ASPECTS OF sn-GLYCEROL-3-PHOSPHATE METABOLISM

BY

PO-JUN CHENG

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.

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ABSTRACT

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by

PO-JUN CHENG

Adviser: Professor Burton E. Tropp

Phenethyl alcohol and the phosphonic acid analogues of glycerol-3-phosphate have been chosen as reagents for studying the sn-glycerol-3-phosphate metabolism. Although 2,3-dihydroxypropyl-1-phosphonate was not recognized by the rabbit muscle NAD-linked glycerol-3-phosphate dehydrogenase, 3,4-dihydroxybutyl-1-phosphonate was oxidized at approximately the same rate and had nearly the same K_m (240 μM for glycerol-3-phosphate compared to 190 μM for 3,4-dihydroxybutyl-1-phosphonate) as the natural substrate. The rate of reduction of dihydroxyacetone phosphate was approximately 25 times faster than that of its analogue, 4-hydroxy-3-oxobutyl-1-phosphonate, even though their K_m values are very similar (130 μM for the natural substrate compared to 182 μM for the analogue).

When experiments were performed to evaluate the ability of the enzymes of Escherichia coli involved in glycerol-3-phosphate metabolism to recognize phosphonic acid analogues

neither the catabolic membrane-bound glycerol-3-phosphate dehydrogenase or the acyl-CoA:glycerol-3-phosphate acyltransferase could use 3,4-dihydroxybutyl-1-phosphonate or 2,3-dihydroxypropyl-1-phosphonate as substrate. Neither analogue exhibited an inhibitory effect upon the catabolic dehydrogenase. The three-carbon analogue, but not the four-carbon analogue appears to have a slight inhibitory effect on the acyltransferase.

Glycerol-3-phosphate and 3,4-dihydroxybutyl-1-phosphonate are competitive inhibitors of the reduction of dihydroxyacetone phosphate by glycerol-3-phosphate:NAD(P) oxidoreductase from E. coli. 2,3-Dihydroxypropyl-1-phosphonate does not appear to be recognized by this enzyme. The apparent K_i for glycerol-3-phosphate is 19 μM and for 3,4-dihydroxybutyl-1-phosphonate it is 42 μM. In addition the glycerol-3-phosphate:NAD(P) oxidoreductase catalyzes the reduction of 4-hydroxy-3-oxobutyl-1-phosphonate (apparent K_m of 182 μM).

3,4-Dihydroxybutyl-1-phosphonate is both a competitive inhibitor (apparent K_i of 740 μM) and a substrate (apparent K_m of 450 μM) for the CDP-diglyceride:glycerol-3-phosphate phosphatidyl transferase but it has no effect upon CDP-diglyceride:serine phosphatidyl transferases from E. coli.

Phenethyl alcohol inhibits glycerol-3-phosphate:NAD(P) oxidoreductase non-competitively with respect to dihydroxyacetone phosphate. At a concentration below 0.3%

phenethyl alcohol exhibits only a slight inhibitory effect upon the catabolic membrane-bound glycerol-3-phosphate dehydrogenase. Phenethyl alcohol not only inhibits acyltransferase activity but also shows different inhibitory effects upon the various acyl-CoAs tested in the assay system for phosphatidic acid and lysophosphatidic acid synthesis in E. coli. The mode of action of 3,4-dihydroxybutyl-1-phosphonate and phenethyl alcohol as inhibitors of phospholipid biosynthesis are discussed.

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TABLE OF CONTENTS

Page

TITLE PAGE..... i

APPROVAL PAGE..... ii

ABSTRACT iii

ACKNOWLEDGMENTS..... vi

TABLE OF CONTENTS..... vii

CHAPTER 1. INTRODUCTION..... 1

A. Enzymes Involved in sn-Glycerol-3-phosphate Metabolism..... 2
B. Genetics..... 5
C. In Vivo Effects of Phosphonic Acid Analogues of Glycerol-3-phosphate on E. coli..... 12
D. In Vivo Effects of Phenethyl Alcohol on Phospholipid Metabolism in E. coli 14
E. Figures..... 15

CHAPTER 2. SUBSTRATE ACTIVITY OF 3,4-DIHYDROXYBUTYL-1-PHOSPHONATE AND 4-HYDROXY-3-OXOBUTYL-1-PHOSPHONATE.... 19

A. Introduction..... 19
B. Materials and Methods..... 21
C. Results..... 24
D. Discussion..... 28
E. Figures and Table..... 30

CHAPTER 3. IN VITRO STUDIES OF 3,4-DIHYDROXYBUTYL-1-PHOSPHONATE METABOLISM..... 61

A. Introduction..... 61
B. Materials and Methods..... 63
C. Results..... 68
D. Discussion..... 74
E. Figures and Tables 77

CHAPTER 4. IN VITRO EFFECTS OF PHENETHYL ALCOHCL..... 89

A. Introduction..... 89
B. Materials and Methods..... 98
C. Results..... 103
D. Figures and Table..... 113

REFERENCE..... 144

CHAPTER 1

INTRODUCTION

The feature common to all phosphoglycerides is a glycerol-3-phosphate backbone. Fatty acid residues are attached to the hydroxy groups at carbon atoms 1 and 2. The phosphate group is usually involved in an ester linkage with various molecules such as ethanolamine, glycerol, choline, serine, and inositol. These phosphoglycerides are important components of membranes and as such may be involved in the following biological processes: oxidative phosphorylation, active transport, replication, or translation. A knowledge of the metabolism of glycerol-3-phosphate appears essential to understanding the control and regulation of phospholipid biosynthesis which is in turn central to progress in related biological problems.

Recently two compounds 3,4-dihydroxybutyl-1-phosphonate (a glycerol-3-phosphate analogue) and phenethyl alcohol (PEA) have been found to affect phospholipid metabolism *in vivo*. This thesis discusses the effects of these two compounds on some isolated enzymes related to *sn*-glycerol-3-phosphate metabolism. It seems appropriate to review some aspects of the synthesis and subsequent metabolism of *sn*-glycerol-3-phosphate. Primary attention will be devoted to bacterial metabolism with special emphasis on *Escherichia coli*. The

reasons for this are as follow: (1) extensive work has been performed on the genetic and biochemical systems of this organism by others and (2) in vivo studies concerning the effects of each of the compounds on E. coli have been reported.

A. Enzymes Involved in sn-Glycerol-3-phosphate Metabolism:

Glycerol and sn-glycerol-3-phosphate are dissimilated by E. coli K-12 through a converging pathway schematized in Fig. 1-1. The glycerol branch begins with an entry process involving facilitated diffusion (Hayashi & Lin, 1965b) which is followed by an adenosine-5'-triphosphate (ATP)-dependent phosphorylation catalyzed by glycerol kinase (Rush et al., 1957). The glycerol-3-phosphate branch begins with an active transport process (Hayashi et al., 1964). Both branches provide the common substrate, sn-glycerol-3-phosphate, for either the aerobic glycerophosphate dehydrogenase or the anaerobic glycerophosphate dehydrogenase. sn-Glycerol-3-phosphate can be a starting substrate for phospholipid biosynthesis (see Fig. 1-2). Unless cells are growing on glycerol or glycerol-3-phosphate as a sole carbon source, sn-glycerol-3-phosphate is made by the reduction of dihydroxyacetone phosphate catalyzed by the anabolic glycerophosphate dehydrogenase. It is believed that the glycerol-3-phosphate for phospholipids biosynthesis is mainly derived from the anabolic pathway.

(a) sn-Glycerol-3-phosphate Dehydrogeanse:

(i) The catabolic enzymes: The dissimilation of glycerol and sn-glycerol-3-phosphate in E. coli K-12 is dependent upon one of two distinct sn-glycerol-3-phosphate dehydrogenases neither of which is linked to pyridine nucleotides. The factor which determines which of the two enzymes is necessary for growth is apparently the nature of terminal electron acceptors available for the respiratory chain. With oxygen as the acceptor, a membrane-associated dehydrogenase which is not stimulated by added flavin (aerobic membrane-bound sn-glycerol-3-phosphate dehydrogenase) is required. With fumarate as the acceptor, a soluble dehydrogenase which is greatly stimulated by added flavin (anaerobic sn-glycerol-3-phosphate dehydrogenase) is required. Both of these enzymes have been purified from E.coli recently (Weiner & Heppel, 1972; Kistler & Lin 1972). The anaerobic enzyme does not appear to function in aerobically cultured cells (Kistler et al., 1969).

(ii) The anabolic enzyme: sn-Glycerol-3-phosphate is one of the building units for phospholipid biosynthesis. The major source of this compound is believed to be the reduction of dihydroxyacetone phosphate, an intermediate in glycolysis. The enzyme (sn-glycerol-3-phosphate:NAD^+ oxidoreductase) (EC. 1.1.8) is a cytoplasmic enzyme, abundant in insect flight muscle and in a number of mammalian tissues including brain and skeletal muscle. This enzyme catalyzes the reversible reduction of dihydroxyacetone phosphate by

reduced NADH to form sn-glycerol-3-phosphate and NAD^+. Among the several purified enzymes from different sources the one from rabbit muscle has been studied most extensively. The enzyme purified from extracts of E.coli (Kito & Pizer, 1969) appears to use NADPH instead of NADH in its reduction of dihydroxyacetone phosphate. The reaction strongly favors the formation of sn-glycerol-3-phosphate.

(b) Enzymes Involved in Phosphoglyceride Metabolism:

(i) Acyltransferase: Studies with a particulate preparation from E.coli showed that phosphatidic acid (diacyl-sn-glycerol-3-phosphate), a key intermediate in the biosynthesis of phospholipids, and lysophosphatidic acid (monoacyl-sn-glycerol-3-phosphate) were formed from sn-glycerol-3-phosphate and palmityl-CoA (Pieringer et al.,1967). In cell free preparations from Clostridium butyricum, using palmityl-ACP as the substrate, lysophosphatidic acid was formed (Goldfine et al., 1969). With this enzyme preparation palmityl-CoA was a poor substrate in the acylation reaction. These results make it apparent that the acylases have some specificity toward the structure of the acyl thioester used. Cronan et al., (1970) have isolated a mutant which contains normal acyl-CoA:monoacyl-glycerol-3-phosphate acyltransferase activity and thermolabile acyl-CoA:sn-glycerol-3-phosphate acyltransferase activity. These results indicate that there are at least two enzymes required for the conver-

sion of sn-glycerol-3-phosphate to phosphatidic acid. For a final clarification of this question a mutant must be isolated with a defect in monoacyl-glycerol-3-phosphate acyltransferase activity.

(ii) CDP-diglyceride:glycerol-3-phosphate phosphatidyl transferase: Although the reaction between CDP-diglyceride and sn-glycerol-3-phosphate is believed to yield phosphatidylglycerol phosphate as an intermediate this intermediate has not been demonstrated in E.coli in vivo. However, Chang and Kennedy have separated the enzymes responsible for phosphatidyl glycerol synthesis into two particulate fractions in E.coli; one catalyzes the synthesis from CDP-diglyceride and sn-glycerol-3-phosphate (Chang & Kennedy, 1967) and the other the dephosphorylation of phosphatidylglycerol phosphate to yield phosphatidylglycerol (Chang & Kennedy, 1967b). Both of these enzymes have been extracted from the particulate fractions and partially purified (Chang & Kennedy, 1967a; and 1967b). The second enzyme is highly specific for its substrate showing little or no activity with phosphatidic acid.

B. Genetics:

The understanding of sn-glycerol-3-phosphate metabolism in recent years is due in large part to the isolation of mutants from E.coli. The mutants to be described are all derived from E.coli K-12.

(a) Mutants as a Means of Studying Metabolic Regulation:

(i) glpF and glpK mutants: Strains with a lesion in the gene for facilitated diffusion (glpF) grow normally on 10 mM glycerol but very slowly on 1 mM glycerol (Richey & Lin, 1972). Mutants which lack glycerol kinase (EC. 2.7. 1.30) ($glpK^-$) are unable to accumulate radioactive material when incubated with [^{14}C]-glycerol, whereas wildtype cells are able to do so (Hayashi et al., 1965b). In addition, mutants lacking glycerol kinase are not capable of growth on synthetic medium supplemented with glycerol, but are able to be grown normally when the medium is supplemented with glycerol-3-phosphate (Lin et al., 1962). In E.coli K-12, the conversion of glycerol to dihydroxyacetone phosphate is regulated by two types of control mechanism: the rate of synthesis of glycerol kinase and the feedback inhibition of the activity of this enzyme by fructose-1,6-diphosphate (Zwaig & Lin, 1966). A strain which has lost both control mechanisims by successive mutations resulting in the constitutive synthesis of a glycerol kinase which is no longer sensitive to feedback inhibition produces a bacteriocidal factor, methylglyoxal, from glycerol. This factor is derived from dihydroxyacetone phosphate. Evidently when the rate of formation of dihydroxyacetone phosphate is excessive, this toxic material is formed (Freedberg et al., 1971).

(ii) glpT, glpD, glpA and glpR mutants: E.coli strain 6, a mutant defective in the transport of glycerol-3-

phosphate (glpT), was obtained from strain 1 which is inducible with respect to glycerol kinase. Strain 6 is able to grow on glycerol but not on glycerophosphate (Hayashi et al., 1964).

Two pathways for the dissimilation of glycerol are known in enteric bacteria, one pathway is mediated by a glycerol kinase and a glycerophosphate dehydrogenase which is not linked to nicotinamide adenine dinucleotide (NAD^+) (Rush et al., 1957), while the other is mediated by NAD^+ linked glycerol dehydrogenase (Burton & Kaplan, 1953) and dihydroxyacetone kinase (Jacob & Vandmark, 1960). In some species such as Streptococcus faecalis (Jacob & Vandemark, 1960) and Aerobacter aerogenes strain 1033 (Lin et al., 1960) both pathways are known to exist; whereas in others such as E.coli K-10 and A. Aerogenes 1041 only the former pathway has been found (as quoted from Koch et al., 1964). Mutants of E. coli that have lost the NAD^+ independent, membrane-bound glycerol-3-phosphate dehydrogenase have been isolated. Such mutants (genotype $glpD^-$) are unable to use either glycerol or glycerol-3-phosphate as the sole carbon source when cultured aerobically. In fact both compounds inhibit the growth of cells that lack this catabolic dehydrogenase. (Koch et al., 1964; Cozzarelli et al., 1963). Meanwhile there is another gene (glpA) which contains the genetic information for the anaerobic, catabolic, glycerol-3-phosphate dehydrogenase.

