).

16. L. C. Meade, W. D. Nunn, and B. E. Tropp, unpublished data.

The 2-ml samples were removed, immediately mixed with an equal volume of chilled carrier cells, and then centrifuged in the cold. After all the samples had been collected, the pellets were washed once with 2 ml of synthetic medium and extracted overnight with 4 ml of 3:1 chloroform-methanol at room temperature. The extracts were then washed three times with 1.0 ml of distilled H_2O, placed in scintillator vials, and dried by overnight evaporation or by heating at temperatures below 50°C. The radioactivity was determined by dissolving the dried extract in toluene scintillator fluid and counting it in the scintillator counter.

Extraction of phospholipids. In experiments in which the effect of PEA, nalidixic acid, valine, or chloramphenicol addition or of thymine starvation on the distribution of labeled acetate was to be studied, potassium acetate (100 µg per ml) and acetate-2-^{14}C (0.04 µCi per ml) were added to the culture medium. Treated and untreated cultures were then incubated at 37°C with shaking, and at various time intervals flasks containing 50 ml of culture medium were removed for the analysis of phospholipids. The extraction method used was essentially that of Tropp, Meade, and Thomas (13). Similar results were obtained with the procedure of Kanfer and Kennedy (17).

Fractionation of phospholipids. After extraction, the chloroform was evaporated. The lipids, redissolved in 1 ml of chloroform, were used for subsequent analysis. The phospholipids were resolved by thin-layer chromatography. The preparation of plates and the thin-layer chromatography were performed using the following system: activated

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17. J. Kanfer and E. P. Kennedy, J. Biol. Chem. 239, 1720 (1964).

Supelcosil silica gel 12A was used as the adsorbent in a two-step developing system with acetone-light petroleum (1:3) as the first solvent and chloroform-methanol-water (65:25:3) as the second solvent. A Desaga-Brinkman spreader was used to apply the adsorbent, 0.5 mm thick, onto 20 x 20 cm glass plates. The adsorbent was prepared as a slurry mixture of 40 grams of silica gel 12A in 100 ml of water. After the adsorbent was applied and had a chance to settle, the plates were activated in an oven at 110°C for 60 minutes. Samples (25 to 50 µl) were applied 2 cm from the bottom of the plate at about 2-cm intervals. The first solvent, acetone-light petroleum, was permitted to run in the ascending direction to 3 cm below the top edge of the chromatography plate. The plate was then removed from the chromatography chamber and dried for 30 minutes at room temperature. After that, the chromatography plate was developed in the second solvent up to a height of 2 to 3 cm below the first solvent. After development of the thin-layer chromatograms, the phospholipids were detected by the exposure of the chromatogram plates to iodine vapors. The radioactivity in the individual spots was determined by quantitatively transferring the gel to scintillation vials. To assure complete recovery of labeled phospholipids, all the silica gel in a lane was routinely assayed by this procedure. A 1-ml sample of glacial acetic acid in absolute ethanol followed by 10 ml of toluene scintillation fluid was added to each vial for counting. The identification of various lipids was established by the simultaneous chromatography of known standards (Fig. 1). The $\underline{R_F}$ values observed for the major lipids were as follows: phosphatidylethanolamine (0.47), phosphatidylglycerol (0.35), and cardiolipin (0.70). These $\underline{R_F}$ values are the same as those previously reported (13). Phosphospray, a product

of Supelcosil, was used for the detection of phospholipids and ninhydrin spray, a product of Sigma Chemicals, was used for detecting phosphatidylethanolamine.

Results

Effect of PEA on relaxed and stringent cells. Some conflicting reports have appeared concerning the effect of PEA on RNA synthesis (4, 5, 7, 8). Such differences might be possible if some studies had been performed on cells with stringent control of RNA synthesis and others on cells with relaxed control. Figure 2 indicates that 0.20% PEA (v/v) inhibits uracil incorporation to the same extent in PA1 (relaxed) and PA2 (stringent) cells. This may be contrasted with the effect of valine, an inhibitor of isoleucine synthesis (18), which inhibits RNA synthesis in stringent but not in relaxed cells (19). Therefore, it appears probable that PEA does not exert its primary effect by inhibiting amino acid synthesis or activation. Furthermore, it is unlikely that PEA is acting in the same fashion as chloramphenicol or other inhibitors of protein synthesis, since these drugs permit continuation of RNA synthesis (19).

Effect of PEA on phospholipid synthesis. Figures 3a and 3b show the effects of various concentrations of PEA on the uptake of labeled precursors into DNA, RNA, protein, and phospholipids in E. coli W3110 after 20 minutes and 90 minutes of incubation, respectively. It is evident from these figures that labeled acetate incorporation into the phospholipid fraction is more sensitive to low concentrations of PEA than is the incorporation of labeled precursors into DNA, RNA, or protein at both incubation times. Similar results were obtained when

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18. T. Ramakrishnan and E. A. Adelberg, J. Bacteriol. 89, 654 (1965).

19. G. Edlin and P. Broda, Bacteriol. Rev. 32, 206 (1968).

labeled phenylalanine was used in place of isoleucine for measuring protein synthesis. The possibility that the effect on phospholipid synthesis might be an indirect one, due to inhibition of one of the other processes, was considered. To exclude such a possibility, phospholipid synthesis was also followed as a function of time in the presence of chloramphenicol (Figure 4a) and nalidixic acid as well as in the absence of thymine (Figure 4b). The addition of 25 μg per ml of chloramphenicol was 98 percent effective in blocking the incorporation of labeled phenylalanine into TCA insoluble material. However, it was less than half as effective as 0.15% PEA as an inhibitor of acetate incorporation into phospholipids. Only one other treatment, the addition of 250 μg per ml of valine, showed an inhibitory effect on acetate incorporation into phospholipids. Since W3110 is stringent, these results are consistent with those reported by Sokawa, Nakao, and Kaziro (20). Recent work in this laboratory (16) indicated that changes in the shaking speed strongly influence phospholipid synthesis in stringent cells and account for the differences reported by Sokawa et al. (20) and by our laboratory (13). Phospholipid synthesis was not inhibited by thymine starvation or by the addition of 10 μg per ml of nalidixic acid. This concentration of nalidixic acid inhibited the uptake of labeled thymine by 86 percent. The present results on the effect of DNA inhibitors on phospholipid metabolism are consistent with those reported by Ballesta and Schaechter (21) for experiments with mitomycin and by Goss, Dietz, and Cook (22) on experiments with nalidixic acid.

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20. Y. Sokawa, E. Nakao, and Y. Kaziro, Biochem. Biophys. Res. Commun. 33, 108 (1968).

21. J. P. G. Ballesta and M. Schaechter, J. Bacteriol. 107, 251 (1971).

22. W. A. Goss, W. H. Dietz, and T. M. Cook, J. Bacteriol. 88, 1112 (1964).

The previous experiments indicate that acetate incorporation into the phospholipid fraction is quite sensitive to the presence of PEA. It was therefore of interest to determine whether PEA exerted a differential effect on the incorporation of acetate into phosphatidylethanolamine, phosphatidylglycerol, or cardiolipin. Cells were cultured in 50 ml of medium containing labeled acetate in the presence and absence of 0.15% PEA for the indicated period of time. The phospholipids were extracted and analyzed by thin-layer chromatography as described in the Materials and Methods section. The results of these experiments are indicated in Figure 5. It is evident that PEA inhibits the labeling of phosphatidylethanolamine and phosphatidylglycerol (Figs. 5a and 5b). The incorporation of acetate into the cardiolipin fraction was, if anything, slightly stimulated by the presence of PEA (Fig. 5c). Changing the concentration of PEA to 0.20% v/v did not significantly affect the distribution pattern.

The distribution of labeled acetate into the phospholipids extracted from cells cultured in the presence of 25 μg per ml chloramphenicol, 10 μg per ml nalidixic acid, or 250 μg per ml valine or in the absence of thymine was determined. The results of these experiments are summarized in Table 1. It should be noted that although valine was greater than 98 percent and 85 percent effective in blocking the incorporation of labeled uracil and phenylalanine, respectively, into the TCA insoluble material, it had only a slight effect on the phospholipid distribution. Chloramphenicol treatment caused a decrease in label found in the cardiolipin fraction. None of the other treatments caused a marked change in the distribution of label into the phospholipids. These results tend to exclude the possibility that the effect of PEA on the distribution of label into phospholipids is a secondary effect.

Discussion

PEA is known to inhibit a new round of DNA replication (4, 5), alter cellular permeability (10, 12), affect RNA and protein synthesis (7, 8), and inhibit the formation of active alkaline phosphatase (9). These diverse effects might be due to the ability of PEA to: (i) prevent normal protein synthesis, (ii) alter some aspect of phospholipid metabolism, or (iii) interact with the membrane or some vital membrane component. The data presented in Figure 2 indicate that PEA inhibits RNA synthesis to the same extent in relaxed and stringent cells. It is therefore quite unlikely that PEA exerts its primary effect by inhibiting either amino acid synthesis or activation. The results presented in Figure 3 show that protein synthesis is relatively resistant to PEA at concentrations that markedly inhibit RNA and lipid synthesis. Thus, it appears unlikely that PEA exerts its primary effect by causing a decrease in the availability of the nucleoside triphosphates or the essential cations required for protein synthesis (11).

PEA clearly has an effect on phospholipid metabolism (Figs. 4a and 5). In particular, the incorporation of labeled acetate into phosphatidylethanolamine or phosphatidylglycerol is quite sensitive to the presence of PEA (Figs. 5a and 5b). The incorporation of label into the cardiolipin fraction does not appear to be inhibited (Fig. 5c). Similar results were obtained by Barbu, Polonovski, Rampini, and Lux (23) with labeled inorganic phosphorus (^{32}P). They did not perform any studies to

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23. E. Barbu, J. Polonovski, C. Rampini, and M. Lux, Comptes Rendus Series D, 270, 2596 (1970).

determine whether the effect exerted by PEA on phospholipid synthesis was a consequence of the inhibition of one of the other cellular processes. They did note, however, that upon removal of PEA, the normal phospholipid labeling pattern was slowly restored (23). Since phosphatidylglycerol is reported to be an intermediate in the pathway to cardiolipin synthesis (24), it is not clear why the apparent inhibition of the synthesis of precursor does not affect the synthesis of product. An explanation for this phenomenon has been proposed by Rampini, Barbu, and Polonovski (25). They postulated another pathway for the synthesis of cardiolipin from phosphatidylglycerol in E. coli which involves the condensation of two molecules of phosphatidylglycerol to form one molecule of cardiolipin and one molecule of free glycerol (25). Evidence in support of their hypothesis has recently been put forth by the findings of Tunaitis and Cronan (26) and by Hirschberg and Kennedy (27). These workers reported the presence of an enzyme in the envelope fraction of E. coli which catalyzes the synthesis of cardiolipin via the pathway postulated by Rampini et al. (25).

The inhibition of phospholipid synthesis may be either the cause

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24. N. Z. Stanacev, Y-Y. Chang, and E. P. Kennedy, J. Biol. Chem. 242, 3018 (1967).

25. C. Rampini, E. Barbu, and J. Polonovski, Comptes Rendus, Series D, 270, 882 (1970).

26. E. Tunaitis and J. E. Cronan, Jr., Biochim. Biophys. Acta, in press.

27. C. B. Hirschberg and E. P. Kennedy, Proc. Nat. Acad. Sci. U.S.A. 69, 648 (1972).

or the effect of altered cellular permeability. The data at this time do not permit the clear distinction.

In Chapters 3 and 4 we will examine and discuss the effects of PEA on the *in vitro* activity of three enzymes involved in phospholipid biosynthesis.

TABLE 1

Treatment	Total CPM per ml of cells	% Total counts per minute				
		Origin	Phosphatidylglycerol	Phosphatidylethanolamine	Cardiolipin	Neutral lipid
Untreated	1967	3	13	63	15	2
PEA	897	4	10	45	34	5
Chloramphenicol	1356	6	16	62	9	4
Nalidixic acid	1800	4	11	62	20	1
Valine	1173	4	18	55	16	5
Thymine starvation	2035	4	11	63	19	2

Table 1: Comparisons of the effects of PEA, 0.15% v/v; chloramphenicol (25 μg per ml); nalidixic acid (10 μg per ml); valine (250 μg per ml); and thymine starvation on the distribution of labeled acetate into phospholipids after 90 minutes of incubation.

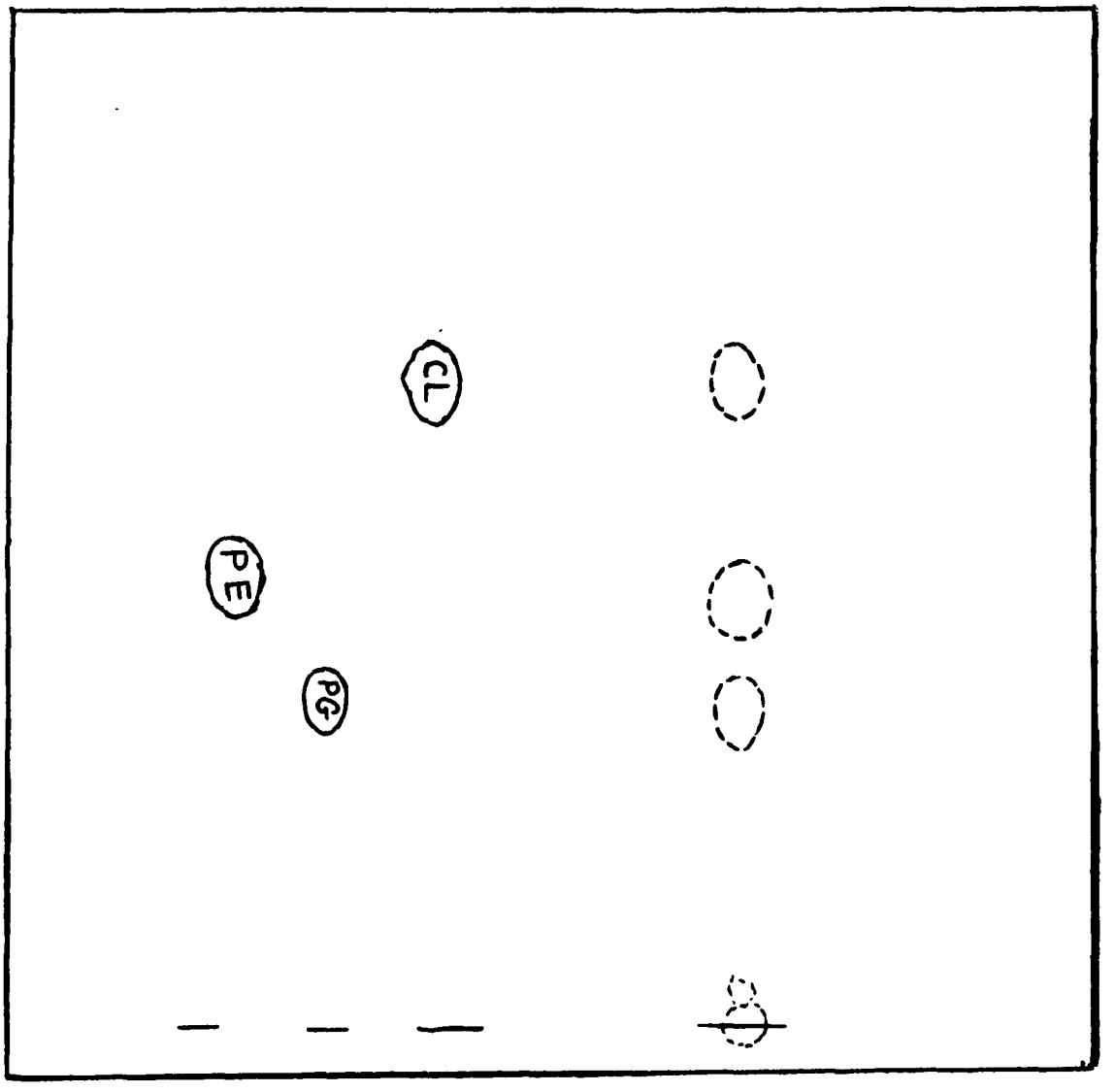

Figure 1

Thin-layer chromatographic separation of phospholipids from chloroform extracts of E. coli (dotted lines). The solid lines indicate the R_F values of the phospholipid standards; phosphatidylethanolamine (PE); phosphatidylglycerol (PG); and cardiolipin (CL).

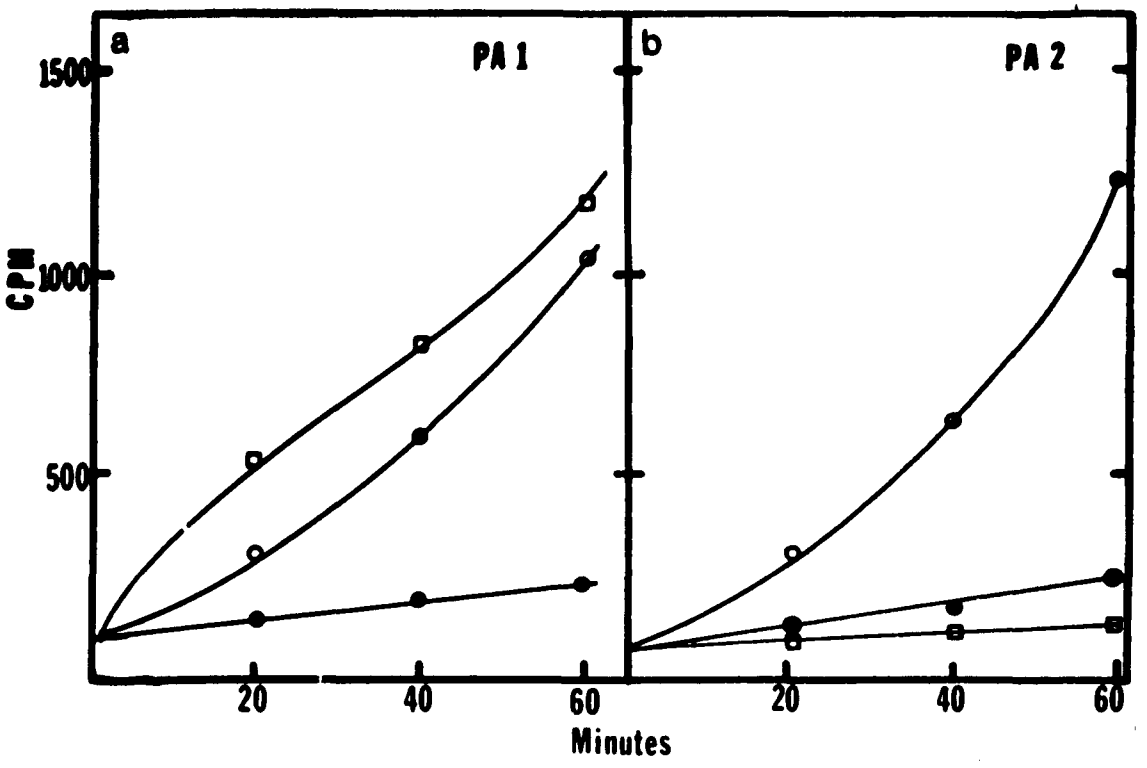

Figure 2

Effect of PEA (0.20%, v/v) and valine (250 µg/ml) on labeled uracil incorporation into (a) relaxed (E. coli PA1) and (b) stringent (E. coli PA2) cells. Exponentially growing cells were cultured in the presence of labeled uracil, labeled uracil and PEA, or labeled uracil and valine. Incorporation of radioisotope was determined as described in Materials and Methods. Symbols: ○, untreated cells; ●, PEA; and □, valine.