Thus even though unable to utilize glycerol aerobically, glpD mutants with anaerobic dehydrogenase could metabolize glycerol anaerobically provided that either nitrate or fumarate was added to the culture medium. These latter two compounds serve as exogenous electron acceptors (Kistler et al., 1969).

It is possible to isolate double mutants of genotype $glpD^{-}$ $glpA^{-}$. Such double mutants cannot utilize glycerol or glycerol-3-phosphate aerobically or anaerobically. The anaerobic dehydrogenase appears to be able to function only when cells are cultured anaerobically (Kistler et al., 1969) even though active anaerobic dehydrogenase can be detected in aerobically cultured E. coli (Kistler & Lin, 1971). The glpD product which is the aerobic, catabolic, NAD-independent, membrane-bound glycerol-3-phosphate dehydrogenase functions both aerobically and anaerobically with nitrate as acceptor but not with fumarate. In summary, a cell with a glpD lesion can utilize glycerol under anaerobic conditions, a cell with glpA lesion can utilize glycerol both aerobically and anaerobically, and double mutants cannot utilize glycerol under either set of conditions.

E. coli strain 8 used as a source for some of the enzymes in the present study is alkaline phosphatase negative; aerobic, catabolic glycerophosphate dehydrogenase negative; and constitutive for the glycerol-3-phosphate transport system. When glycerol-3-phosphate is added to growing cultures of this organism greater than 98% of the radioactivity incorporated

is found in the phospholipid fraction (Farrow et al., 1968).

The glpA, glpD, glpT, glpF (facilitated diffusion), and glpK (glycerol kinase) are part of a regulon and therefore under the control of the same repressor protein. It is possible to isolate mutants with a defective repressor that are constitutive for all of the above genes. Such mutants are referred to as $glpR^-$. In addition to the repressor system, two other kinds of control mechanisms govern the expression of the members of the glp regulon in E. coli K-12: catabolic repression and respiratory repression (the effect exerted by exogenous hydrogen acceptors). The operons of the glp system show different patterns of response to each control. By growing in parallel a mutant strain with temperature-sensitive repressor ($glpR^{ts}$) and an isogenic control with a deletion in the regulator gene at progressively higher temperatures, it can be shown that the synthesis of the aerobic glycerol-3-phosphate dehydrogenase (glpD product) is far more sensitive to specific repression than that of either glycerol kinase (glpK product) or glycerol-3-phosphate transport (glpT product). In addition, in a strain with a deletion in the regulator gene, the synthesis of glycerol kinase and glycerol-3-phosphate transport are more sensitive to catabolite repression than that of the aerobic glycerol-3-phosphate dehydrogenase. The level of the two flavoprotein glycerol-3-phosphate dehydrogenases vary in opposite directions in

response to changes of exogenous hydrogen acceptors (Freedberg & Lin, 1973).

(iii) Mutants of anabolic glycerol-3-phosphate dehydrogenase: A glycerol-requiring mutant of E. coli K-12 with a specific defect in anabolic glycerol-3-phosphate dehydrogenase was isolated. In this mutant, removal of glycerol from the growth medium results in the immediate inhibition of the synthesis of phospholipids (Hsu and Fox, 1970). Bell (1974) has isolated mutants lacking the anabolic glycerol-3-phosphate dehydrogenase from E. coli strain 8 and such mutants require glycerol-3-phosphate for growth. Glycerol cannot substitute for glycerol-3-phosphate. The inability of glycerol to replace glycerol-3-phosphate is thought to be due to the sensitivity of glycerol kinase to the fructose-1,6-diphosphate which accumulates when these cells are cultured on glucose. Glucose must be added because these cells also lack the catabolic glycerol-3-phosphate dehydrogenase and cannot grow on glycerol-3-phosphate alone.

It is possible to isolate further mutants lacking a fructose-1,6-diphosphate sensitive glycerol kinase. These mutants which lack the anabolic and catabolic dehydrogenase and have a glycerol kinase which is insensitive to allosteric inhibition can be successfully cultured on synthetic medium supplemented with glucose and glycerol or glycerol-3-phosphate.

(iv) Mutants of glycerol-3-phosphate acyltrans-

ferase: E. coli mutants possessing temperature-sensitive lesions for glycerol-3-phosphate acyltransferase have been isolated and characterized genetically. Such mutants are defective in phospholipid biosynthesis as measured by [^{32}P]-phosphate incorporation into the phospholipid fraction at 37°C but contain normal 1-acyl-sn-glycerol-3-phosphate acyltransferase activity (Cronan & Vagelos, 1972). Bell (1974) has recently isolated K_m mutants for the glycerol-3-phosphate acyltransferase.

(b) Genetic Map

All the mutations cited in the previous sections have been mapped (see Fig. 1-3). The structural gene coding for the transport system and enzymes required for the metabolism of glycerol and sn-glycerol-3-phosphate are found in three clustered regions of the E. coli map (Cozzarelli et al., 1968) The glpF gene controlling facilitated diffusion of glycerol (Sanno et al., 1968) and the glpK gene specifying the structure of glycerol kinase, the first enzyme of the catabolic pathway (see Fig. 1-1) (Koch et al., 1964), appear to be part of a single operon located at minute 76 on the chromosome according to Taylor (Cozzarelli & Lin, 1966). The gene for the glycerol-3-phosphate transport system (glpT) and the gene for the catabolic anaerobic glycerol-3-phosphate dehydrogenase (glpA) are located adjacent to one another at minute 43 (Hayashi & Lin, 1965) of the E. coli genetic map (Berman et al., 1969; Zwaig et al., 1970) and may also belong to a single

operon. The gene for catabolic aerobic glycerol-3-phosphate dehydrogenase (glpD) is found in a third region at minute 66 adjacent to the glpR locus (Hayashi & Lin, 1965) which specifies the repressor. The glpR gene regulates the expression of all the operons of the glp system and responds to sn-glycerol-3-phosphate as the effector (see Fig. 1-3). In addition, mutants for sn-glycerol-3-phosphate acyltransferase, the enzyme catalyzing the first step in phospholipid biosnythesis, have also been characterized genetically. They map in a single locus called plsA, which is located at minute 13 on the E.coli genetic map. The anabolic glycerol-3-phosphate dehydrogenase mutants have been mapped at minute 71 on the E. coli genetic map (Bell, 1974).

C. In Vivo Effect of Phosphonic Acid Analogues of Glycerol-3-phosphate on E. coli.

The growth of cultures of E. coli strains that are constitutive for the glycerol-3-phosphate transport system and that lack the membrane-bound glycerol-3-phosphate dehydrogenase is more sensitive to the inhibition caused by the phosphonate, 3,4-dihydroxybutyl-1-phosphonate, than that caused by the natural metabolite sn-glycerol-3-phosphate. The growth inhibition caused by this phosphonate differs from that caused by glycerol-3-phosphate in that it is not offset by the presence of glucose or high concentrations of phosphate in the growth medium and it is not dependent upon the absence of the membrane-

bound glycerol-3-phosphate dehydrogenase. (Shopsis et al., 1972)

Studies on the accumulation of labeled precursor incorporated into DNA, RNA, protein, and lipid revealed that the phosphonate inhibits phospholipid synthesis more effectively than it inhibits either DNA, RNA, or protein synthesis. In contrast glycerol-3-phosphate was found to have its strongest inhibitory effect on RNA synthesis (Shopsis et al., 1973). The distribution of labeled acetate incorporated into the phospholipid fraction was altered by the presence of the phosphonate in the incubation medium (Shopsis et al., 1972). The accumulation of [^{32}P]-phosphate into different fractions of phospholipid indicates that phosphatidylglycerol is very strongly inhibited, while phosphatidylethanolamine is less inhibited. There is almost no effect on cardiolipin biosynthesis. It has been suggested that the limited amount of phosphatidylglycerol synthesis that takes place in the presence of the analogue is sufficient to account for the de novo synthesis of cardiolipin. (Shopsis et al., 1974)

Studies on the rate of disappearance of [^{32}P]-phosphate from the phosphoglycerides of prelabeled cells indicate that the addition of phosphonate analogue to a culture does not alter the rate of turnover of the phospholipids. Labeled phosphonic acid analogue was readily incorporated into a chloroform extractable fraction of E. coli strain

8 (Shopsis, et al., 1974). These results indicate that 3,4-dihydroxybutyl-1-phosphnate is a metabolic regulator exerting a particular effect on phospholipid metabolism. Nunn (1972) has demonstrated that 3,4-dihydroxybutyl-1-phosphonate is a substrate for CDP-diglyceride:glycerol-3-phosphate phosphatidyl transferase.

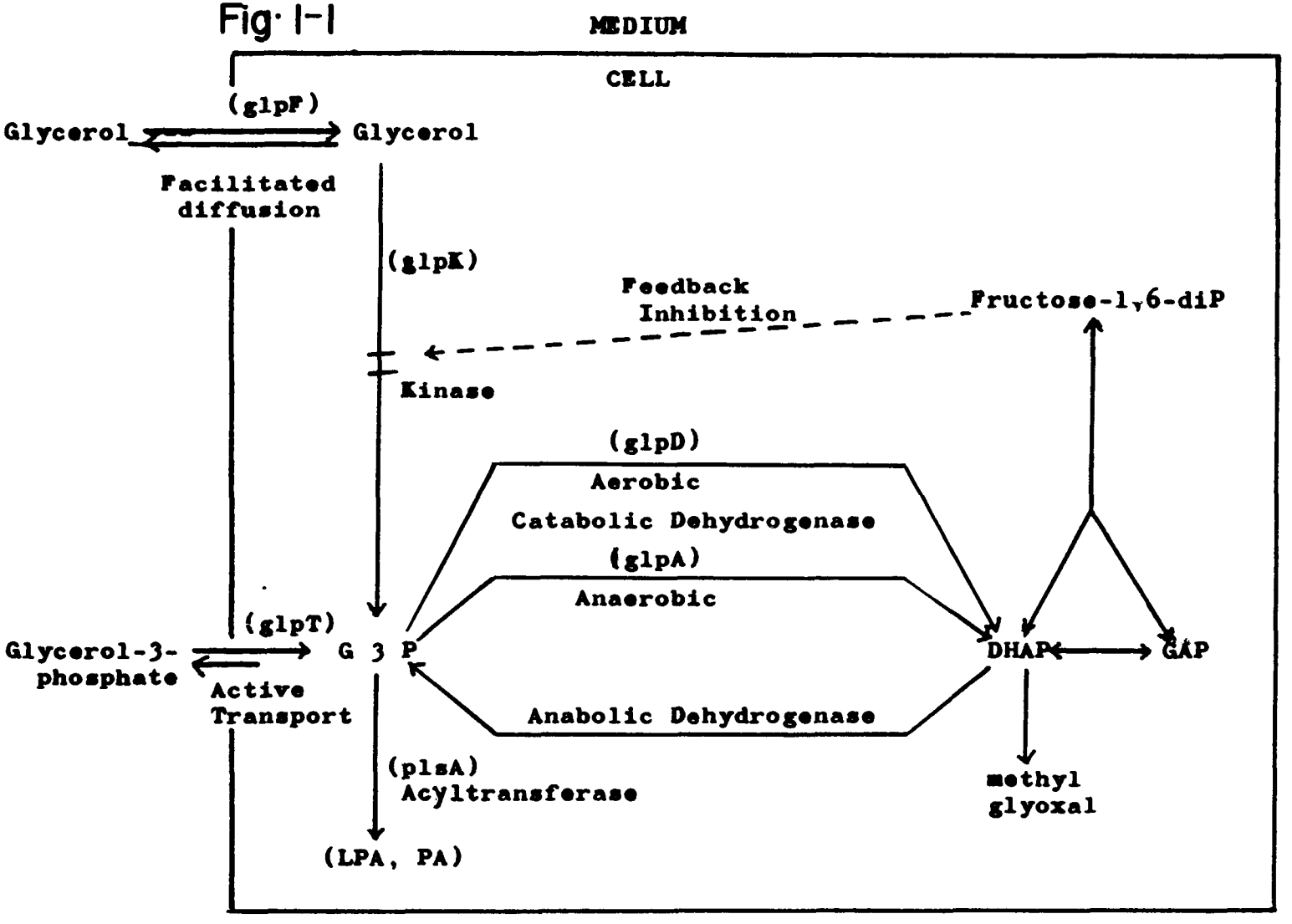
D. In Vivo Effects of Phenethyl Alcohol on Phospholipid Metabolism in E. coli.

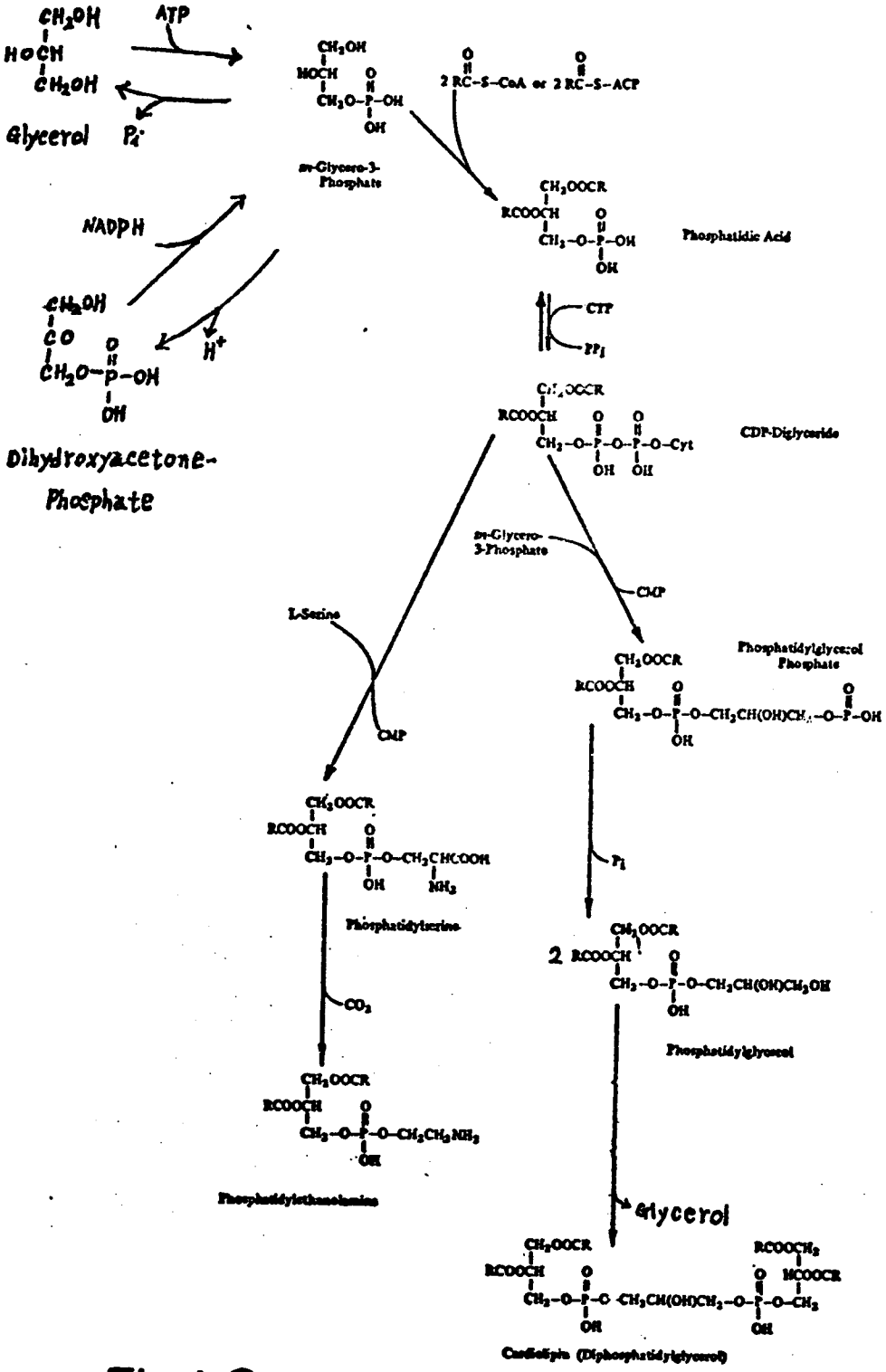
Nunn and Tropp (1972) reported that the biosynthesis of phospholipid was more sensitive to the inhibition by low concentrations of PEA than the biosynthesis of RNA, DNA or protein.