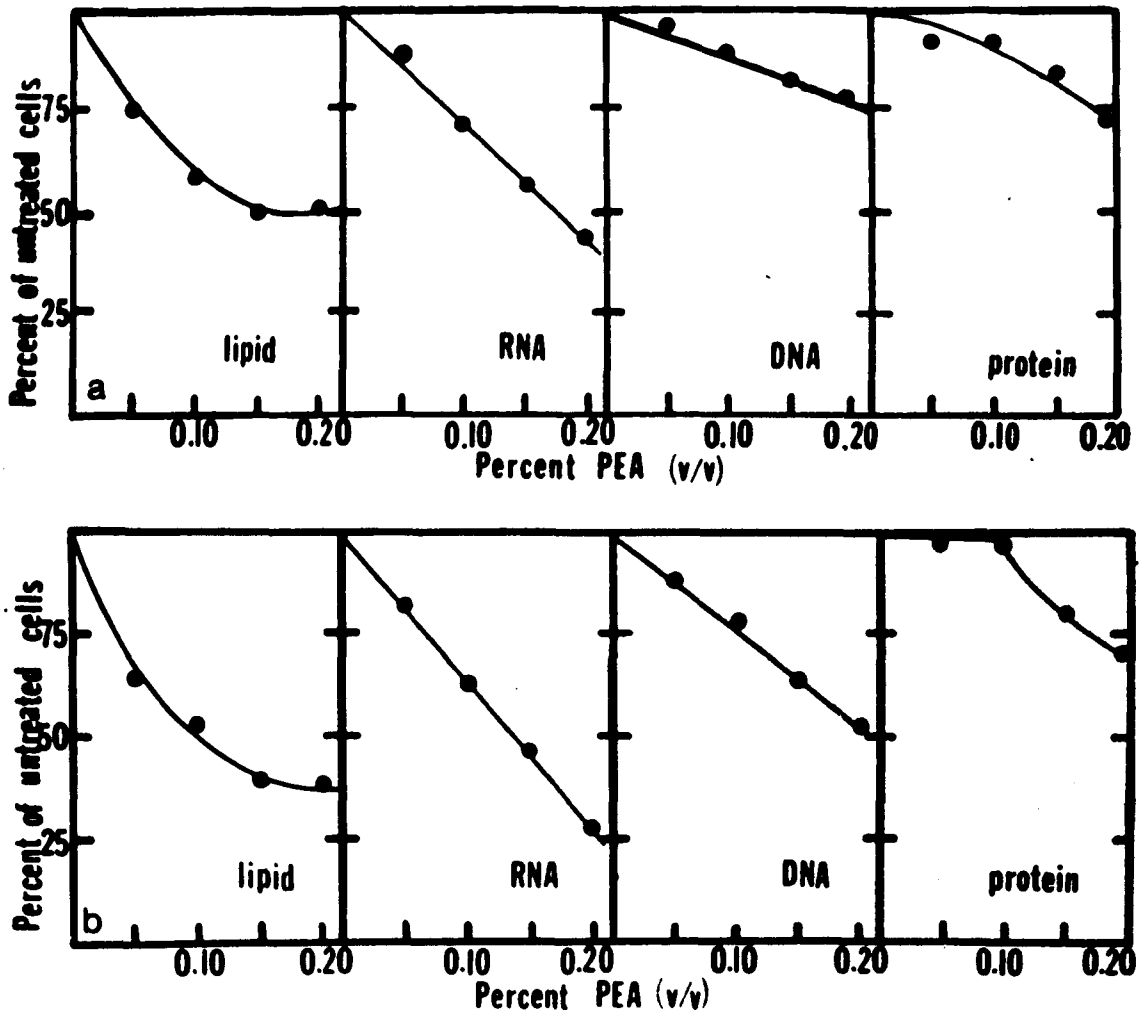

Figure 3

Lipid, RNA, DNA, and protein synthesis by E. coli W3110 as a function of phenethyl alcohol concentration. Macromolecular synthesis was measured by incorporation of radioactive precursors into lipid, RNA, DNA, and protein for 20 and 90 minutes. Isotope incorporation was determined as described in Materials and Methods. The incorporation values were then expressed as percentages of the level observed with an untreated culture at that time point: (a) 20 minutes; (b) 90 minutes.

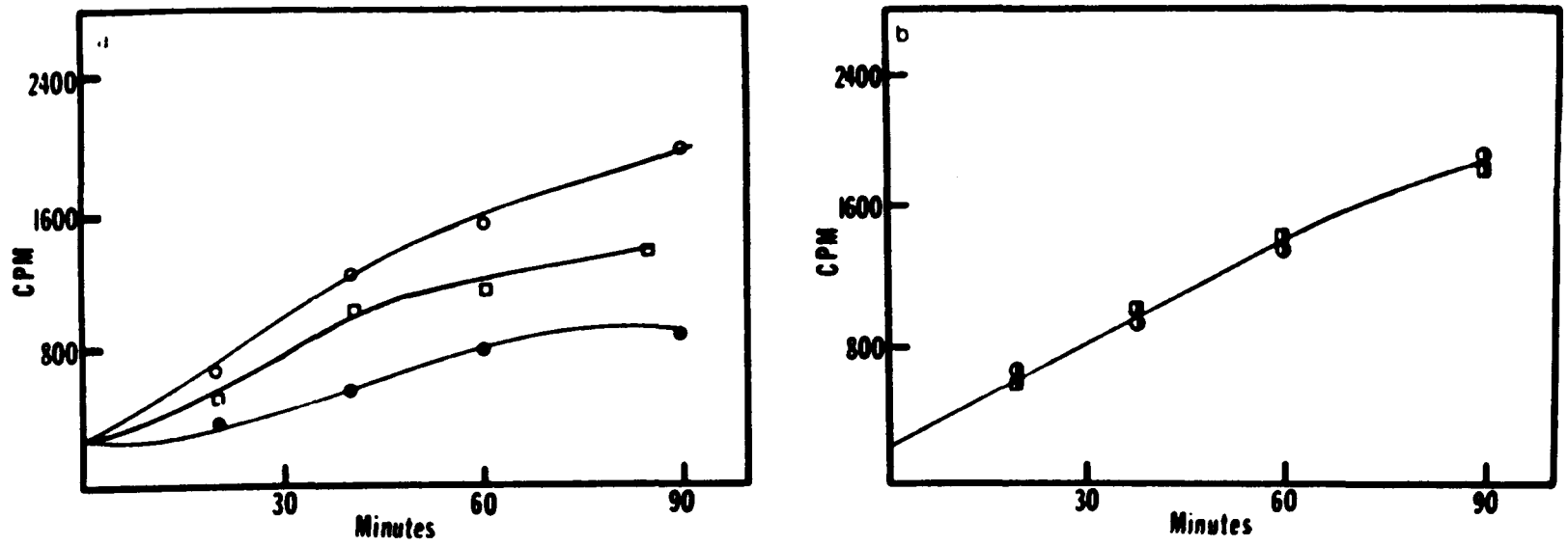

Figure 4

Effect of PEA (0.15%, v/v), chloramphenicol (25 μg/ml), nalidixic acid (10 μg/ml), or thymine starvation on the incorporation of labeled acetate into total phospholipids of E. coli W3110. Experimental details were as described in Materials and Methods. Symbols: ○, untreated cells; ●, PEA; □, chloramphenicol; ■, nalidixic acid; and ◑, thymine starvation.

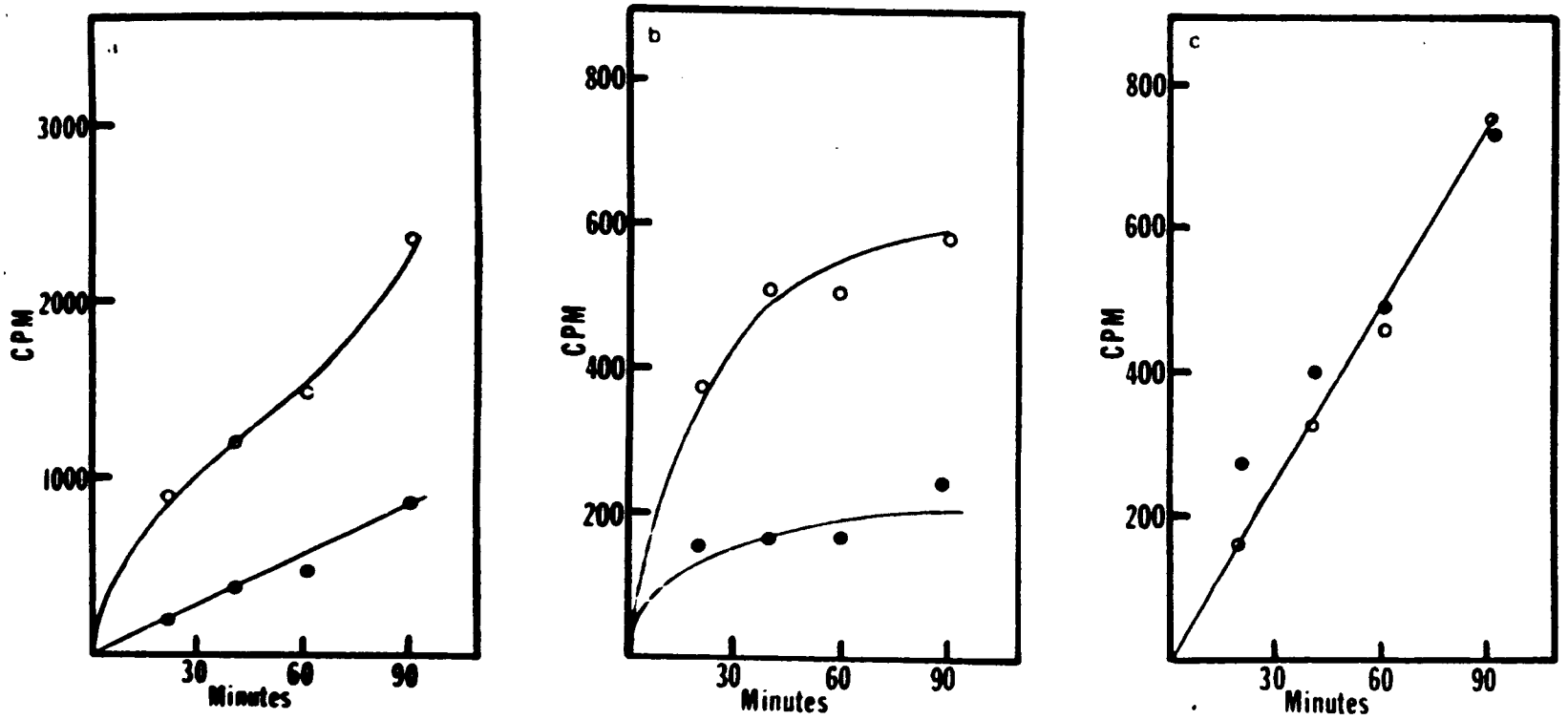

Figure 5

Incorporation of labeled acetate into fractionated phospholipids of E. coli W3110 in the presence and absence of phenethyl alcohol (0.15%, v/v). Symbols: ○, untreated cells; ●, PEA (0.15%). (a) Phosphatidylethanolamine; (b) phosphatidylglycerol; (c) cardiolipin.