The distribution of labeled acetate incorporated into phospholipids was markedly affected by the presence of PEA. The uptake of acetate into both phosphatidylethanolamine and phosphatidylglycerol was inhibited (Barbu et al., 1970; Nunn & Tropp. 1972). More extensive reviews concerning the mode of action of 3,4-dihydroxybutyl-1-phosphonate and PEA on cultures of Escherichia coli will be presented in subsequent chapters.

Fig. 1-1: Metabolic pathway for sn-glycerol-3-phosphate and related compounds in E. coli K-12. It also lists the genes for coded proteins (see also Fig. 1-3 Genetic map) DHAP stands for dihydroxyacetone phosphate, GAP, D-glyceraldehyde-3-phosphate, LPA, lysophosphatidic acid and PA, phosphatidic acid (modified from Freedberg and Lin, 1973).

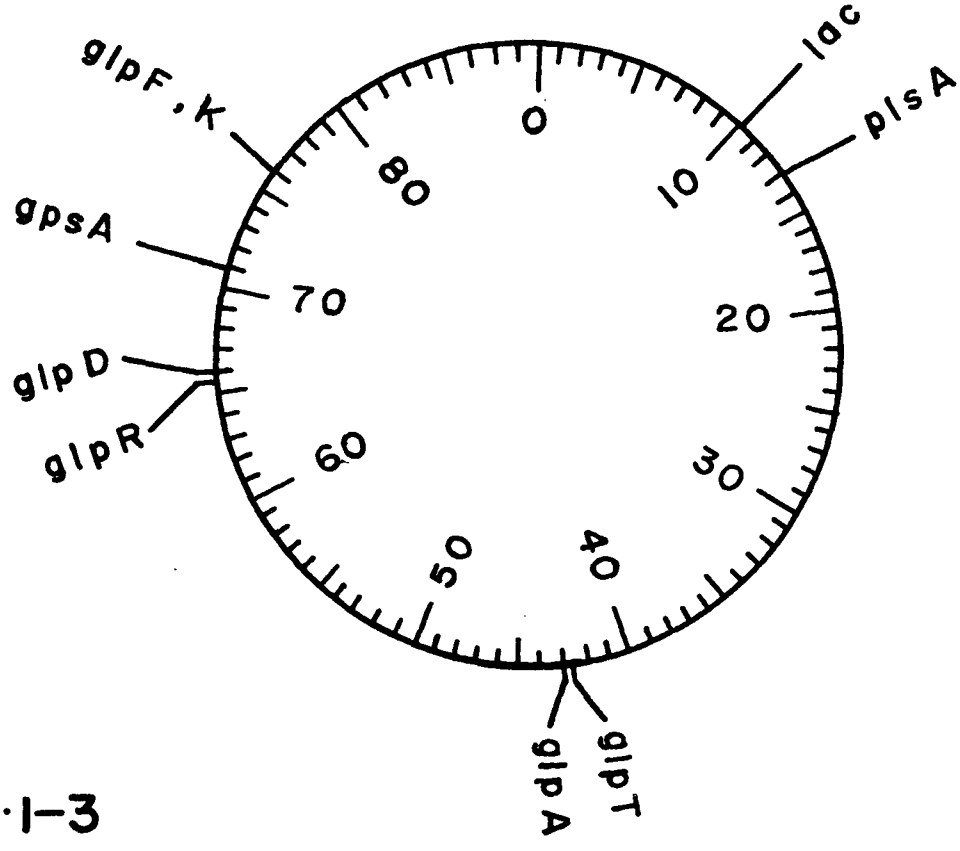
Fig. I-I


MEDIUM
CELL
(glpF)
Glycerol
Glycerol
Facilitated diffusion
(glpK)
Feedback Inhibition
Fructose-1,6-diP
Kinase
(glpD)
Aerobic
Catabolic Dehydrogenase
(glpA)
Anaerobic
(glpT)
Glycerol-3-phosphate
G 3 P
DHAP
GAP
Active Transport
Anabolic Dehydrogenase
(plsA)
Acyltransferase
methyl glyoxal
(LPA, PA)



Fig·1-2

The biosynthesis of the phospholipids of E. coli modified from Cronan and Vagelos. (1972)



Fig·1-3

Genetic map of E. coli modified from Cozzarelli et al. (1968)

Gene symbols	Activity affected
glpA	anaerobic sn-glycerol-3-phosphate dehydrogenase
glpD	aerobic sn-glycerol-3-phosphate dehydrogenase
glpF	glycerol transport facilitator
glpK	glycerol kinase
glpT	glycerol-3-phosphate transport
plsA	sn-glycerol-3-phosphate acyltransferase
gpsA	anabolic sn-glycerol-3-phosphate dehydrogenase
glpR	repressor of glp regulon

CHAPTER 2

SUBSTRATE ACTIVITY OF 3,4-DIHYDROXYBUTYL-1-PHOSPHONATE AND 4-HYDROXY-3-OXOBUTYL-1-PHOSPHONATE[1]

A. Introduction

Shopsis et al., (1972) have previously described conditions under which 3,4-dihydroxybutyl-1-phosphonate, an analogue of glycerol-3-phosphate, inhibits the growth of Escherichia coli. More recent investigations revealed that the four-carbon phosphonate has a profound effect upon phospholipid metabolism (Shopsis et al., 1973). These observations led the author to explore whether the dihydroxybutyl-1-phosphonate could serve as a substrate or an inhibitor for several reactions catalyzed by enzymes isolated from E. coli and other sources. In the course of these studies attention became focused on glycerol-3-phosphate dehydrogenase activities. This chapter describes work performed with the rabbit muscle NAD-linked glycerol-3-phosphate dehydrogenase (L-glycerol-3-phosphate:NAD oxidoreductase EC. 1.1.1.8.).

Compounds containing the $-CH_2PO_3H_2$ group in place of

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1. The studies reported in this chapter have been published in Biochimica et Biophysica Acta, 341:85-92 (1974)

the $-OPO_2H_2$ group are structurally similar but not identical. Structural differences involving bond lengths and angles have been reported among compounds of the P-X-P type, where X is either O, NH, or CH_2 (Larsen et al., 1969). Furthermore, the phosphonic acid analogues are weaker acids than their phosphate counterparts. These differences are on the order of 2.5 pK_a units for each of the dissociation constants. From a biological point of view it is obvious that enzymes responsible for phosphoester cleavage would not be able to perform their catalytic function on a phosphonic acid analogue. It is less clear as to whether an enzyme catalyzing a reaction at some site other than the phosphoester bond would be able to function with such an analogue.

2,3-Dihydroxypropyl-1-phosphonate, an analogue of glycerol-3-phosphate, has been tested as a substrate for the rabbit muscle dehydrogenase and was observed to be inert (Baer et al., 1969). One possible conclusion from such an observation is that the phosphoester bond of glycerol-3-phosphate is required for activity. An alternative conclusion is that the steric dissimilarities between glycerol-3-phosphate and the three-carbon analogue are too great and therefore preclude any activity. This chapter presents evidence indicating that 3,4-dihydroxybutyl-1-phosphonate and 4-hydroxy-3-oxobutyl-1-phosphonate, an analogue of dihydroxyacetone phosphate, serve as substrates for the rabbit muscle glycerol-3-phosphate dehydrogenase.

B. Materials and Methods

Materials: NAD^+ (grade III), NADH (grade III), DL-glycerol-3-phosphate, disodium salt (grade X), di-monocyclohexylamine salt of dihydroxyacetone phosphate dimethylketal, glycine, triethanolamine-HCl, bovine serum albumin, and L-glycerol-3-phosphate:NAD oxidoreductase (EC. 1.1.1.8) were purchased from the Sigma Chemical Co., St. Louis, Mo. Hydrazine sulfate was purchased from the Fisher Scientific Co., Pittsburgh, Pa. Dihydroxyacetone phosphate was generated using the Dowex 50 x 4-200R provided by the manufacturer for this purpose and following the manufacturer's instructions.

The dilithium salt of 3,4-dihydroxybutyl-1-phosphonic acid, the monosodium salt of 4-hydroxy-3-oxobutyl-1-phosphonic acid, and the dilithium salt of 2,3-dihydroxypropyl-1-phosphonic acid were a generous gift of Professor Engel. The preparation of these compounds have been described by Kabak et al., (1972), Goldstein et al., (1974) and Rosenthal and Geyer (1968).

Enzyme Assay: The rabbit muscle glycerol-3-phosphate dehydrogenase was diluted as described by Black (1966). The dehydrogenase activity was determined spectrophotometrically by measuring the rate of formation or disappearance of NADH at 340 nm using either a Gilford model 2400 spectrophotometer or Gilford model 240 fitted with a constant temperature cuvette holder and a Honeywell recorder. Reaction rates

were determined during the first 30 seconds for the oxidation of glycerol-3-phosphate and 3,4-dihydroxybutyl-1-phosphonate. The assay procedure used in all kinetic experiments was that described by Black (1966). Although racemic mixtures of glycerol-3-phosphate and its phosphonic acid analogue were used throughout the present studies, the results are expressed in terms of L or D isomer[2]. All assays were performed at 26°C.

The reaction mixture for assaying the rate of oxidation of glycerol-3-phosphate and 3,4-dihydroxybutyl-1-phosphonate contained 33 μmoles triethanolamine buffer at pH 9.0, 2 μmoles EDTA, 2 μmoles β-mercaptoethanol, 1.0 mg bovine serum albumin, and the indicated concentrations of glycerol-3-phosphate, 3,4-dihydroxybutyl-1-phosphonate, NAD^+, and enzyme per ml. The Li^+ concentration was kept constant at 10 mM for the reaction. The reaction mixture used for following the reduction of dihydroxyacetone phosphate and 4-hydroxy-3-oxobutyl-1-phosphonate contained 50 μmoles triethanolamine buffer at pH 7.5 and no Li^+ but was identical in all other respect to that used for measuring the oxidation of glycerol-3-phosphate. The pH of all components were preadjusted to

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2. L-glycerol-3-phosphate, D-glycerol-1-phosphate and sn-glycerol-3-phosphate are the same. The corresponding phosphonic acid isomer is D-3,4-dihydroxybutyl-1-phosphonate.

7.5 individually. Solutions of NAD^+ and NADH were prepared freshly before use. Calculations were made by using 6.22 x $10^3 M^{-1} cm^{-1}$ as the molar extinction coefficient for NADH at 340 nm. The extent of oxidation of sn-glycerol-3-phosphate and D-3,4-dihydroxybutyl-1-phosphonate was determined enzymatically according to the method of Hohorst (1963).

C. Results

Baer, Nazir, and Basu (1969) reported that 2,3-dihydroxypropyl-1-phosphonate cannot serve as either a substrate or an inhibitor for rabbit muscle glycerol-3-phosphate dehydrogenase. Experiments performed in the course of the present investigations completely support these conclusions (date not shown). However, preliminary experiments revealed that 3,4-dihydroxybutyl-1-phosphonate could serve as a substrate for the muscle dehydrogenase. The fact that the substrate activity is not due to a contamination present in the preparation of the four-carbon phosphonate was revealed by measuring the extent of oxidation of glycerol-3-phosphate and 3,4-dihydroxybutyl-1-phosphonate under assay conditions favoring the completion of the reaction. Figure 2-1 indicates that greater than 95% of the optically active isomer of each of the compounds was converted to the oxidized form.

A series of experiments were initiated to determine the true K_m values for sn-glycerol-3-phosphate, D-3,4-dihydroxybutyl-1-phosphonate, and NAD^+. Figure 2-2a is a Lineweaver-Burk plot (Lineweaver & Burk, 1934) of the reciprocal of the initial velocity (v) versus the reciprocal of the millimolar concentration of sn-glycerol-3-phosphate at serveral different concentrations of NAD^+. As expected (Black, 1966), the results indicate that the K_m for sn-

glycerol-3-phosphate is dependent upon the NAD^+ concentration. Figure 2-2b is a Lineweaver-Burk plot of the reciprocal of the initial velocity (v) versus the reciprocal of the millimolar concentration of NAD^+ at serveral different concentrations of sn-glycerol-3-phosphate. Again, the results are consistent with those of Black (1966) and indicate that the K_m for NAD^+ is dependent upon the glycerol-3-phosphate concentration. A similar series of experiments was performed for D-3,4-dihydroxybutyl-1-phosphonate and NAD^+. The results, as depicted in Figure 2-3, are qualitatively similar to those obtained for the natural substrate.

The true Michaelis constants for sn-glycerol-3-phosphate and NAD^+ were obtained by treating the data in Figure 2-2 according to the method used by Florini and Vestling (1957). The results are shown in Figure 2-4b. In a similar fashion the data in Figure 2-3 was treated to obtain the true Michaelis constants for D-3,4-dihydroxybutyl-1-phosphonate and NAD^+, as shown in Figure 2-4a. The true K_m values reported by Black (1966) for the natural substrates as well as the values obtained in the present study are summarized in Table 2-1.