CHAPTER 3

IN VITRO STUDIES

Introduction

Since glycerol-3-phosphate is a precursor to phospholipid synthesis, a study of the effects of phosphonic acid analogues of this compound on lipid synthesis *in vivo* and *in vitro* is of special interest. The functional groups of the phosphonic acid isosteres, $-OPO_3H_2$ and $-CH_2PO_3H_2$, have approximately equal steric properties. However, cleavage of the phosphorus moiety from the $-CH_2PO_3H_2$ functional group does not readily occur. For this reason, investigations involving the effect of phosphonic acid isosteres on the metabolic processes of *E. coli* are now being carried out in this laboratory. The phosphonic acid analogues of glycerol-3-phosphate which are being used in these studies are 3,4-dihydroxybutyl-1-phosphonate and 2,3-dihydroxypropyl-1-phosphonate.

The syntheses of 2,3-dihydroxypropyl-1-phosphonate and 3,4-dihydroxybutyl-1-phosphonate have recently been accomplished in this laboratory. Studies involving the effect of these phosphonate analogues on the growth of *E. coli* show that 3,4-dihydroxybutyl-1-phosphonate but not 2,3-dihydroxypropyl-1-phosphonate inhibits cell growth (1). This inhibition appears to be bacteriostatic (1). Preliminary investigations of the effect of the four-carbon phosphonate analogue on

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1. C. Shopsis, R. Engel, and B. E. Tropp, manuscript submitted for publication.

in vivo metabolic processes indicate that lipid synthesis is inhibited to a greater extent than DNA, RNA, and protein synthesis (2). In addition, this analogue has been found to inhibit the transport of glycerol-3-phosphate in an *E. coli* mutant which is constitutive for permease activity (1). These findings prompted us to study the effects of these analogues on the *in vitro* activity of the following two phospholipid biosynthetic enzymes: L-glycerol-3-phosphate:CMP phosphatidyltransferase and L serine:CMP phosphatidyltransferase.

The biosynthesis of phosphatidylglycerol and phosphatidylethanolamine in *E. coli* has been shown by Kennedy and co-workers (3, 4) to follow two pathways, both dependent on CDP-diglyceride.

[1] CDP-diglyceride + L-glycerol-3-phosphate → phosphatidylglycerophosphate + CMP

[2] CDP-diglyceride + L-serine → phosphatidylserine + CMP

Reaction [1] is catalyzed by L-glycerol-3-phosphate:CMP phosphatidyltransferase (4). Phosphatidylglycerophosphate formed in reaction [1] is dephosphorylated to yield phosphatidylglycerol (4). Reaction [2] is catalyzed by L-serine:CMP phosphatidyltransferase (3). Phosphatidylserine formed in reaction [2] is decarboxylated to yield phosphatidylethanolamine (3).

In this chapter, the effects of PEA and the phosphonate analogues of glycerol-3-phosphate on the components of the enzyme-catalyzed reactions ([1] and [2]) are reported.

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2. C. Shopsis, R. Engel, W. Nunn, and B. E. Tropp, unpublished data.
3. J. Kanfer and E. P. Kennedy, *J. Biol. Chem.* 239, 1720 (1964).
4. Y-Y. Chang and E. P. Kennedy, *J. Lipid Res.* 8, 447 (1967).

Materials and Methods

Chemicals. DL-α-Glycerol-3-phosphate (Grade X) and the nonionic detergent, Triton X-100 (octylphenoxypolyethoxyethanol) were purchased from Sigma Chemical Co., St. Louis, Mo. ^{14}C-L-Glycerol-3-phosphate (26 μCi per μmole) and DL-serine-3-^{14}C (4.17 μCi per μmole) were obtained from ICN Corporation, Irvine, Calif. CDP-dipalmitin was purchased from Serdary Research Laboratories, London, Ontario, Canada. The organic synthesis of 2,3-dihydroxypropyl-1-phosphonate and 3,4-dihydroxybutyl-1-phosphonate was carried out in this laboratory by De Filippe, Kaback, Engel, and Tropp (5). They used the procedure of Rosenthal and Geyer (6) to synthesize the dilithium salt of 2,3-dihydroxypropyl-1-phosphonate. A modification of this procedure was used to synthesize the dilithium salt of 3,4-dihydroxybutyl-1-phosphonate (5). PEA was obtained from Matheson, Coleman and Bell, Norwood, Ohio. Phosphatidylserine, phosphatidylethanolamine, and phosphatidylglycerol were purchased from Supelco, Inc., Bellfonte, Pa. All other chemicals were of reagent grade and all solvents were distilled before use.

Bacterial strain. E. coli strain 8, a mutant devoid of the aerobic L-glycerol-3-phosphate dehydrogenase activity and constitutive for the L-glycerol-3-phosphate transport system (7, 8), was a gift of J. E. Cronan, Jr.

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5. L. De Filippe, J. Kaback, R. Engel, and B. E. Tropp, unpublished data.
6. A. Rosenthal and R. Geyer, J. Amer. Chem. Soc. 80, 5240 (1958).
7. W. S. Kistler and E. C. C. Lin, J. Bacteriol. 108, 1224 (1971).
8. S. Hayashi, J. Koch, and E. C. C. Lin, J. Biol. Chem. 239, 3098 (1964).

Growth of bacteria and preparation of enzymes. E. coli strain 8 cultures were incubated at 37°C in a New Brunswick model G25 controlled environment incubator shaker at 200 r.p.m. The bacteria were grown in the synthetic medium of Davis and Mingioli (9) with glucose as the carbon source. Cells were grown overnight from a small inoculum (0.2 ml of broth culture in 50 ml of minimal medium) and diluted 20-fold into the same medium. Cell growth was determined at 660 nm in a Klett-Summerson colorimeter. When the turbidity reached 70 to 80 Klett units, the cells were harvested and centrifuged at 4°C in a Sorvall RC-2 with an SS-34 rotor at 10,000 r.p.m. for 5 minutes. The cells were washed once with ice-cold 0.1 M Tris-HCl buffer (pH 8) containing 10 mM mercaptoethanol. The washed cells from 800 ml of culture were then suspended in 15 ml of ice-cold buffer. Cell-free preparations were made by sonic irradiation of the cell suspension in an ice-bath for eight to ten 30-second bursts with a Bronson model W140D sonifier at a setting of 8. The final suspension was cooled to 0°C and the intact cells were removed by centrifugation at 3000 x g for 10 minutes. The resulting supernatant was divided into two equal portions. One portion of this crude extract was used for assaying L-serine:CMP phosphatidyltransferase activity. The other portion of crude extract was centrifuged for 60 minutes at 40,000 x g in a Spinco model L ultracentrifuge. The pellet was suspended in cold buffer and recentrifuged at 40,000 x g for 60 minutes, and finally resuspended in 10 ml of cold buffer. This suspension contained the particulate enzyme L-glycerol-3-phosphate:CMP

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9. B. D. Davis and E. S. Mingioli, J. Bacteriol. 60, 17 (1950).

phosphatidyltransferase. The method of Lowry et al. (10) was used for determining the protein concentration of the enzyme preparations.

Assay for L-glycerol-3-phosphate:CMP phosphatidyltransferase activity. L-Glycerol-3-phosphate:CMP phosphatidyltransferase activity was measured by monitoring the conversion of ^{14}C-L-glycerol-3-phosphate into chloroform extractable material as described by Chang and Kennedy (4). The reaction mixture (0.5 ml) for this assay contained 0.25 M Tris-HCl buffer (pH 8.5), 0.8 mM DL-glycerol-3-phosphate, 1.0 μM ^{14}C-L-glycerol-3-phosphate (26 μCi per μmole), 0.08 mM CDP-dipalmitin, 10 mM $MgCl_2$, 5 mM mercaptoethanol, 2 mg per ml Triton X-100, and 100 to 200 μg of particulate enzyme. The reaction mixture has an absolute requirement for CDP-dipalmitin. The reaction mixtures were incubated for 30 minutes at 37°C. At the end of the incubation, 2 ml of methanol was added, followed by 4 ml of chloroform. Each solution was agitated with a vortex mixer and washed twice with 3 ml of 2 M $MgCl_2$. After separation of the phases, the upper aqueous phase was carefully drawn off with a capillary pipette and the lower chloroform phase washed twice more with 3 ml of distilled water. The chloroform extract was then placed in a scintillator vial and dried by overnight evaporation or by heating at temperatures below 50°C. The contents were dissolved in toluene scintillator fluid and counted in the scintillation counter. The incorporation of ^{14}C-L-glycerol-3-phosphate into lipid extractable material, under these conditions, was observed to be linear with time up to 50 minutes. Less than 10 percent of the initial concentration

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10. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, J. Biol. Chem. 193, 265 (1951).

of ^{14}C-L-glycerol-3-phosphate was converted into chloroform extractable material during this time. One unit of enzyme activity is that amount which catalyzes the conversion of 1 μmole of L-glycerol-3-phosphate to lipid per minute under these conditions (4).

Determination of the effect of phosphonic acid isosteres and PEA on L-glycerol-3-phosphate:CMP phosphatidyltransferase activity. PEA at final concentrations between 0.0 and 0.3 percent was added to reaction mixtures containing 40 mμmoles of CDP-dipalmitin, 125 μmoles Tris-HCl (pH 8.5), 5 μmoles $MgCl_2$, 2.5 μmoles mercaptoethanol, 0.4 μmoles DL-glycerol-3-phosphate, 0.013 μCi ^{14}C-L-glycerol-3-phosphate (26 μCi per μmole), and 1 mg Triton X-100. When the effect of either DL-3,4-dihydroxybutyl-1-phosphonate or DL-2,3-dihydroxypropyl-1-phosphonate on L-glycerol-3-phosphate:CMP phosphatidyltransferase activity was studied, the final concentrations of the dilithium salts of these phosphonic acid analogues added to the above reaction mixtures varied between 0 and 10 mM. Another set of identical reaction mixtures was supplemented with LiCl at final concentrations between 0 and 20 mM. These concentrations of lithium do not appear to affect the enzyme's activity. One hundred micrograms of particulate enzyme were added, resulting in a final volume of 0.5 ml for each mixture. The reaction mixtures were incubated at 37°C for 30 minutes, during which time duplicate samples were removed at 0, 15, and 30 minutes. Incorporation of ^{14}C-L-glycerol-3-phosphate into lipid extractable material was measured by the procedure described for the assay of L-glycerol-3-phosphate:CMP phosphatidyltransferase.

Determination of the K_m for CDP-dipalmitin. Reaction mixtures were prepared in duplicate containing 4.0 to 40 mμmoles of CDP-dipalmitin,

125 μmoles Tris-HCl (pH 8.5), 5 μmoles $MgCl_2$, 2.5 μmoles mercaptoethanol, 0.4 μmoles DL-glycerol-3-phosphate, 0.013 μCi ^{14}C-L-glycerol-3-phosphate (26 μCi per μmole), and 1 mg Triton X-100. These mixtures were stored in the cold room until ready for incubation. One hundred fifty micrograms of particulate enzyme were added to each of the reaction mixtures. The total volume in each mixture was 0.5 ml. The mixtures were incubated at 37°C for 30 minutes. The reaction was then stopped, and the ^{14}C-L-glycerol-3-phosphate converted into chloroform extractable material was measured by the procedure described in the assay for L-glycerol-3-phosphate:CMP phosphatidyltransferase activity. A Lineweaver and Burk plot (11) was drawn (Fig. 1) of $1/\underline{S}$ versus $1/\underline{v}$, where $\underline{S}$ is moles per liter of DL-glycerol-3-phosphate and $\underline{v}$. the initial velocity, is the number of counts per minute incorporated in 30 minutes.

Determination of the K_m for L-glycerol-3-phosphate and K_i for the phosphonic acid isosteres of glycerol-3-phosphate. Two sets of reaction mixtures, stored in either the cold room or an ice-bath, were prepared in duplicate. One set of tubes contained 0.04 to 0.4 μmoles of DL-glycerol-3-phosphate, 1.3 to 13 mμCi ^{14}C-L-glycerol-3-phosphate (26 μCi per μmole), 40 mμmoles CDP-dipalmitin, 125 μmoles Tris-HCl (pH 8.5), 5 μmoles $MgCl_2$, 2.5 μmoles mercaptoethanol, and 1 mg Triton X-100. The other set of tubes contained the same constituents in addition to 2.5 μmoles of either DL-3,4-dihydroxybutyl-1-phosphonate or DL-2,3-dihydroxypropyl-1-phosphonate. One hundred fifty micrograms of particulate enzyme were added to each mixture to bring the final volume to 0.5 ml. The reaction mixtures were incubated at 37°C for 30 minutes.

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11. H. Lineweaver and D. Burk, J. Amer. Chem. Soc. 56, 658 (1934).

The reaction was stopped, and the conversion of ^{14}C-L-glycerol-3-phosphate into lipid extractable material was measured by the procedure described for assaying L-glycerol-3-phosphate:CMP phosphatidyltransferase activity. The K_m and K_i values were calculated based upon the concentration of the L-form of these isosteric compounds.

The two-step thin-layer chromatography system, described in Chapter 2, was used to resolve the chloroform extractable products of the L-glycerol-3-phosphate:CMP phosphatidyltransferase assay. Two spots were found that contained over 90 percent of the total radioactivity. One spot (55 percent of the counts) cochromatographed with the phosphatidylglycerol standard which had an R_F value of 0.35. The other spot (37 percent of the counts) streaked somewhat with an approximate R_F value of 0.18. Since the position of this spot is indicative of the type of migration a very polar component would have in the solvent system used, it was assumed to be phosphatidylglycerolphosphate.

Assay for L-serine:CMP phosphatidyltransferase activity. L-serine:CMP phosphatidyltransferase activity was measured by monitoring the conversion of DL-serine-3-^{14}C to a lipid as described by Kanfer and Kennedy (3). The reaction mixture (0.3 ml) for the assay of this enzyme contained 0.04 M Tris-HCl buffer (pH 8.0), 0.1 M Na_2SO_4, 10 mM mercaptoethanol, 2 mM EDTA, 0.08 mM CDP-dipalmitin, 2 mg per ml Triton X-100, 2 mM DL-serine-3-^{14}C (0.2 µCi per µmole), and 200 to 300 µg of crude extract. The system was incubated at 37°C for 60 minutes. The procedure for measuring the incorporation of labeled serine into lipid was as previously described for the assay of L-glycerol-3-phosphate:CMP phosphatidyltransferase. Under the conditions of the assay, incorporation of DL-serine-3-^{14}C into chloroform extractable material was

entirely dependent upon added CDP-dipalmitin and required the addition of Na_2SO_4 (Table 1). There was essentially little or no activity when the surface active agent, octanol or Triton X-100, was omitted from the reaction mixture (Table 1). The conversion of DL-serine-3-^{14}C into chloroform extractable material was observed to be linear with time for 90 minutes. At 60 minutes, less than 10 percent of the initial DL-serine-3-^{14}C concentration had been converted into lipid.

Determination of the effect of phosphonic acid isosteres and PEA on L-serine:CMP phosphatidyltransferase activity. 3,4-Dihydroxybutyl-1-phosphonate (0.8 µmoles), 2,3-dihydroxypropyl-1-phosphonate (0.8 µmoles) or PEA (5.5 µmoles) were added to reaction mixtures prepared in duplicate containing 0.7 µmoles of DL-serine-3-^{14}C (0.2 µCi per µmole), 33 µmoles Na_2SO_4, 3 µmoles mercaptoethanol, 40 µmoles CDP-dipalmitin, 13 µmoles Tris-HCl (pH 8.0), 0.7 µmoles EDTA, and 0.5 mg Triton X-100. Two hundred micrograms of crude extract were added to the reaction mixtures to bring the final volume to 0.3 ml. The mixtures were incubated for 60 minutes at 37°C. The conversion of DL-serine-3-^{14}C to lipid extractable material was measured by the procedure described for the assay of L-glycerol-3-phosphate:CMP phosphatidyltransferase.

Determination of the K_m for L-serine. Reaction mixtures, stored in an ice-bath, were prepared in duplicate containing 0.2 to 0.7 µmoles of DL-serine-3-^{14}C (0.2 µCi per µmole), 33 µmoles Na_2SO_4, 3 µmoles mercaptoethanol, 40 µmoles CDP-dipalmitin, 13 µmoles Tris-HCl (pH 8.0), 0.7 µmoles EDTA, and 0.5 mg Triton X-100. Two hundred micrograms of

crude enzyme extract were added, making the final volume of each reaction mixture 0.3 ml. The mixtures were incubated for 60 minutes at 37°C. The reaction was stopped and assayed for the conversion of DL-serine-3-^{14}C to lipid extractable material as described for the assay of L-glycerol-3-phosphate:CMP phosphatidyltransferase. Since Kanfer and Kennedy (4) were unable to obtain classical Michaelis-Menton kinetics when they varied the CDP-dipalmitin concentration (at constant L-serine concentration), no attempts were made to ascertain the $\underline{K}_{\underline{m}}$ for this compound.

When the products of the L-serine:CMP phosphatidyltransferase reaction were fractionated by the two-step thin-layer chromatography procedure described in Chapter 2, it was found that over 88 percent of the label was recovered in the phosphatidylethanolamine fraction. This result is not surprising in view of the fact that the crude extracts used in this assay contained not only L-serine:CMP phosphatidyltransferase but also phosphatidylserine decarboxylase. Approximately 5 percent of the label was recovered in the phosphatidylserine fraction. The identification of these phospholipids was established by cochromatography with known standards and by the positive reaction these spots gave when sprayed with ninhydrin.

Results

Particulate fractions obtained from sonically disrupted E. coli incorporate ^{14}C-L-glycerol-3-phosphate into lipid. Under the conditions of the assay, incorporation of radioactivity into chloroform extractable material was entirely dependent upon CDP-dipalmitin and Triton X-100. When the concentration of DL-glycerol-3-phosphate was fixed at a non-saturating concentration and the concentration of CDP-dipalmitin was varied, the apparent K_m for CDP-dipalmitin was 2.5×10^{-4} M (Fig. 1). These results are in agreement with those reported by Chang and Kennedy (4).

Since preliminary studies in our laboratory with the phosphonate analogues of glycerol-3-phosphate seem to indicate that lipid synthesis in vivo is more sensitive to inhibition than DNA, RNA, and protein synthesis, the effect of these analogues on glycerol-3-phosphate:CMP phosphatidyltransferase activity was determined. Enzyme activity was measured, in the presence and absence of the phosphonic acid isosteres, by monitoring the uptake of ^{14}C-L-glycerol-3-phosphate into lipid extractable material. The results of these experiments show that the four-carbon phosphonate, 3,4-dihydroxybutyl-1-phosphonate, was more effective than the three-carbon phosphonate analogue, 2,3-dihydroxypropyl-1-phosphonate, in reducing the activity of this enzyme (Figs. 2a and 2b). When the enzyme activity was measured as a function of L-glycerol-3-phosphate concentration alone and in the presence of 3,4-dihydroxybutyl-1-phosphonate (5.0 mM), the four-carbon phosphonate acted as a competitive inhibitor toward glycerol-3-phosphate (Fig. 3a). The K_m for L-glycerol-

3-phosphate obtained from these studies was 2.5×10^{-4} M and the K_i for L-3,4-dihydroxybutyl-1-phosphonate was 6.8×10^{-4}. The same experiments carried out with the three-carbon phosphonate analogue (5.0 mM) indicate that this compound does not act as effectively as a competitive inhibitor toward glycerol-3-phosphate (Fig. 3b). The K_i for 2,3-dihydroxypropyl-1-phosphonate was 2.7×10^{-3} M. Since the CDP-dipalmitin was not present in a saturating concentration, the K_m and K_i are the apparent values under the conditions reported. The K_m value obtained for L-glycerol-3-phosphate agreed with that reported by Chang and Kennedy (4).

The effect of PEA on the enzymatic activity of L-glycerol-3-phosphate:CMP phosphatidyltransferase was also determined. Although PEA inhibits the synthesis of phosphatidylglycerol and phosphatidylethanolamine *in vivo* (12), this compound elicited no effect on the *in vitro* uptake of labeled glycerol-3-phosphate into chloroform extractable material (Fig. 4).

The effect of the phosphonic acid analogues and PEA on L-serine:CMP phosphatidyltransferase activity was studied by measuring the incorporation of DL-serine-3-^{14}C into lipid extractable material from reaction mixtures containing these compounds. The results of these studies, as indicated in Table 2, show that neither the phosphonic acid analogues nor PEA had any appreciable effect on the incorporation of labeled serine into the lipid extractable fraction. When the effect of various concentrations of PEA on L-serine:CMP phosphatidyltransferase activity was studied in the absence of Triton X-100, it was observed that PEA at final concentrations between 0.3 and 1.0 percent had a linear stimulatory

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12. W. D. Nunn and B. E. Tropp, J. Bacteriol. 109, 162 (1972).

effect on this enzyme's activity. This stimulatory effect is attributed to the enzyme's requirement for an added surface active agent (3).