The rabbit muscle glycerol-3-phosphate dehydrogenase catalyzed the oxidation of glycerol-3-phosphate and 3,4-dihydroxybutyl-1-phosphonate at nearly the same rate (The values of V_{max} are 59 and 66 mmoles/min. per g of enzyme

for sn-glycerol-3-phosphate and D-3,4-dihydroxybutyl-1-phosphonate, respectively). However, when the reaction was assayed in the reverse direction the reduction of dihydroxyacetone phosphate proceeded at twenty-five times the rate of the reduction of 4-hydroxy-3-oxobutyl-1-phosphonate (V_{max} is 610 mmoles/min per g of enzyme for dihydroxyacetone phosphate compared to 24 mmoles/min per g of enzyme for 4-hydroxy-3-oxobutyl-1-phosphonate). This result was not due to contamination of the analogue since the reduction of both the analogue and dihydroxyacetone phosphate proceeded to approximately the same extent (data not shown).

One possible explanation for the difference in activity might be a difference in K_m values. For this reason the true K_m values were determined. Figure 2-5a is a Lineweaver-Burk plot of the reciprocal of the initial velocity (v) versus the reciprocal of the millimolar concentration of dihydroxyacetone phosphate at several different concentrations of NADH. Figure 2-5b is a Lineweaver-Burk plot of the reciprocal of the initial velocity (v) versus the reciprocal of the millimolar concentration of NADH at several different concentrations of dihydroxyacetone phosphate. A similar series of experiments was undertaken for 4-hydroxy-3-oxobutyl-1-phosphonate and NADH. The results, as depicted in Figure 2-6, are qualitatively similar to those obtained for the natural substrate with the important exception of a greatly diminished

rate. The true Michaelis constants for dihydroxyacetone phosphate and NADH were obtained by treating the data according to Florini and Vestling (1957) and are obtained from Figure 2-7b. In a similar fashion, the true K_m values for 4-hydroxy-3-oxobutyl-1-phosphonate and NADH are obtained from Figure 2-7a. The true K_m values for the substrates examined along with the values for the natural substrates reported by Black (1966) are summarized in Table 2-1.

It is evident that the K_m values for the natural substrates and their corresponding analogues are quite similar (Table 2-1). There is a major difference in pK_a values for phosphates and phosphonates. This difference could be responsible for the observation that the rate of oxidation of glycerol-3-phosphate and 3,4-dihydroxybutyl-1-phosphonate at pH 9.0 are similar while the rate of reduction of dihydroxyacetone phosphate was approximately 25-fold greater than that for 4-hydroxy-3-oxobutyl-1-phosphonate at pH 7.5. A higher pH optimum was expected for the phosphonic acid analogues because they are weaker acids than the natural substrates. When the effect of pH on rate was determined (Figure 2-8), contrary to expectation, 4-hydroxy-3-oxobutyl-1-phosphonate had a lower pH optimum than the natural substrate.

D. Discussion

The phosphonic acid analogues of natural organic phosphates may have some potential as metabolic regulators and chemotherapeutic agents. It was previously demonstrated that 3,4-dihydroxybutyl-1-phosphonate can inhibit the growth of E. coli (Shopsis et al., 1972) and has a profound effect upon phospholipid metabolism (Shopsis et al., 1973). In vitro studies on systems derived from E. coli have revealed that 3,4-dihydroxybutyl-1-phosphonate can serve as a substrate or an inhibitor of glycerol-3-phosphate:CDP-diglyceride phosphatidyltransferase (Nunn, 1972) and the anabolic glycerol-3-phosphate:NAD(P) oxidoreductase (see next chapter). However, it is not a substrate or an inhibitor for the glycerol-3-phosphate acyltransferase reaction or for the membrane-bound catabolic glycerol-3-phosphate dehydrogenase. It is not clear at this time why the phosphonic acid analogues serve as substrates for certain enzymes but not for others. However, the steric and pK_a factors are undoubtedly important in certain cases. Our present working hypothesis is that 3,4-dihydroxybutyl-1-phosphonate inhibits cell growth by virtue of its ability to serve as a substrate or an inhibitor for the glycerol-3-phosphate:CDP-diglyceride phosphatidyltransferase.

The rabbit muscle dehydrogenase is the only enzyme studied to date that does not appear to distinguish between

glycerol-3-phosphate and 3,4-dihydroxybutyl-1-phosphonate in the sense that both the K_m values and the rates of oxidation for each substrate are quite similar. However, this enzyme does distinguish between dihydroxyacetone phosphate and 4-hydroxy-3-oxobutyl-1-phosphonate. While the K_m values for these compounds are similar, there is a 25-fold greater rate of reduction of the natural substrate. The pH profiles (Figure 2-8) do not appear to be consistent with the hypothesis that the differences in the rate of reduction are due to differences in the charge on the substrates. Studies with the muscle dehydrogenase indicate the importance of steric factors since 2,3-dihydroxypropyl-1-phosphonate is neither substrate nor inhibitor for this enzyme. There may be many additional factors involved in explaining the differences in the substrate activity of the phosphonic acid analogues and their natural counterparts. Such explanations must await further experimentation. It seems clear that the phosphonic acid analogues have a tremendous potential in studies concerning enzyme-substrate interactions and also as antimetabolites. The role of phosphonic acids as antimetabolites is dependent upon their ability to enter cells. This permeability problem remains to be studied.

Figure 2-1: Extent of oxidation of various concentrations of sn-glycerol-3-phosphate, ●——●——● and D-3,4-dihydroxybutyl-1-phosphonate, ■——■——■ as determined by the reduction of NAD^+ according to the method of Hohorst (1963).

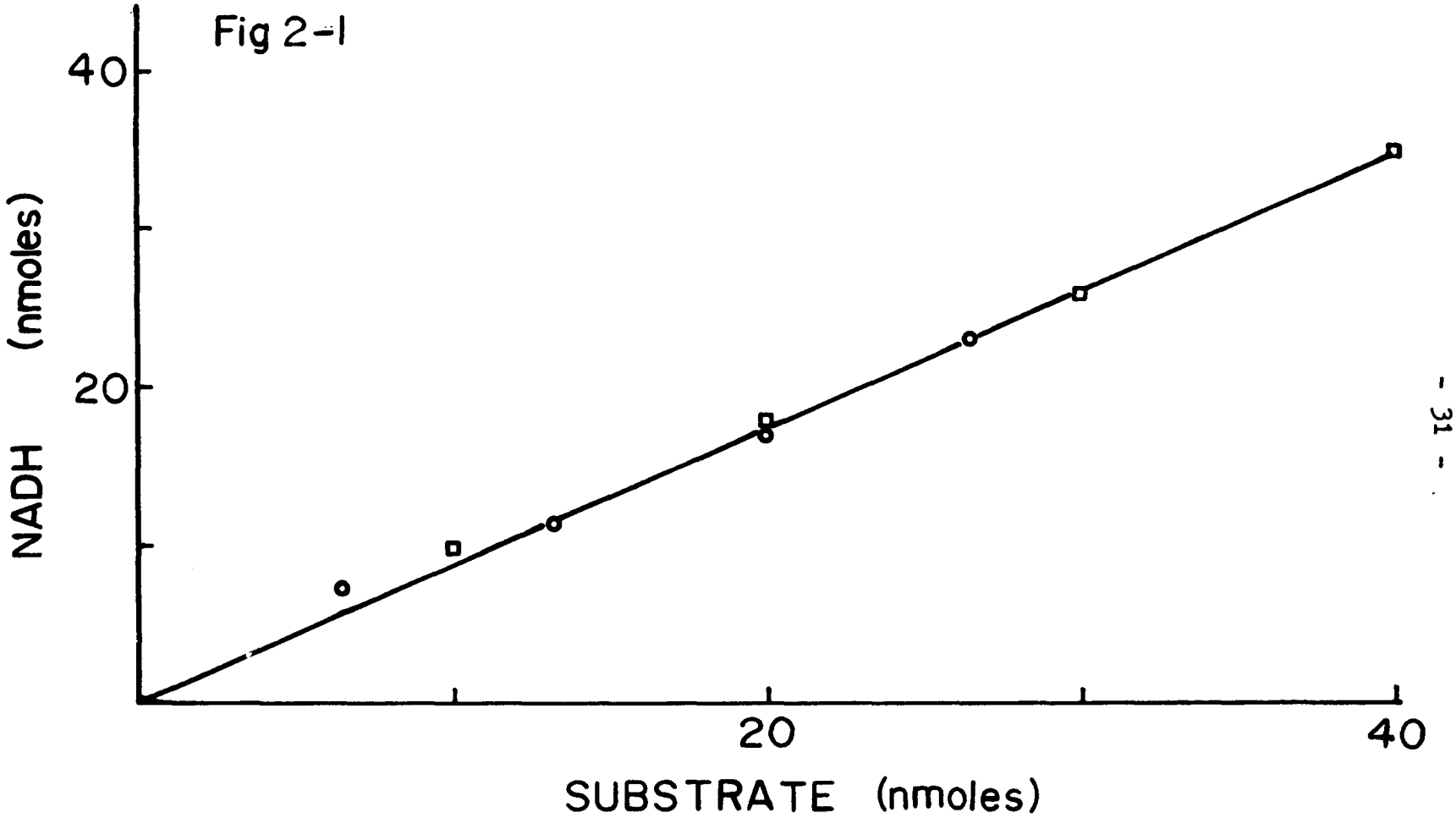
Fig 2-1
40
20
NADH (nmoles)
20
40
SUBSTRATE (nmoles)

Figure 2-2a : Plot of reciprocal of initial velocity (1/v) versus reciprocal of the millimolar concentreation of sn-glycerol-3-phosphate. The following concentrations of NAD^+ were used: ✖—✖—✖ 0.25 mM; □—□—□ , 0.40 mM; ⊙—⊙—⊙ , 0.75 mM; and +—+—+ , 2.0 mM. The reaction mixture and conditions for incubation were as described in the Materials and Methods section. Although the initial rates were actually measured during the first 30 seconds of reaction the rates are expressed as the change in μmoles per minute of NADH formed. The reaction was initiated by the addition of 250 ng of sn-glycerol-3-phosphate dehydrogenase (95 units per mg of protein) per ml.

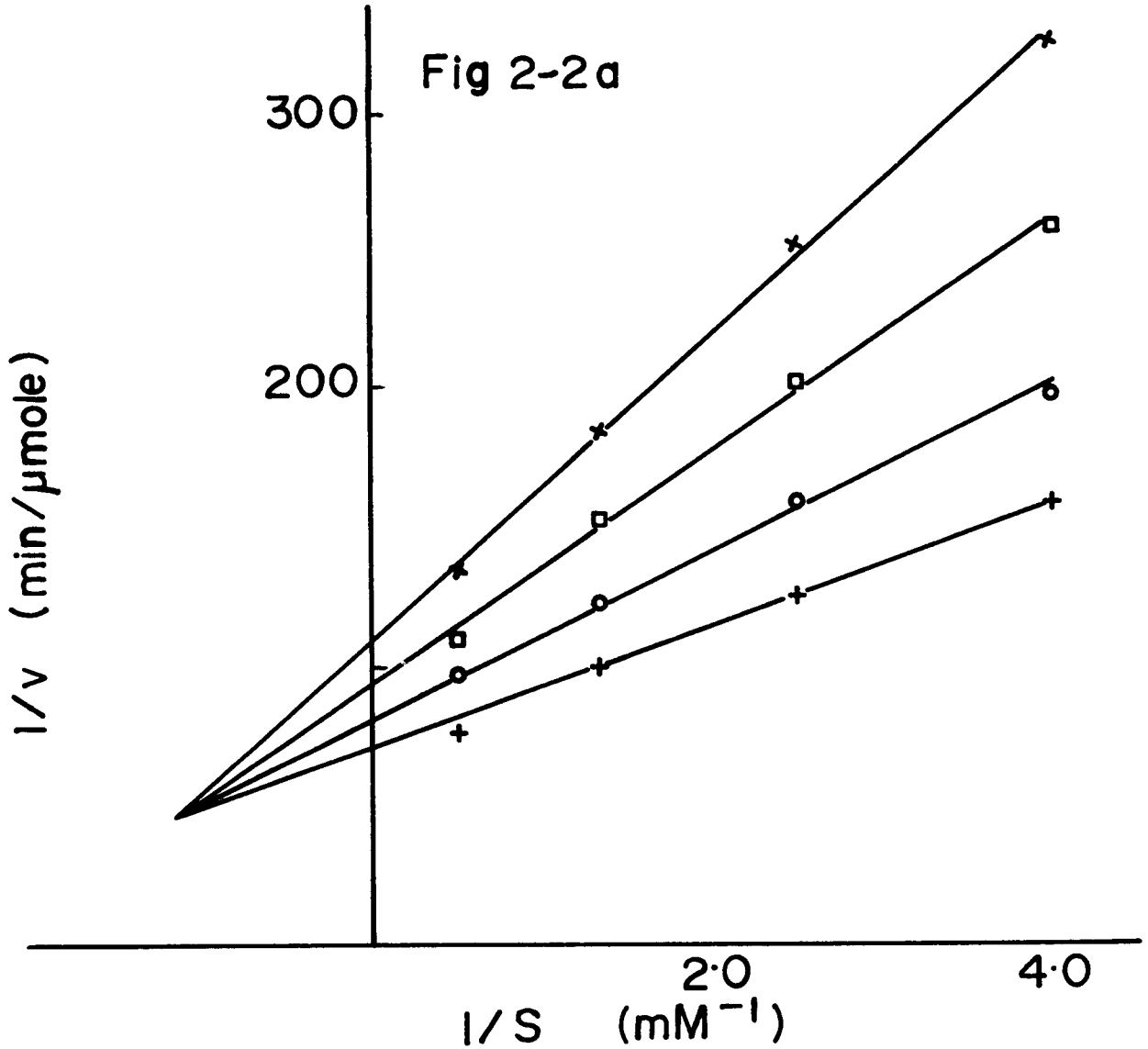
Fig 2-2a
300
200
1/v (min/μmole)
2·0
4·0
1/S (mM⁻¹)

Figure 2-2b : Plot of reciprocal of initial velocity (1/v) versus reciprocal of the millimolar concentration of NAD^+. The following concentrations of sn-glycerol-3-phosphate were used: ✖——✖——✖ , 0.25 mM; ◘——◘——◘ , 0.40 mM; ●——●——● , 0.75 mM; and ✚——✚——✚ 2.0 mM. The reaction mixture and conditions for incubation were as described in the Materials and Methods section. Although the initial rates were actually measured during the first 30 seconds of reaction the rates are expressed as the change in μmoles per minute of NADH formed. The reaction was initiated by the addition of 250 ng of sn-glycerol-3-phosphate dehydrogenase (95 units per mg of protein) per ml.

Fig. 2-2b

Gigure 2-3a : Plot of reciprocal of initial velocity (1/v) versus reciprocal of the molar concentrantion of D-3,4-dihydroxybutyl-1-phosphonate. The following concentrations of NAD^+ were used: ✖——✖——✖ 0.25 mM; ◘——◘——◘ , 0.40 mM; ●——●——● 0.75 mM; and ✚——✚——✚ 2.0 mM. All experimental concitions were the same as in Figure 2-2.

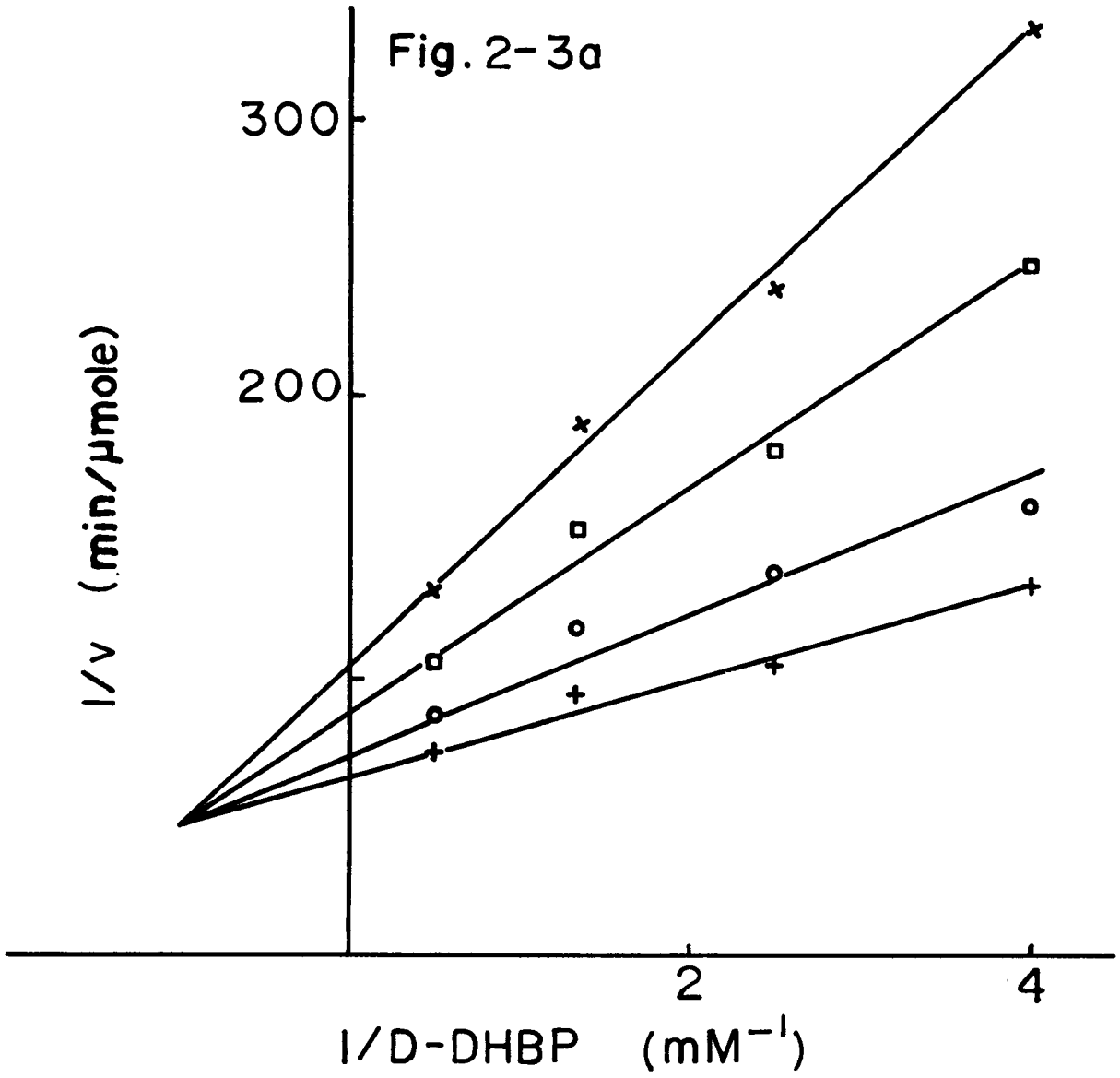
Fig. 2-3a
300
200
1/v (min/μmole)
2
4
1/D-DHBP (mM⁻¹)

Figure 2-3b : Plot of reciprocal of initial velocity (1/v) versus reciprocal of molar concentration of NAD^+. The following concentrations of D-3,4-dihydroxybutyl-1-phosphonate were used: ✖——✖——✖ , 0.25 mM; ◘——◘——◘ , 0.40 mM; ●——●——● , 0.75 mM; and +——+——+ , 2.0 mM. All experimental conditions were the same as in Figure 2-2.

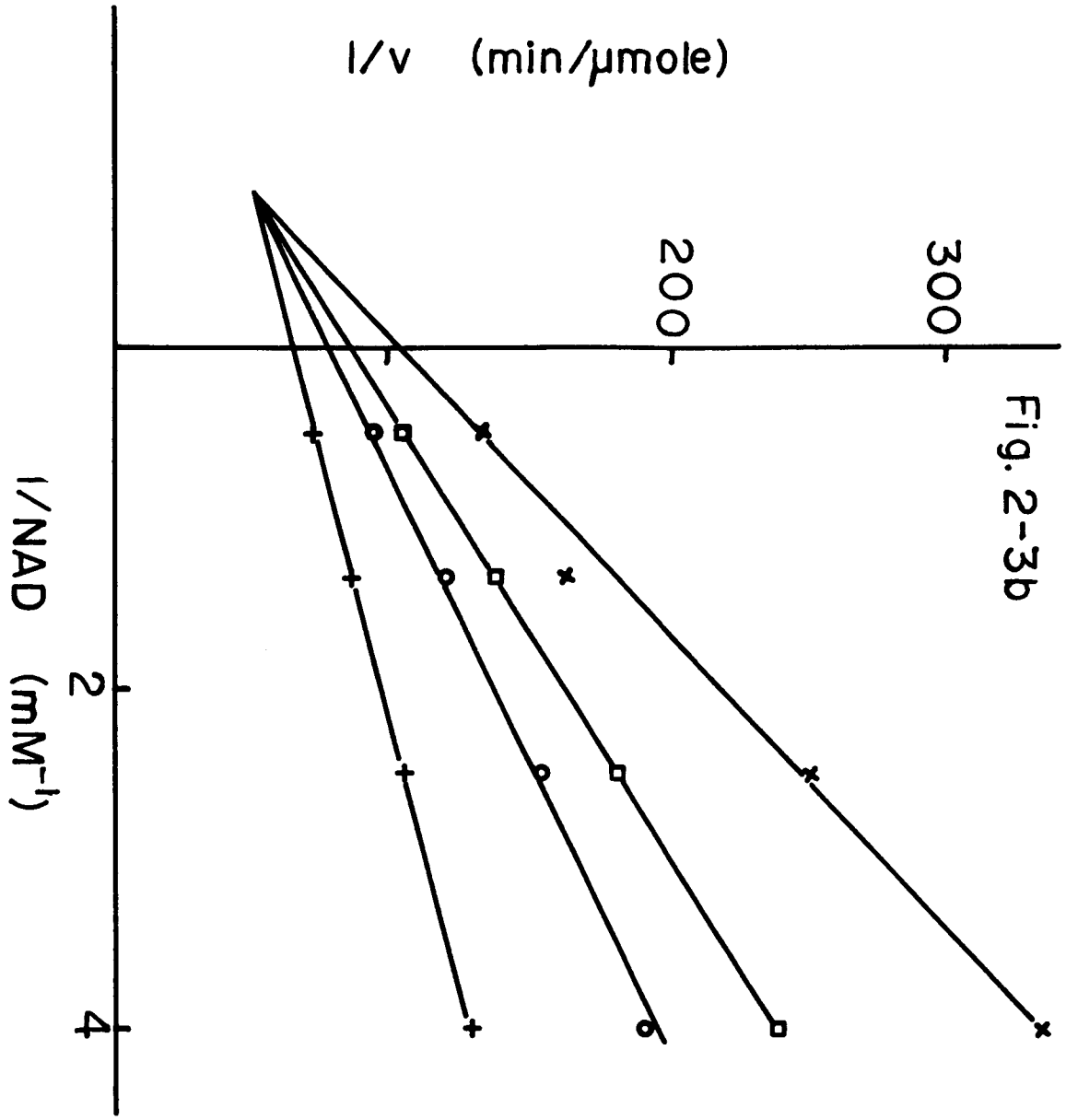

Fig. 2-3b

Figure 2-4a : Plot of the reciprocal of the maximal reaction velocity ($1/V_{max}$) versus reciprocal of substrate concentration (1/S) or ($1/NAD^+$) for the oxidation of sn-glycerol-3-phosphate and 3,4-dihydroxybutyl-1-phosphonate — secondary plot of the data obtained from Figure 2-3: ✚——✚——✚ NAD^+ and ◘——◘——◘ , D-3,4-dihydroxybutyl-1-phosphonate.

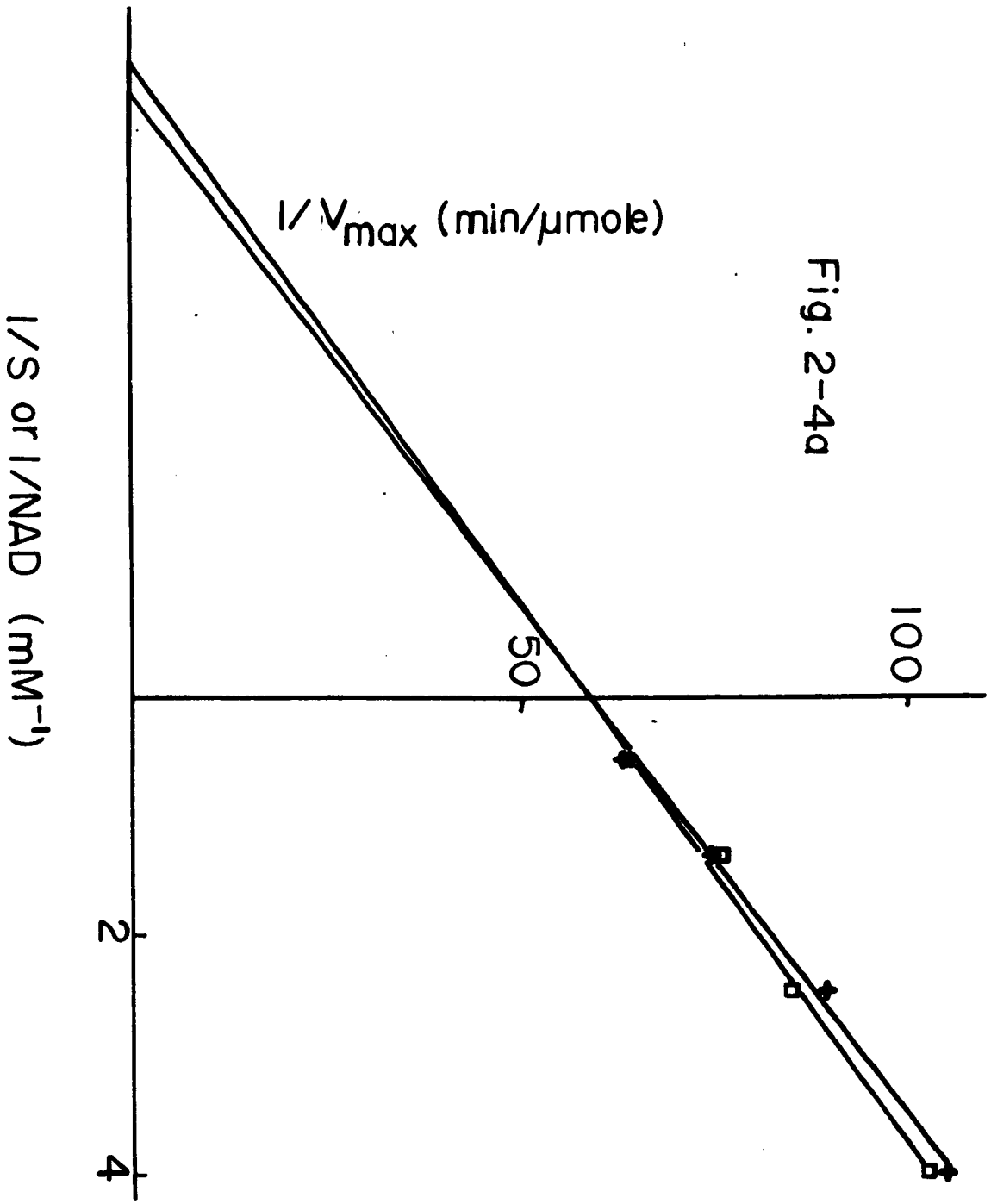

Fig. 2-4a

Figure 2-4b : Plot of the reciprocal of the maximal reaction velocity ($1/V_{max}$) versus reciprocal of substrate concentration (1/S) or ($1/NAD^+$) for the oxidation of sn-glycerol-3-phosphate and 3,4-dihydroxybutyl-1-phosphonate — secondary plot of the data obtained from Figure 2-2: ✚——✚——✚ NAD^+ and ⊙——⊙——⊙ , sn-glycerol-3-phosphate.