When the concentration of DL-serine in the reaction mixture was varied while the CDP-dipalmitin was held constant at a nonsaturating concentration, the apparent $\underline{K_m}$ for L-serine was found to be 7.0×10^{-4} M (Fig. 5). This value compared favorably with that obtained by Kanfer and Kennedy (3).

Discussion

The primary observation described in this chapter is that 3,4-dihydroxybutyl-1-phosphonate inhibits the *in vitro* activity of glycerol-3-phosphate:CMP phosphatidyltransferase. The inhibition is competitive with glycerol-3-phosphate. The inhibition of this enzyme's activity by the four-carbon phosphonate analogue might be sufficient and specific enough to account for the bacteriostatic action exerted by this compound (1). It is hoped that the isolation of *E. coli* mutants resistant to this phosphonic acid analogue will clarify whether this is the case or not. These problems are presently being investigated in this laboratory.

Since PEA was observed not to affect either of the enzymes studied, it is apparent that this compound does not exert its primary effect on the enzyme-substrate reactions of these phospholipid biosynthetic enzymes. This, of course, does not exclude the possibility that PEA affects these enzymes by altering the membrane environment needed for optimal activity.

TABLE 1

System	DL-serine-3-^{14}C (CPM)	Relative incorporation (%)
Complete	8887	100
Octanol (20 µl) or Triton X-100 omitted	1340	15
CDP-dipalmitin omitted	132	1.4
Na_2SO_4 omitted	286	3.2

TABLE 2

Treatment	DL-serine-3-^{14}C (CPM)	Relative incorporation (%)
Untreated	9,824	100
2,3-Dihydroxypropyl-1-phosphonate (2.5 mM)	9,709	99
3,4-Dihydroxybutyl-1-phosphonate (2.5 mM)	10,836	110
Phenethyl alcohol (0.20%)	9,125	93

Table 2. The enzymatic incubations were carried out at 37°C for 60 minutes under the conditions described in the experimental section of this chapter. The incubation mixtures (0.3 ml) contained 200 µg of crude enzyme extract.

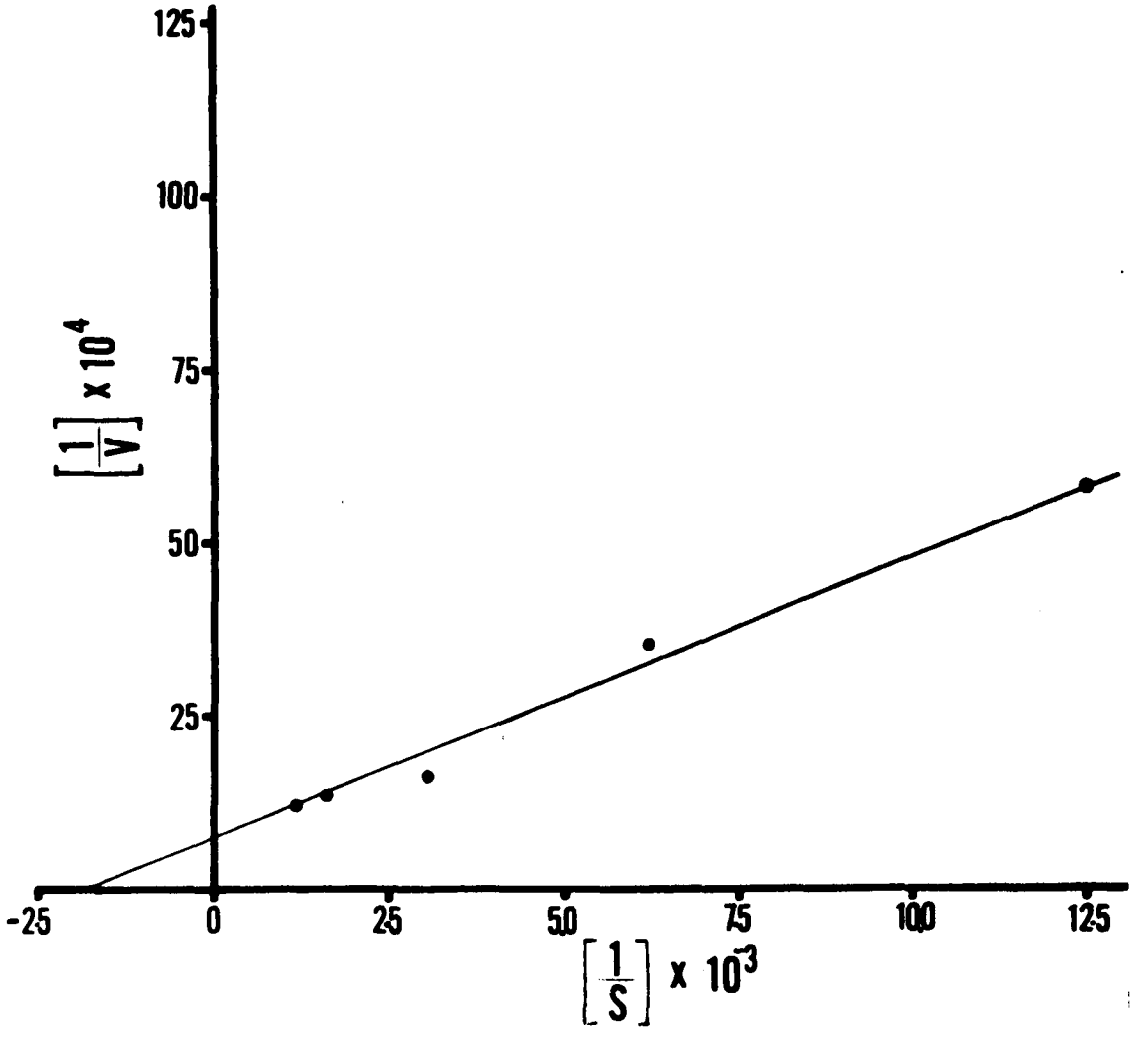

Figure 1

Lineweaver-Burk plot of the data obtained to determine the K_m for CDP-dipalmitin. The assay was performed as described in Materials and Methods.

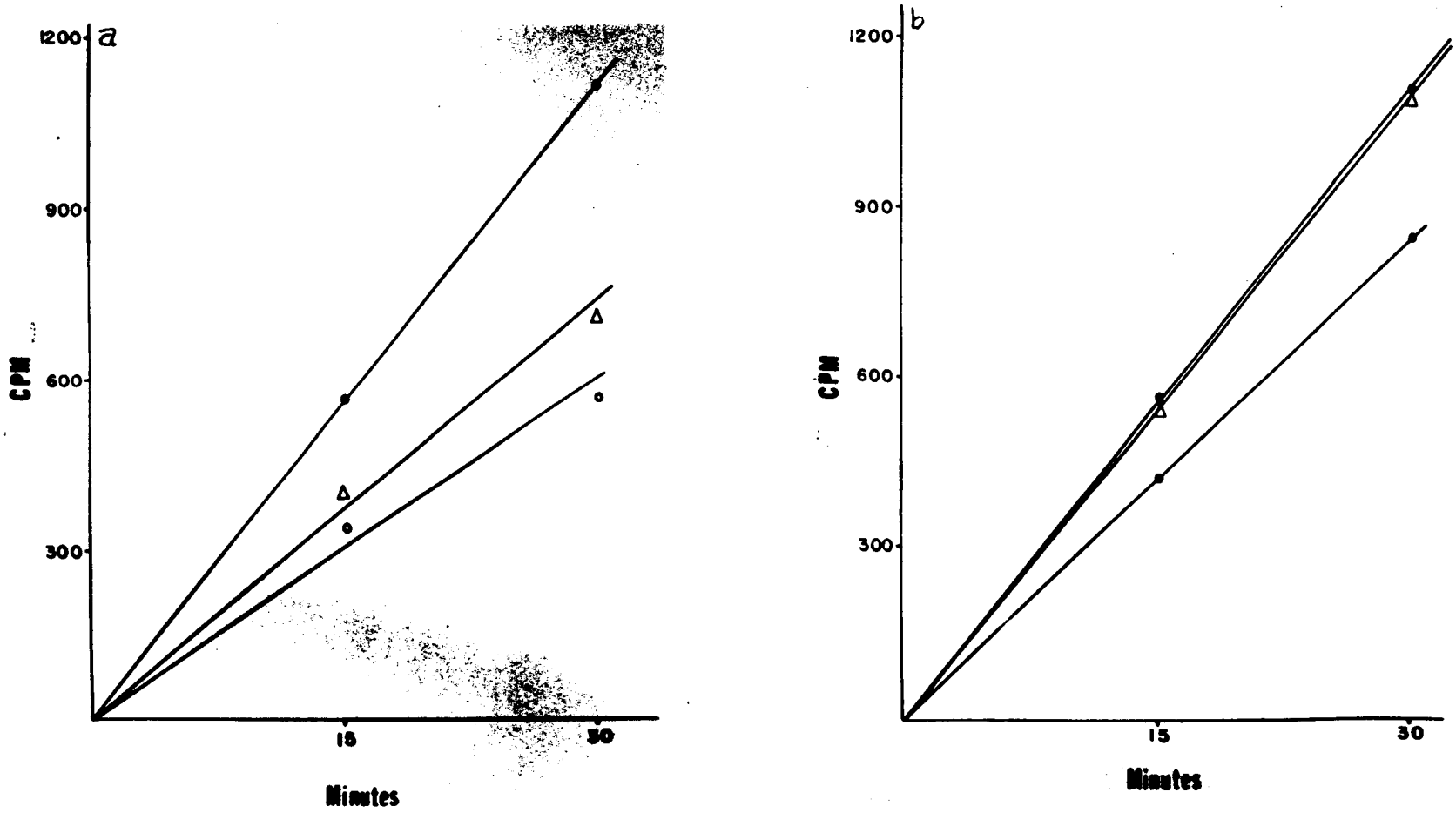

Figure 2

Effect of the dilithium salts 3,4-dihydroxybutyl-1-phosphonate and 2,3-dihydroxypropyl-1-phosphonate on L-glycerol-3-phosphate:CMP phosphatidyltransferase activity. The assays were performed with the DL-3,4-dihydroxybutyl-1-phosphonate or DL-2,3-dihydroxypropyl-1-phosphonate concentrations indicated. Incorporation of ^{14}C-L-glycerol-3-phosphate by the reaction mixtures at 37°C was determined as described in Materials and Methods. ●, untreated; △, 2.5 mM; ○, 10 mM. (a) DL-3,4-dihydroxybutyl-1-phosphonate. (b) DL-2,3-dihydroxypropyl-1-phosphonate.