Fig. 2-4b

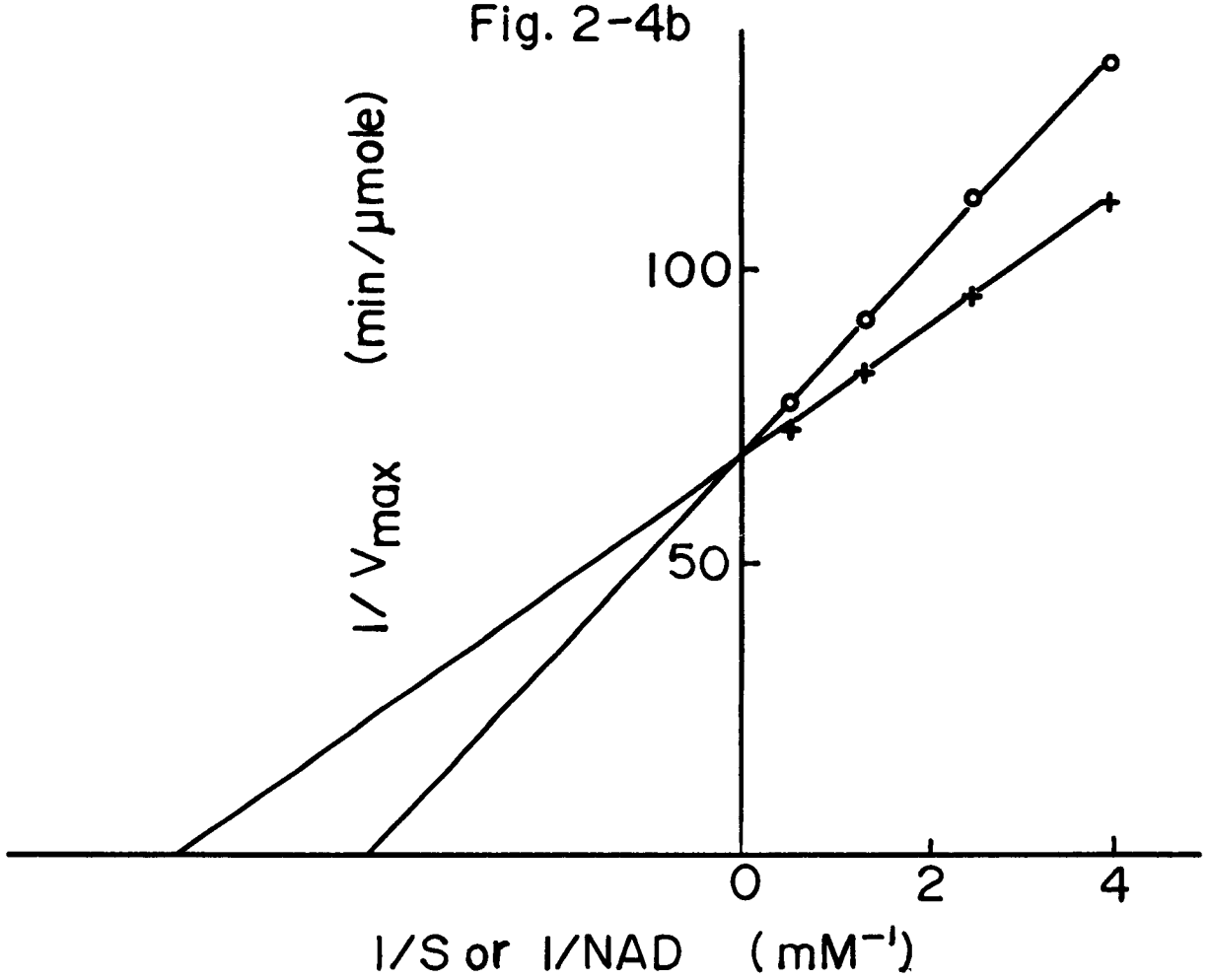

Figure 2-5a : Plot of reciprocal of initial velocity (1/v) versus reciprocal of the millimolar concentration of dihydroxyacetone phosphate. The following concentration of NADH were used: ✕——✕——✕ 2 μM; □——□——□, 3 μM; ●——●——●, 6 μM and +——+——+, 12.5 μM. The reaction mixture and conditions for incubation were as described in the Materials and Methods section. The initial rates are expressed as the change in μmoles per minute of NADH disappeared. The reaction was initiated by the addition of 9.4 ng of sn-glycerol-3-phosphate dehydrogenase (134 units per mg protein) per ml.

Fig. 2-5a

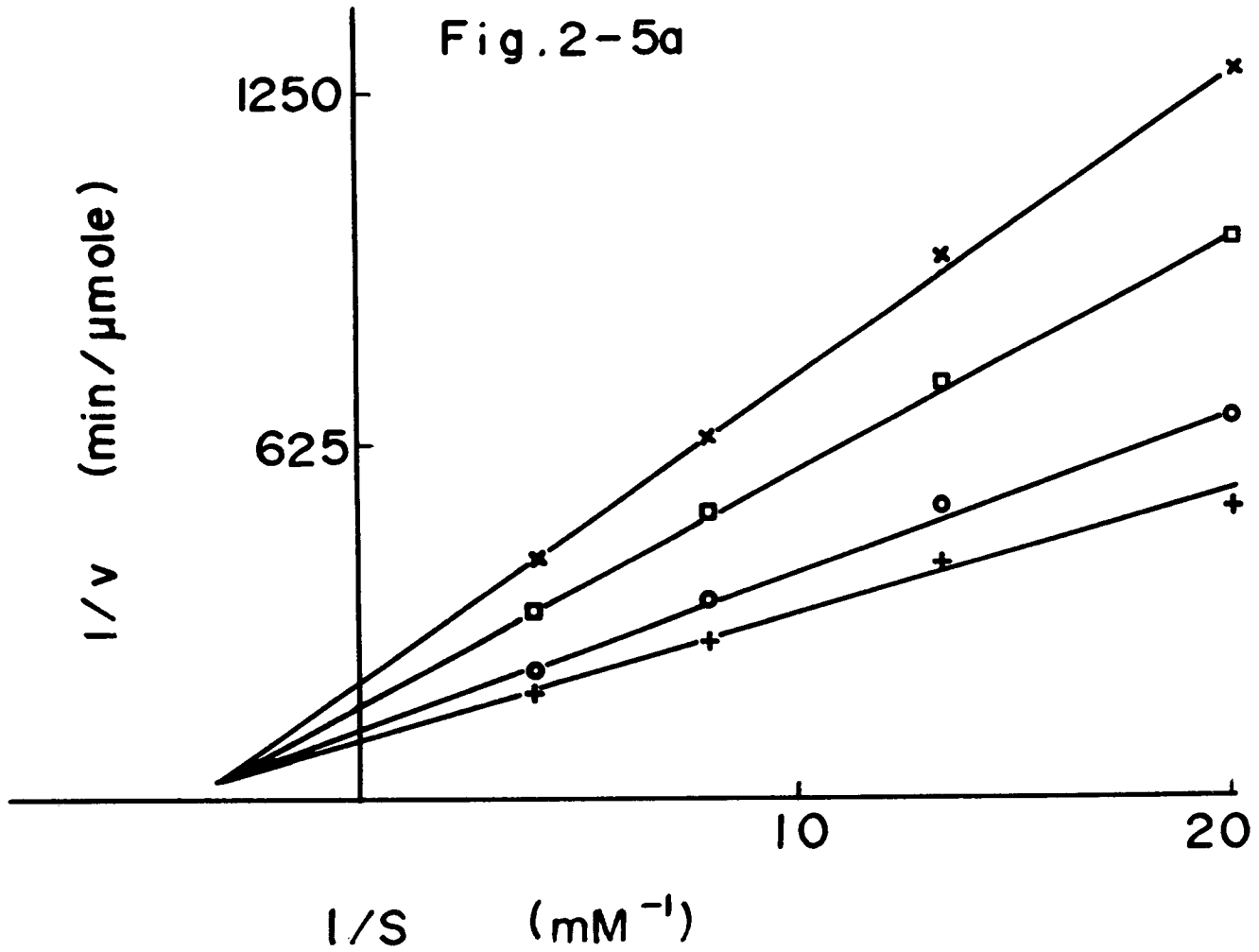

Figure 2-5b : Plot of reciprocal of initial velocity (1/v) versus reciprocal of millimolar concentration of NADH. The following concentrations of dihydroxyacetion phosphate were used; ✖——✖——✖ , 40 μM; ◘——◘——◘ , 60 μM; ⊙——⊙——⊙ , 100 μM; and ✚——✚——✚. 200 μM. The reaction mixture and conditions for incubation were as described in the Materials and Methods section. The initial rates are expressed as the change in μmoles per minute of NADH disappeared. The reaction was initiated by the addition of 9.4 ng of sn-glycerol-3-phosphate dehydrogenase (134 units per mg protein) per ml.

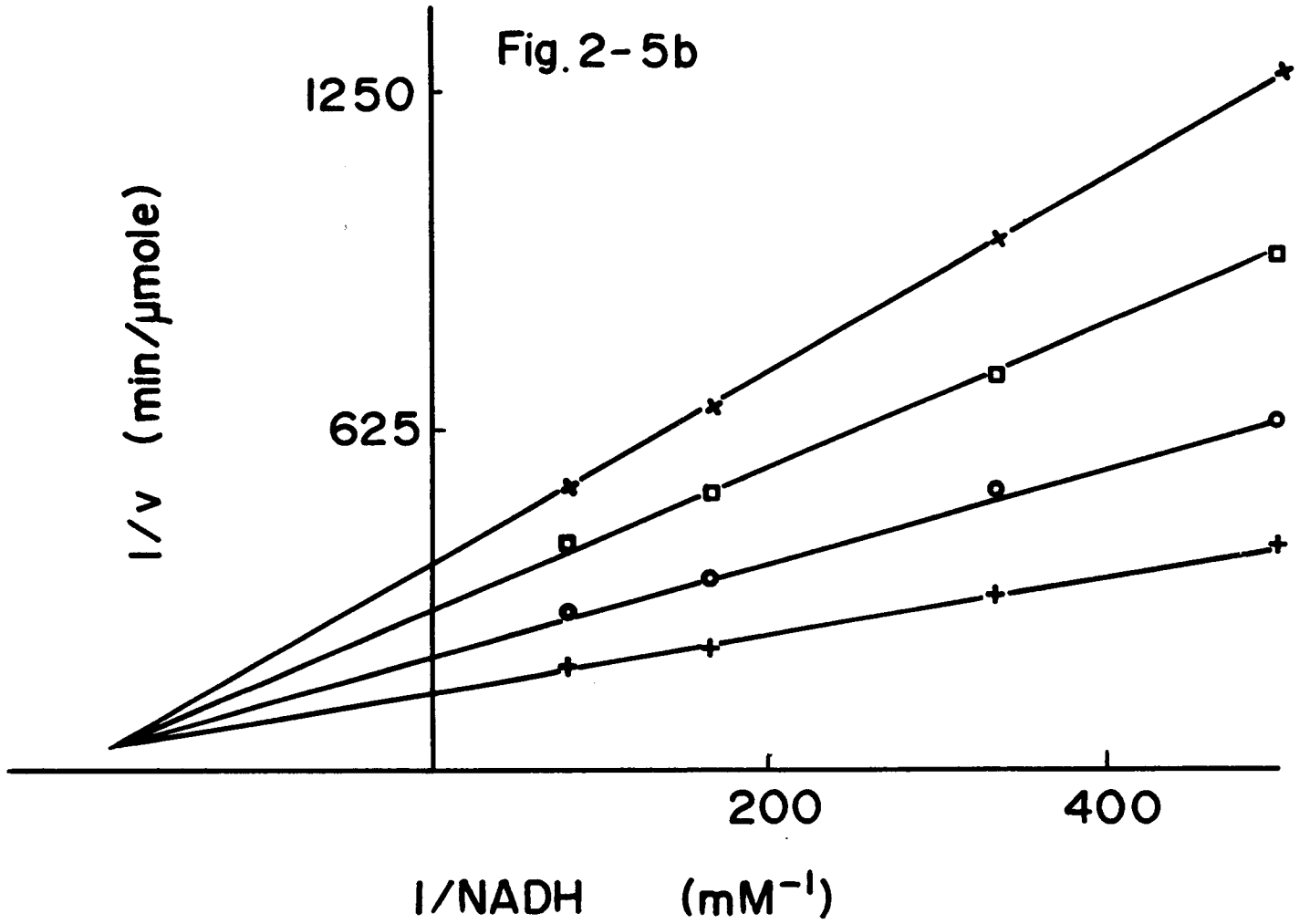
Fig. 2-5b
1250
625
1/v (min/μmole)
200
400
1/NADH (mM⁻¹)

Figure 2-6a : Plot of reciprocal of initial velocity (1/v) versus reciprocal of millimolar concentration of 4-hydroxy-3-oxobutyl-1-phosphonate. The following concentrations of NADH were used: ✖——✖——✖ 2 μM; ◘——◘——◘ 3 μM; ⊙——⊙——⊙ , 5 μM; and ✚——✚——✚ , 8 μM. All experimental conditions were the same as in Figure 2-5 except that 188 ng of enzyme per ml was used to initiate the reaction.

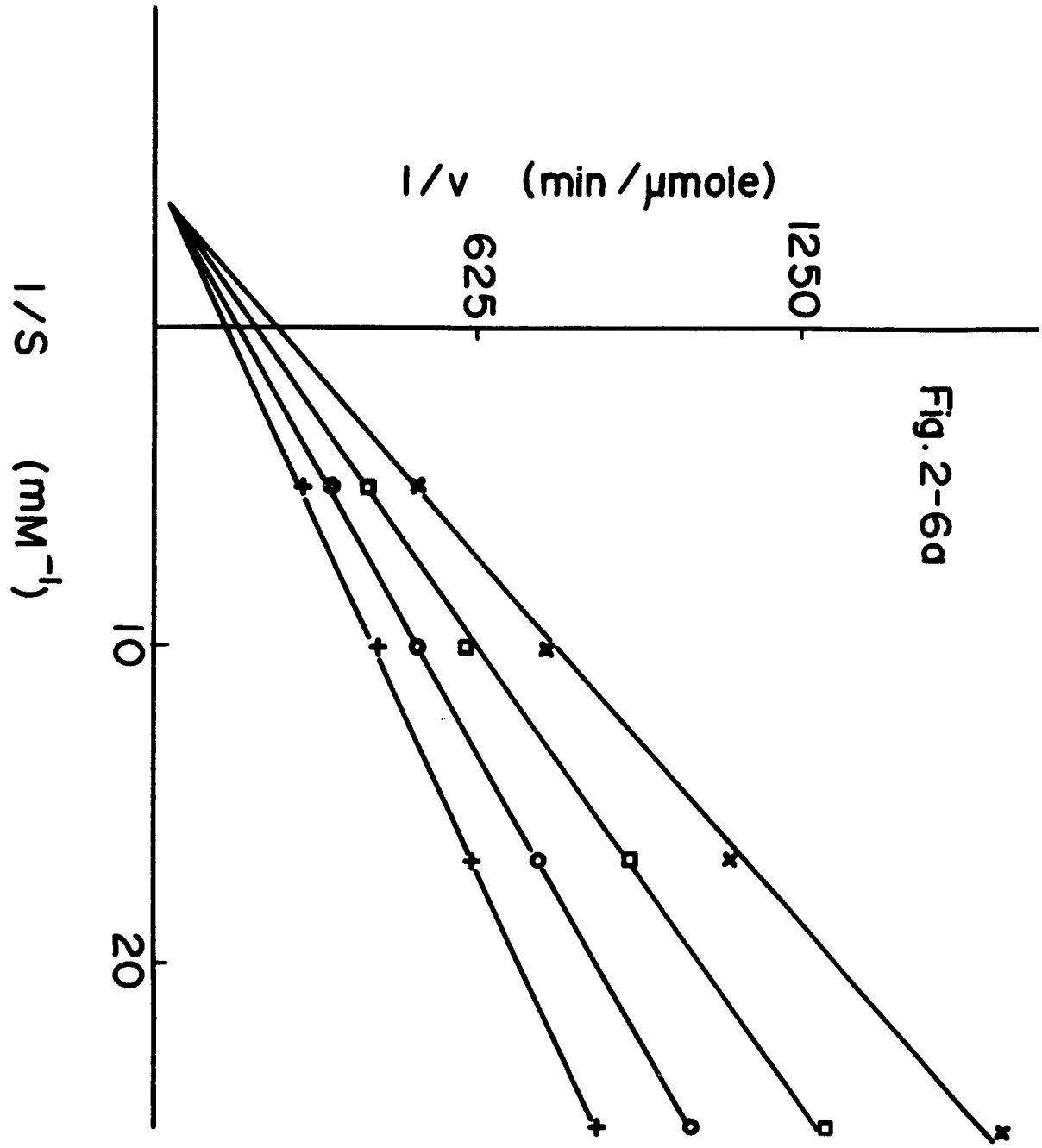

Fig. 2-6a

Figure 2-6b : Plot of reciprocal of initial velocity (1/v) versus reciprocal of the millimolar concentration of NADH. The following concentrations of 4-hydroxy-3-oxobutyl-1-phosphonate were used: ✖——✖——✖ , 40 μM; ◘——◘——◘ 60 μM; ⊙——⊙——⊙ 100 μM; and ✚——✚——✚ 200 μM. All experimental conditions were the same as in Figure 2-5 except that 188 ng of enzyme per ml was used to initiate the reaction.

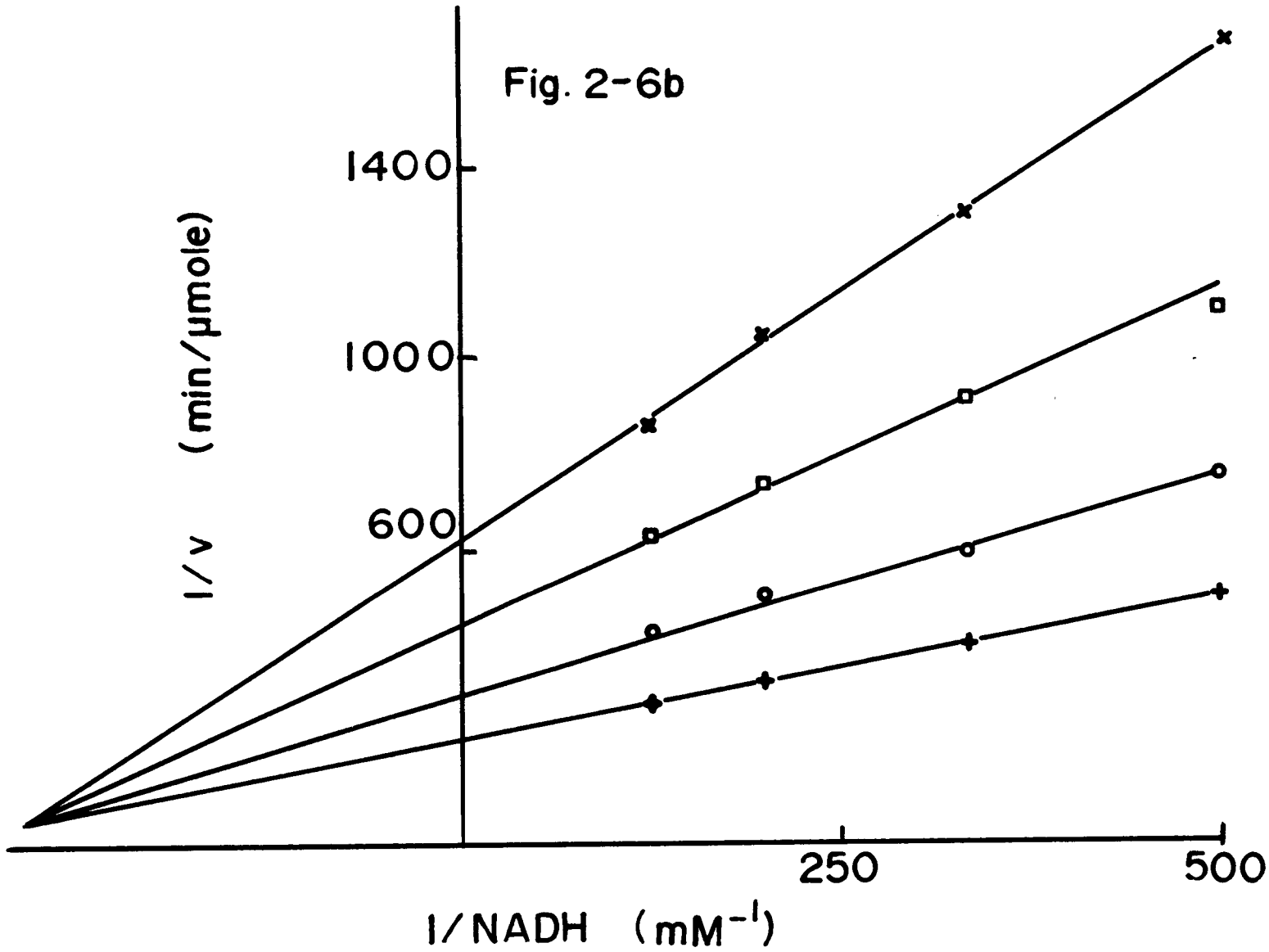
Fig. 2-6b
1400
1000
600
1/v (min/μmole)
250
500
1/NADH (mM⁻¹)

Figure 2-7a : Plot of the reciprocal of the maximal reaction velocity ($1/V_{max}$) versus reciprocal of substrate concentration (1/S) or (1/NADH) for the reduction of 4-hydroxy-3-oxobutyl-1-phosphonate and dihydroxyacetone phosphate--secondary plot of the data obtained from Figure 2-6: ▫——▫——▫ 4-hydroxy-3-oxobutyl-1-phosphonate and +——+——+ , NADH.

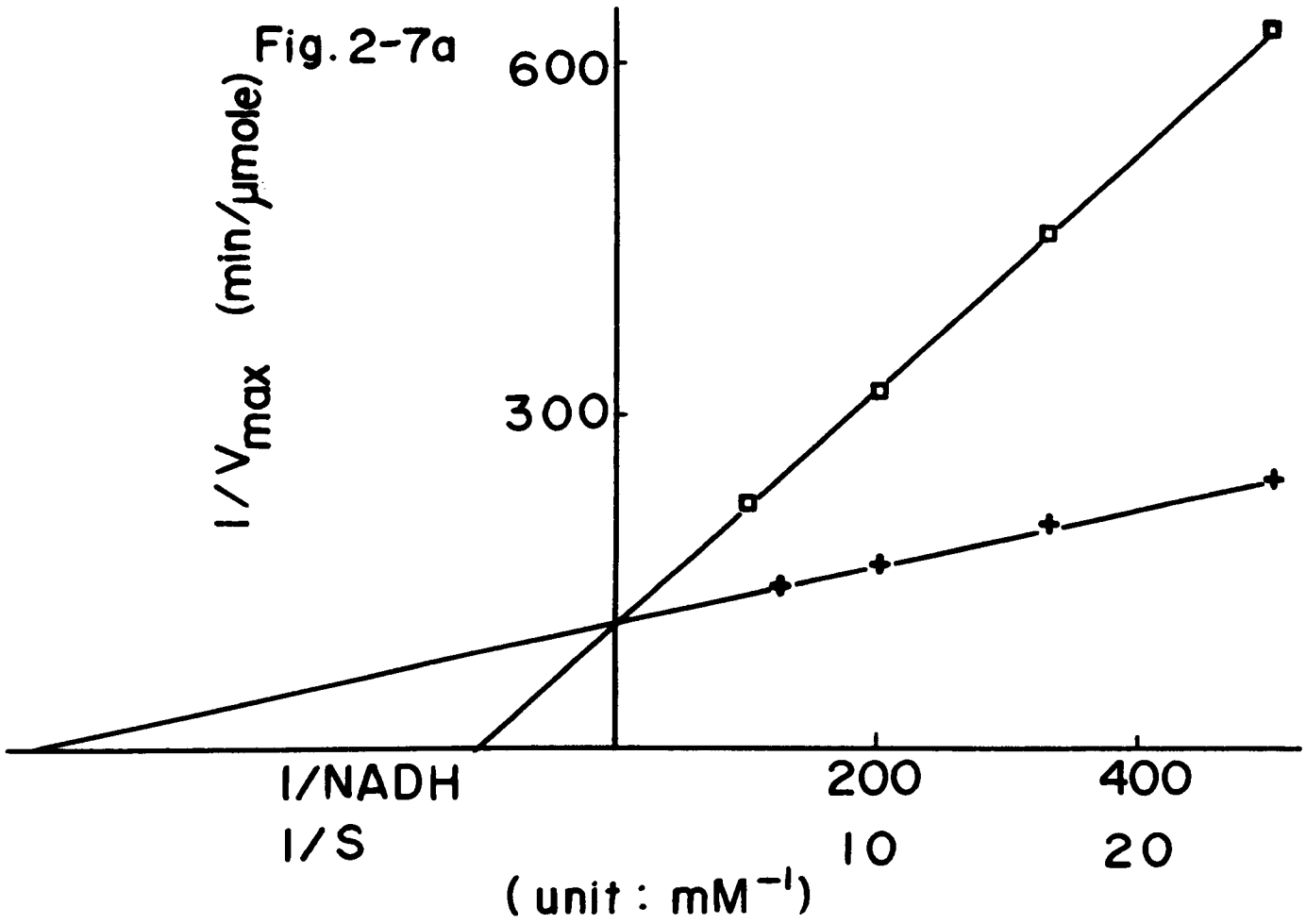
Fig. 2-7a
600
300
1/Vmax (min/μmole)
1/NADH
1/S
200
400
10
20
(unit : mM⁻¹)

Figure 2-7b : Plot of the reciprocal of the maximal reaction velocity ($1/V_{max}$) versus reciprocal of substrate concentration (1/S) or (1/NADH) for the reduction of 4-hydroxy-3-oxobutyl-1-phosphonate and dihydroxyacetone phosphate —secondary plot of the data obtained from Figure 2-5: o——o——o , dihydroxyacetone phosphate and +——+——+ , NADH.

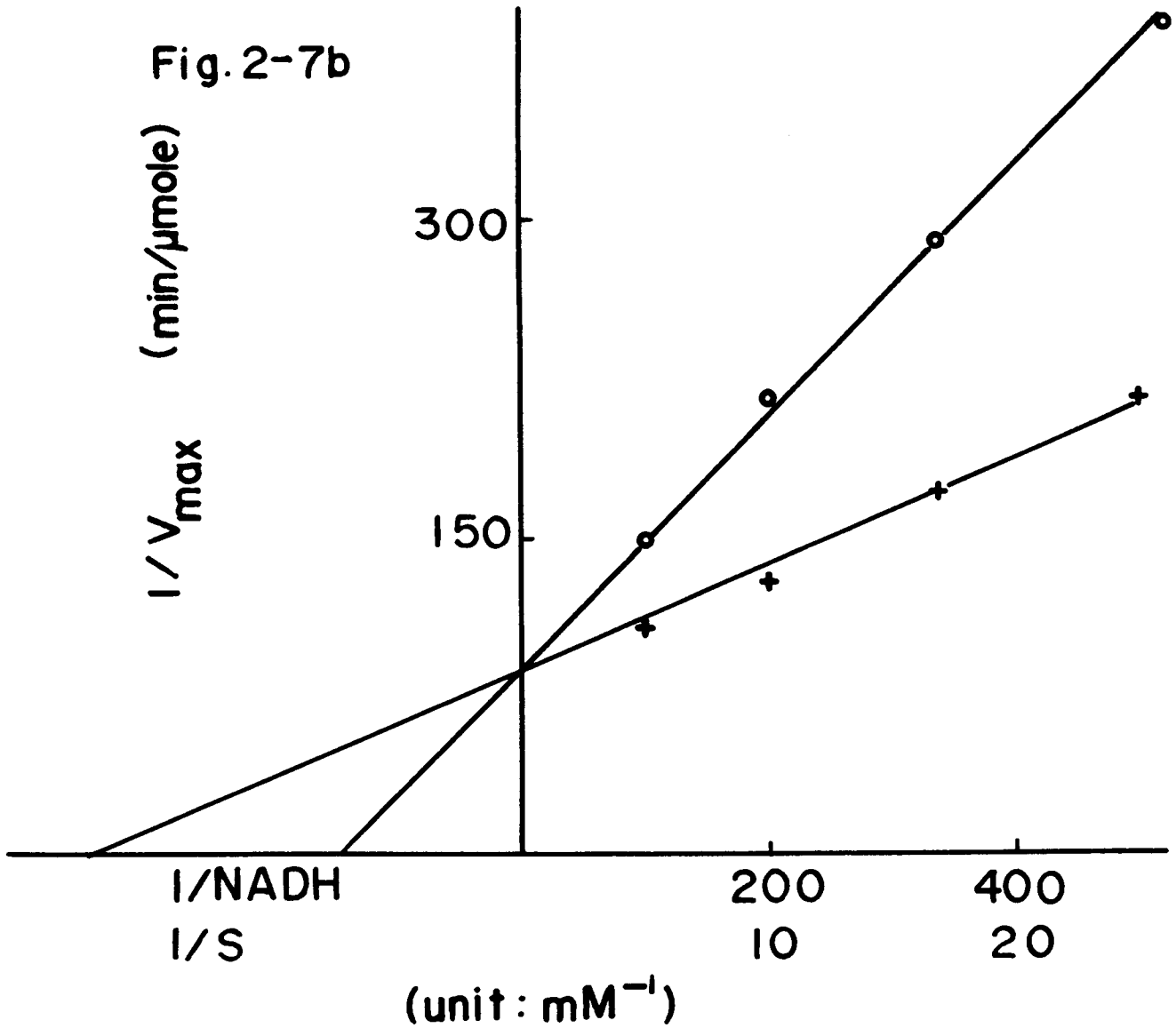
Fig. 2-7b
1/V_max (min/μmole)
300
150
1/NADH
1/S
200
10
400
20
(unit : mM⁻¹)

Figure 2-8a : Effect of pH on the initial rate of oxidation of sn-glycerol-3-phosphate ●——●——● and 3,4-dihydroxybutyl-1-phosphonate □——□——□ The reaction mixture consisted of NAD^+, 50 μM; sn-glycerol-3-phosphate or D-3,4-dihydroxybutyl-1-phosphonate, 5 mM; triethanolamine buffer, 50 mM; EDTA, 1mM; mercaptoethanol, 1 mM; and 1 mg of bovine serum albumin per ml. The reactions were initiated by the addition of 150 ng of glycerol-3-phosphate dehydrogenase (134 units per mg) per ml. The initial rated are expressed as the change in nmoles per minute of NADH formed or disappeared. The incubation conditions were as described in the Materials and Methods section.