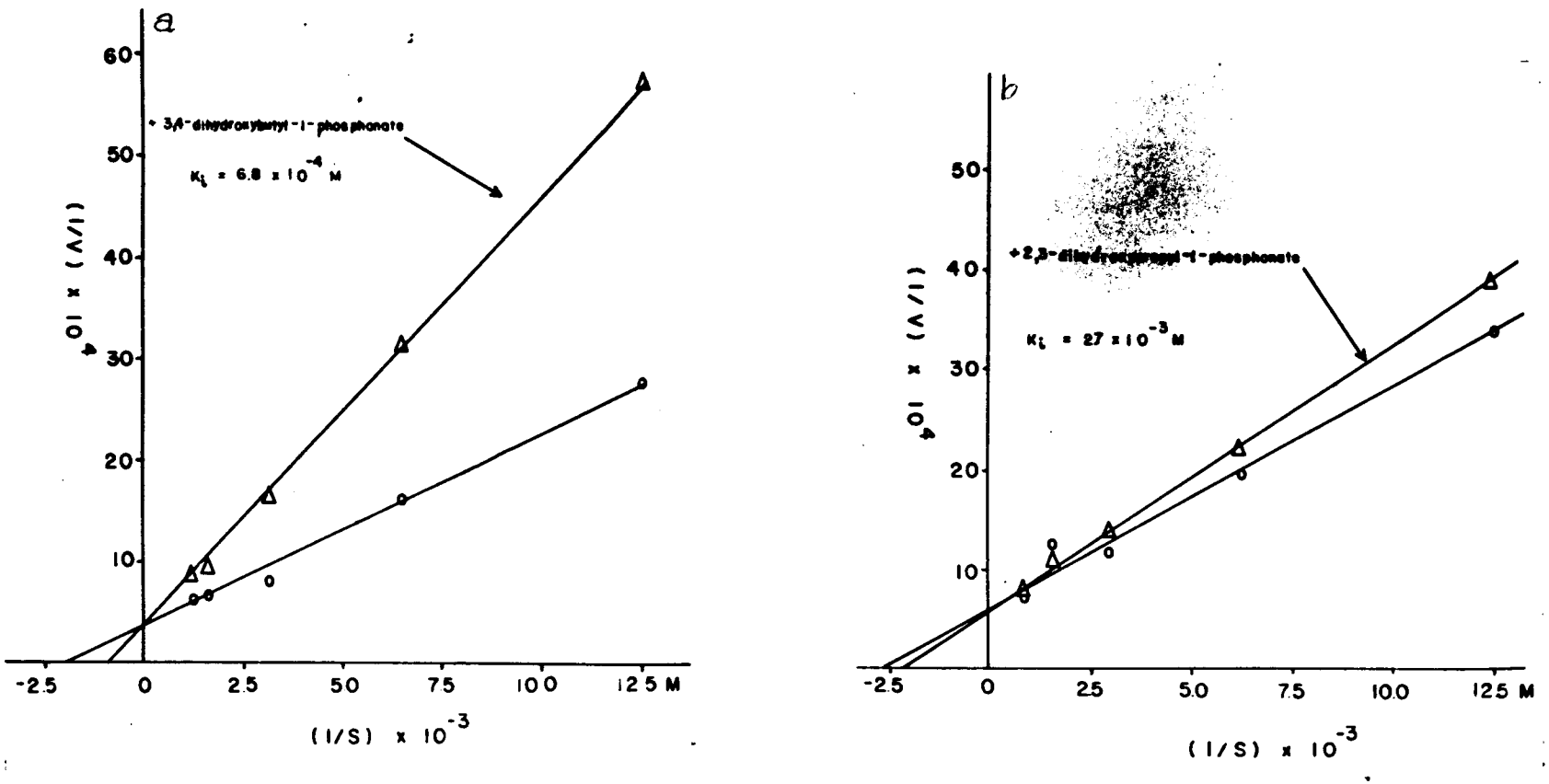

Figure 3

Double reciprocal plot for the conversion of ^{14}C-L-glycerol-3-phosphate into lipid extractable material by L-glycerol-3-phosphate:CMP phosphatidyltransferase in the presence and absence of DL-3,4-dihydroxybutyl-1-phosphonate (5.0 mM) or 2,3-dihydroxypropyl-1-phosphonate (5.0 mM). The assays were performed as described in Materials and Methods.

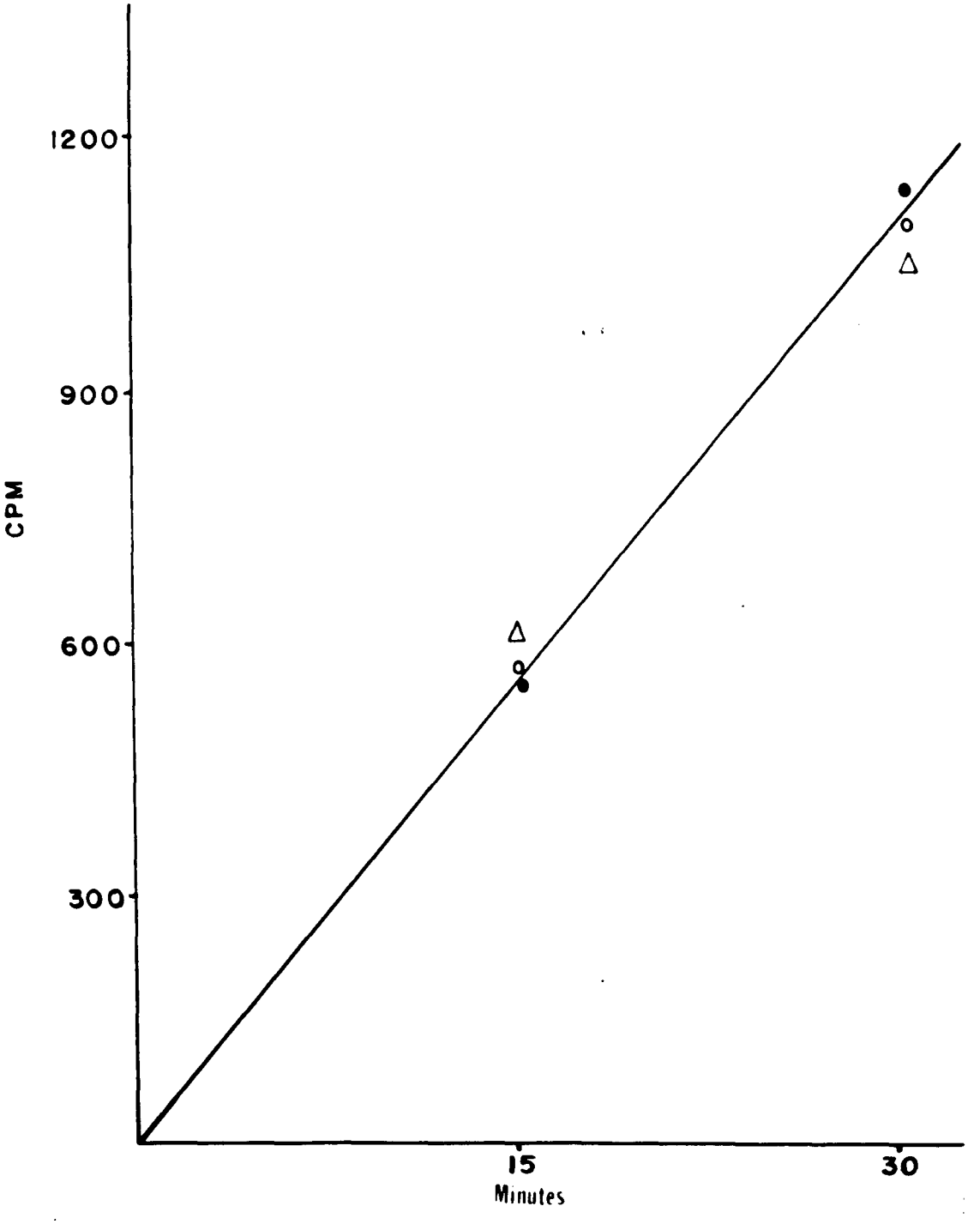

Figure 4

The effect of PEA on L-glycerol-3-phosphate:CMP phosphatidyltransferase activity. PEA was added at the final concentration indicated. The assay was performed as described in Materials and Methods. ●, untreated; ○, 0.15%; △, 0.30%.

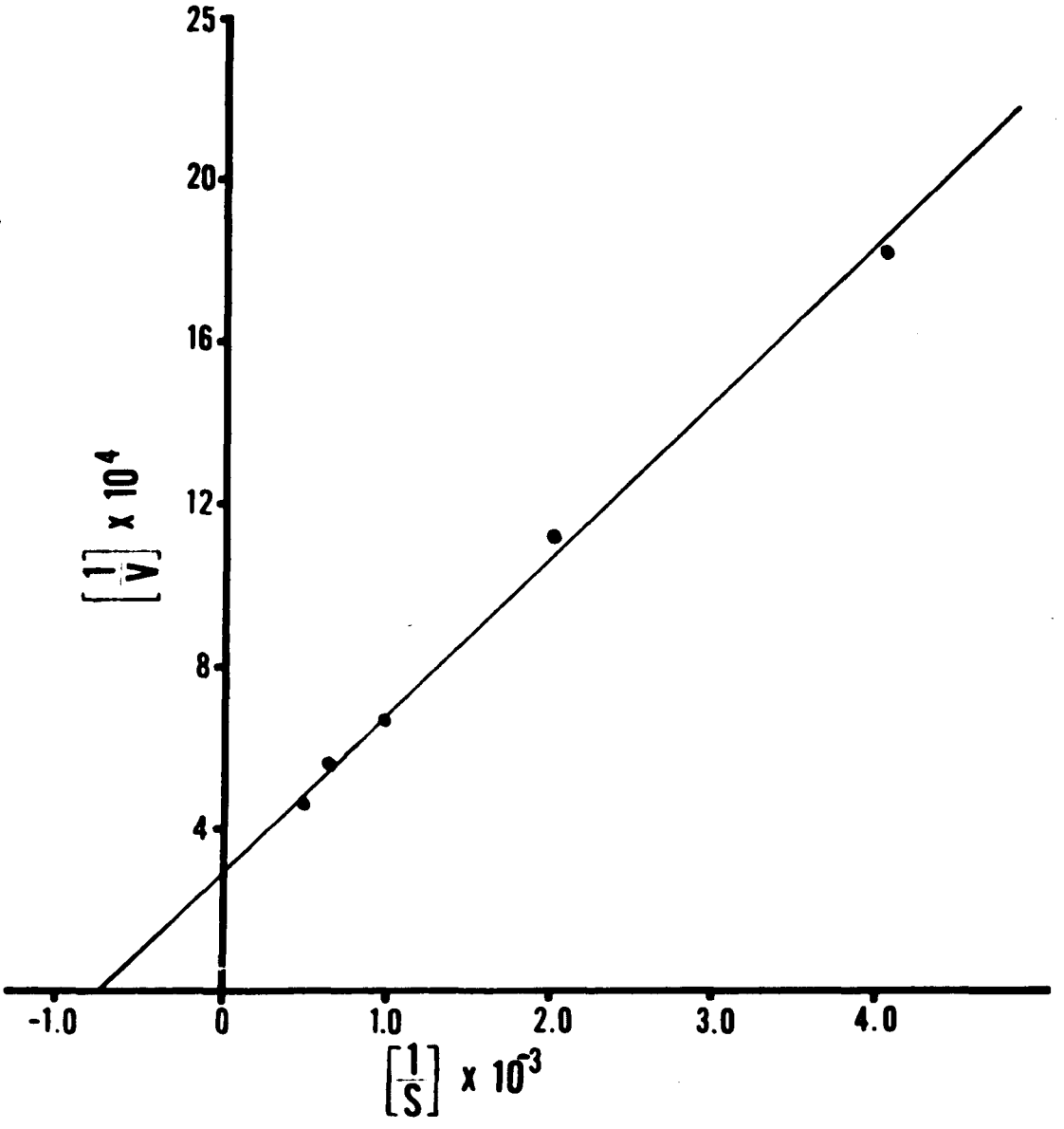

Figure 5

Lineweaver-Burk plot of the data obtained to determine the K_m for L-serine. The assay was performed as described in Materials and Methods.

CHAPTER 4

SUMMARY

The effect of PEA on phospholipid synthesis in E. coli was investigated with particular emphasis on relating the results of these studies to the diverse biochemical effects this compound exerts on other cellular processes. The work of Lester (1) with N. crassa and Silver and Wendt (2) with E. coli showing that PEA alters cellular permeability characteristics led to the proposal that the primary action of PEA is at the level of the cell membrane with resultant breakdown of the permeability barriers. Furthermore, these workers suggested that the inhibition of DNA synthesis and other cellular processes was a secondary consequence of the alteration in membrane structure (2). Our studies, involving the incorporation of labeled precursors into DNA, RNA, protein, and phospholipids of E. coli, indicate that PEA preferentially inhibits the synthesis of phospholipids (3). To exclude the possibility that this effect of PEA on lipid synthesis was due to the inhibition of DNA or protein synthesis, the uptake of labeled acetate into the phospholipid fraction of E. coli W3110 was studied in the presence of nalidixic acid and chloramphenicol, as well as in the absence of thymine. The results of these studies indicated that none of the above treatments inhibited lipid synthesis as effectively as PEA did. In addition, preliminary

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1. G. Lester, J. Bacteriol. 90, 29 (1965).
2. S. Silver and L. Wendt, J. Bacteriol. 93, 560 (1967).
3. These studies were reported in Chapter 2.

studies involving the effect of rifamycin on the uptake of labeled acetate showed that rifamycin, at concentrations that inhibit RNA synthesis by 50 percent, had no effect on lipid synthesis. Further studies on the nature of the phospholipids synthesized in the presence of PEA revealed that this compound markedly reduced the incorporation of labeled acetate into phosphatidylethanolamine and phosphatidylglycerol while apparently eliciting no effect on the uptake of labeled acetate into cardiolipin. These changes in the labeling of membrane phospholipids were not observed when the bacteria were deprived of thymine or a required amino acid or when chloramphenicol or nalidixic acid was present in the culture medium. These results are therefore in line with Silver and Wendt's proposal that the primary site of action of PEA is the cell membrane. However, at this time, it is not known whether the inhibition of phospholipid synthesis by PEA is the cause or effect of changes in cellular permeability. It is hoped that studies involving the effect of PEA on lipid synthesis in the PEA resistant mutants of E. coli such as those isolated by Yura and Wada (4) will result in the clarification of this problem.

Several workers (5, 6) have shown that PEA inhibits the initiation of a new cycle of DNA replication. Studies in our laboratory, involving the effect of PEA on DNA synthesis, are in agreement with the results obtained by Lark and Lark (6). On the basis of their findings, these

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4. T. Yura and C. Wada, Genetics 59, 177 (1968).
5. R. W. Treick and W. A. Konetzka, J. Bacteriol. 88, 1580 (1964).
6. K. G. Lark and C. Lark, J. Mol. Biol. 20, 9 (1966).

workers proposed that two proteins were required for the initiation of DNA synthesis (6). They suggested that the synthesis of one of these proteins was sensitive to PEA and insensitive to chloramphenicol. The other protein was postulated to be sensitive to chloramphenicol but insensitive to PEA. Our studies, involving treatment of E. coli cultures with 0.15% PEA, indicate that although phospholipid synthesis was inhibited by 55 percent, the synthesis of proteins was not affected. On the basis of this data, it appears that the PEA sensitive component postulated by Lark and Lark (6) may be a phospholipid(s) and not a protein. If this is the case, the inhibition of this phospholipid(s) sensitive component might result in altering the membrane in such a manner that subsequent initiation of DNA synthesis is prevented.

Several laboratories, including ours, have reported the marked inhibition of RNA synthesis by PEA (3, 7, 8). The studies of Wurster, Elsbach, Rand, and Simon (9) with levorphanol indicate that this drug affects both RNA and lipid synthesis in a manner similar to PEA. These authors suggested that the effect of levorphanol on lipid synthesis might be related to the inhibition of the synthesis of ribosomal RNA by this drug. They based their assumption on the evidence provided by Haywood (10) that ribosomal RNA is synthesized on the E. coli membrane.

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7. H. S. Rosenkranz, H. S. Carr, and H. M. Rose, J. Bacteriol. 89, 1354 (1965).

8. C. Prevost and V. Moses, J. Bacteriol. 91, 1446 (1966).

9. N. Wurster, P. Elsbach, J. Rand, and E. J. Simon, Biochim. Biophys. Acta 248, 252 (1971).

10. A. M. Haywood, Proc. Nat. Acad. Sci. U.S.A. 68, 435 (1971).

Although no attempts in this laboratory have been made to establish which species of RNA is inhibited by PEA, it is nevertheless obvious that a marked inhibition of total RNA synthesis must eventually result in a decrease in ribosomal RNA. It is reasonable to assume that if ribosomal RNA transcription is a membrane mediated process, the effect of PEA on RNA synthesis is a secondary consequence of the perturbation of the cell membrane.

Since PEA was shown to inhibit the synthesis of phosphatidylethanolamine and phosphatidylglycerol in E. coli, the effects of this compound on the in vitro activity of L-serine:CMP phosphatidyltransferase and L-glycerol-3-phosphate:CMP phosphatidyltransferase were investigated. The results of these studies showed that PEA exerted no effect on either of these enzymes. Thus, it is apparent that this compound does not exert its primary effect on the enzyme-substrate reactions of these phospholipid biosynthetic enzymes. Recently, Cheng (11) performed some preliminary experiments involving the effect of PEA on the in vitro activity of sn-glycerol-3-phosphate acyltransferase. The results he obtained seem to indicate that PEA exerts a profound inhibitory effect on this enzyme's activity. However, evaluation of this inhibition must await more extensive studies.

The effects of the phosphonic acid isosteres of glycerol-3-phosphate, 3,4-dihydroxybutyl-1-phosphonate and 2,3-dihydroxypropyl-1-phosphonate on the activity of the above phospholipid biosynthetic enzymes were also studied. The reasons for investigating the effects

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11. P. J. Cheng, unpublished data.

of the phosphonic acid isosteres on the *in vitro* activity of these enzymes were twofold: (i) the four-carbon phosphonate was found to inhibit the growth of *E. coli* (12), and (ii) tracer studies of the incorporation of labeled precursors of DNA, RNA, protein, and lipids into *E. coli*, in the presence of the phosphonic acid isosteres, indicate that the synthesis of lipids was affected prior to the synthesis of the macromolecules (13). The results of the *in vitro* studies showed that 3,4-dihydroxybutyl-1-phosphonate was more effective than 2,3-dihydroxypropyl-1-phosphonate in inhibiting the activity of L-glycerol-3-phosphate:CMP phosphatidyltransferase. The inhibition was competitive with glycerol-3-phosphate. The K_m for L-glycerol-3-phosphate obtained from these studies was 2.5×10^{-4} M and the K_i for L-3,4-dihydroxybutyl-1-phosphonate was 6.8×10^{-4} M. The K_i for L-2,3-dihydroxypropyl-1-phosphonate was experimentally found to be 2.7×10^{-3} M. The effect of the phosphonic acid analogues on L-serine:CMP phosphatidyltransferase activity was studied by measuring the uptake of labeled serine into chloroform extractable material from reaction mixtures containing these compounds. The data obtained from these studies revealed that the phosphonate analogues elicited no appreciable effect on the *in vitro* activity of this enzyme. Preliminary studies by Cheng (11), involving the effect of these phosphonic acid analogues on sn-glycerol-3-phosphate acyltransferase activity, indicate that 3,4-dihydroxybutyl-1-phosphonate but not 2,3-dihydroxypropyl-1-phosphonate inhibits the *in vitro* activity

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12. C. Shopsis, R. Engel, and B. E. Tropp, manuscript submitted for publication.

13. C. Shopsis, R. Engel, and B. E. Tropp, unpublished data.

of this enzyme. Further studies will have to be performed before any conclusion can be drawn from the latter experiments. Recently, we examined the effects of the phosphonate analogues on the uptake of labeled acetate into the major phospholipids of E. coli. These studies showed that the uptake of ^{14}C-acetate into the phosphatidylglycerol fraction was inhibited by 40-60 percent in the presence of 3,4-dihydroxybutyl-1-phosphonate. These results support the in vitro findings of reduced L-glycerol-3-phosphate:CMP phosphatidyltransferase activity in the presence of the four-carbon phosphonate analogue.

Although it is still not clear what roles the major phospholipids play in the various biochemical processes, the present studies of the effects of PEA and the phosphonic acid isosteres on lipid synthesis represent starting points from which more sophisticated probes of unbalanced growth conditions, resulting from the perturbation of phospholipid metabolism, can be performed.