Fig. 2-8a

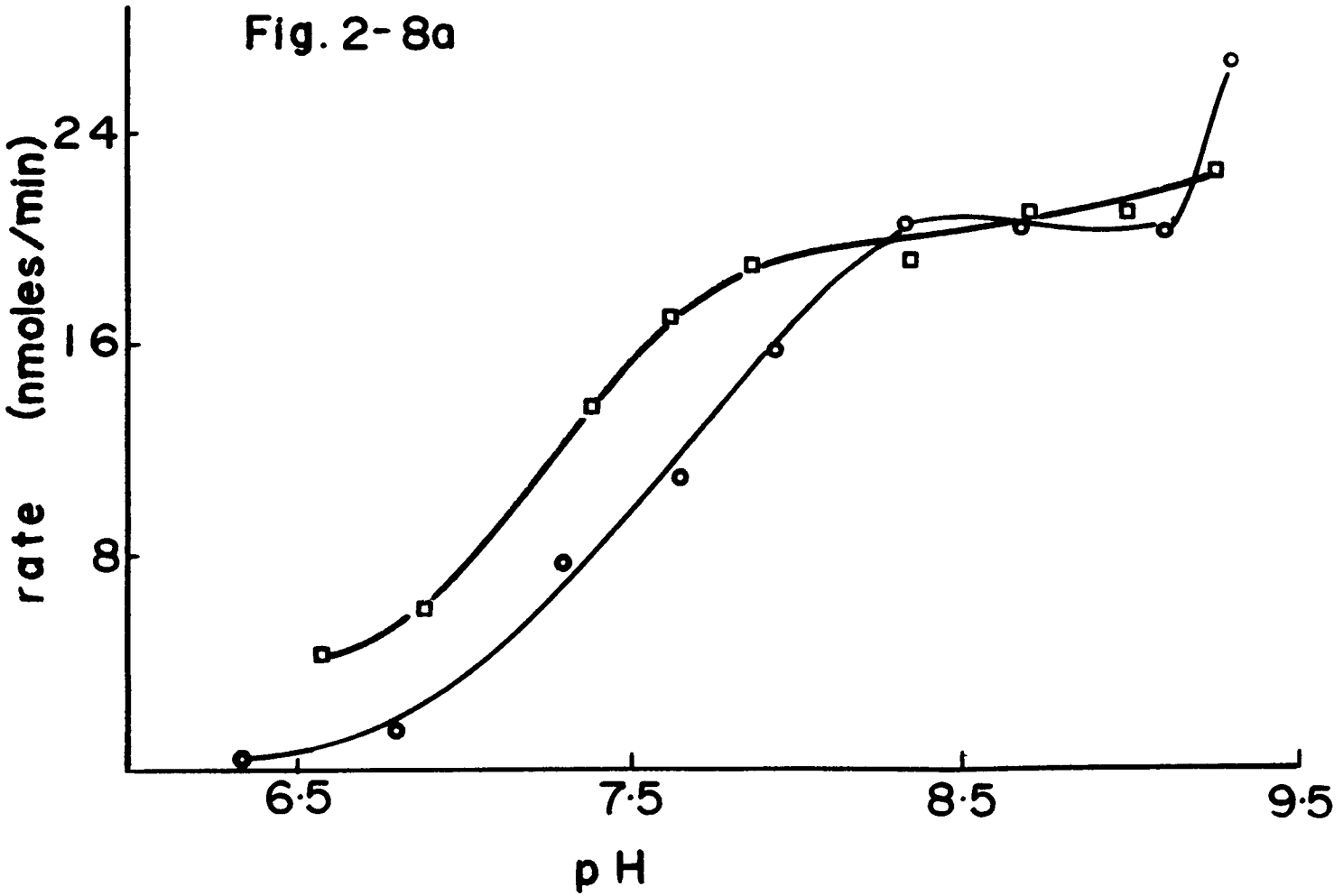

Figure 2-8b : Effect of pH on the initial rate of reduction of dihydroxyacetone phosphate  and 4-hydroxy-3-oxobutyl-1-phosphonate  The reaction mixture consisted of NADH. 60 μM; dihydroxyacetone phosphate or 4-hydroxy-3-oxobutyl-1-phosphonate, 2 mM; triethanolamine buffer, 50 mM; EDTA, 1.0 mM; mercaptoethanol, 1.0 mM, and 1 mg bovine serum albumin per ml. The reduction of dihydroxyacetone phosphate was initiated by the addition of 30 ng of sn-glycerol-3-phosphate dehydrogenase (134 units per ng) per ml and the reduction of 4-hydroxy-3-oxobutyl-1-phosphonate by addition of 150 ng of enzyme.

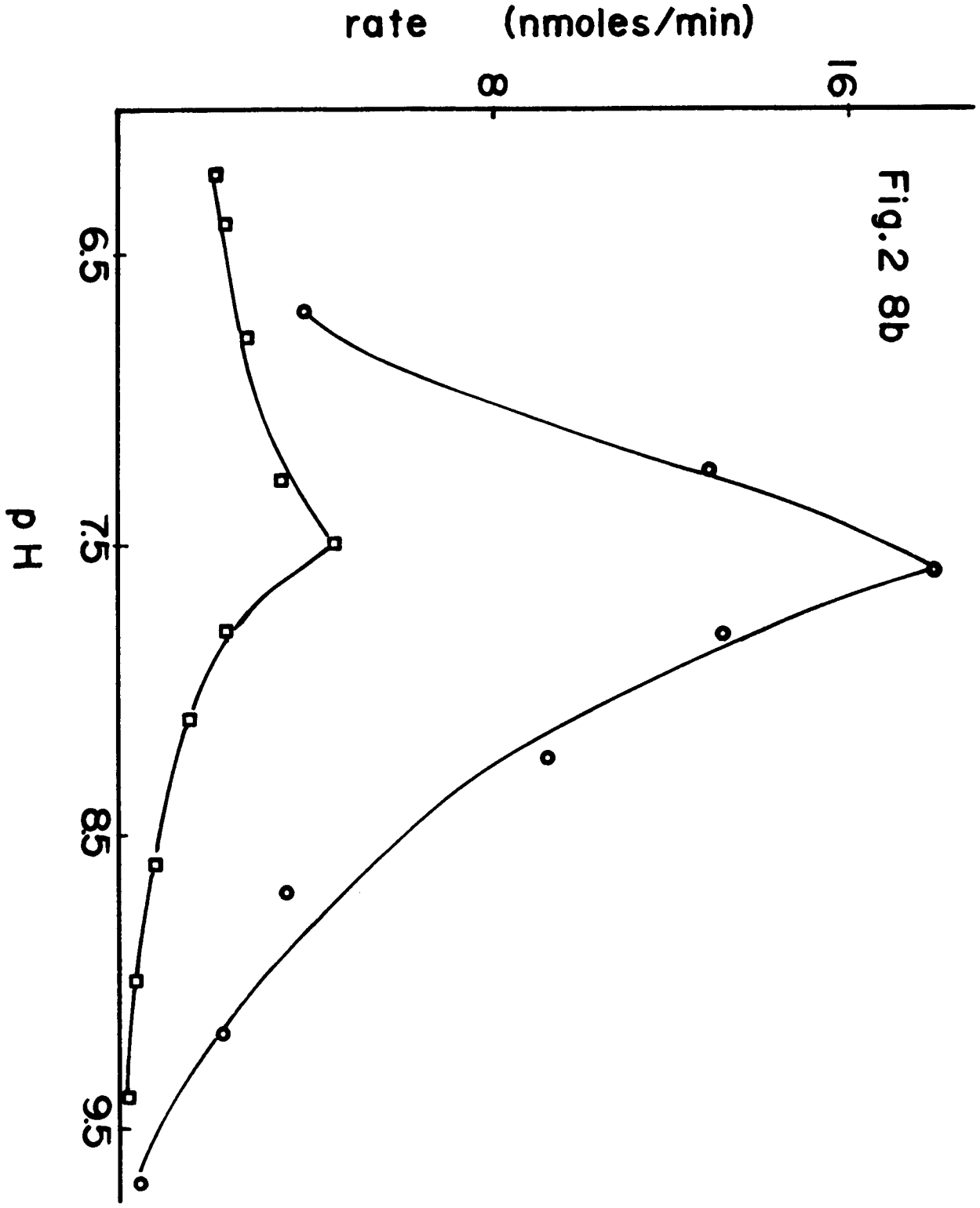

Fig.2 8b

Table 2-1

Kinetic constant of natural substrates and their analogues

for rabbit muscle sn-glycerol-3-phosphate dehydrogenase

	True Km Values observed	True Km Values Reported by Black (1966)
Glycerol-3-phosphate	240 µM	260 µM
NAD^+	160 µM	160 µM
3,4-Dihydroxybutyl-1-phosphonate	190 µM	------
NAD^+	200 µM	------
Dihydroxyacetone phosphate	130 µM	80 µM
NADH	2.9 µM	6.3 µM
4-Hydroxy-3-oxobutyl-1-phosphonate	182 µM	------
NADH	2.2 µM	-------

CHAPTER 3

IN VITRO STUDIES OF 3,4-DIHYDROXYBUTYL-1-PHOSPHONATE METABOLISM

A. Introduction

3,4-Dihydroxybutyl-1-phosphonate, an analogue of glycerol-3-phosphate was oxidized at approximately the same rate and had the same K_m as the natural substrate in a reaction catalyzed by rabbit muscle NAD-linked glycerol-3-phosphate dehydrogenase *in vitro* (see chapter 2). It has also been demonstrated to inhibit the growth of *Escherichia coli* (Shopsis *et al.*, 1972) and to have a profound effect upon phospholipid metabolism in this organism (Shopsis *et al.*, 1973). In particular, 30 μM 3,4-dihydroxybutyl-1-phosphonate, a concentration, which slows down the growth of *E. coli* strain 8 (Shopsis *et al.*, 1973), has a very marked inhibitory effect on phosphatidylglycerol synthesis of this strain (Shopsis *et al.*, 1974). In addition, when 3,4-dihydroxy[3-^{3}H]butyl-1-phosphonate was added to cultures of *E. coli* strain 8, it was incorporated into a very polar chloroform soluble material.

The *in vivo* studies raised many new questions. Perhaps the most important of these questions is the one concerning the primary site of action of the phosphonic acid analogue. It seems likely that 3,4-dihydroxybutyl-1-phosphonate acts

by interfering with the metabolism of glycerol-3-phosphate. For this reason some insights into the mode of action of the analogue might be revealed by *in vitro* experiments designed to evaluate the ability of enzymes involved in glycerol-3-phosphate metabolism to recognize 3,4-dihydroxybutyl-1-phosphonate. This chapter describes such experiments involving the enzymes of *E. coli*. In particular the following enzyme activities were investigated: (i) the catabolic membrane-bound glycerol-3-phosphate dehydrogenase, (ii) the anabolic glycerol-3-phosphate:NAD(P) oxidoreductase, (iii) the acyl-CoA : glycerol-3-phosphate acyltransferase.

B. Materials and Methods

Chemicals: 3,4-Dihydroxybutyl-1-phosphonate, 3,4-dihydroxy-[3-^{3}H]-butyl-1-phosphonate, 4-hydroxy-3-oxobutyl-1-phosphonate, and 2,3-dihydroxypropyl-1-phosphonate were kindly provided by Professor Engel and Dr. Goldstein, (see previous chapter).

NADPH, Type II; 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT); phenazine methosulfate (PMS); bovine serum albumin (BSA); DL-glycerol-3-phosphate, disodium salt, grade X; coenzyme A, grade I; DEAE-Sephadex A-50; Sephadex G-25; streptomycin sulfate; tris-(hydroxymethyl)-aminomethane (Tris); 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB); and the nonionic detergent, Triton X-100 (octyl-phenoxypolyethoxyethanol) were purchased from the Sigma Chemical Company, St. Louis, Mo.. Palmityl-CoA was synthesized by the method of Seubert (1960).

The di-monocyclohexylamine salt of dihydroxyacetone phosphate dimethylketal, prepared by the method of Ballou and MacDonald (1963), was a generous gift of L. Gelbaum. Dihydroxyacetone phosphate was generated from this compound by treating it with Dowex 50 (hydrogen ion form) as described by Ballou and MacDonald (1963). Triethanolamine was a product of the Aldrich Chemical Co., Milwauke, Wisconsin. sn-[^{14}C]-Glycerol-3-phosphate was purchased from the New England Nuclear Corp., Boston, Mass.. All other chemicals used were of reagent grade.

Bacterial Strains: E. coli strain 7 and its derivative strain 8 were originally isolated and described by Hayashi, Koch, and Lin (1964). Strain 7 was kindly provided by E. C. C. Lin and strain 8 by J. Cronan Jr. The genotype of strain 7 as expressed by the genetic symbols described by Taylor and Trotter (1972) is as follows: Hfr C glp R^c2, phoA8, ton A22, $T2^R$, rel-1 (λ). Strain 8 has an additional lesion, glp D3. The strains were cultured on the synthetic medium described by Davis and Mingioli (1950). E. coli B harvested from enriched culture medium in the stationary phase of growth was purchased from the Grain Processing Corp., Muscatine, Iowa.

Assay of the Membrane-Bound and Pyridine Nucleotide-Independent L-Glycerol-3-phosphate Dehydrogenase: The catabolic glycerol-3-phosphate dehydrogenase was assayed with the purified enzyme which was very generously provided for these studies by Dr. L. Heppel. The purification of this enzyme was described by Weiner and Heppel (1972) (also see the following chapter). The assay was performed by the procedure of Kistler and Lin (1971). The reaction mixture contained, in a final volume of 1 ml: 100 µmoles potassium phosphate, pH 7.5; 33 µg MTT; 200 µg PMS; 6 µmoles KCN; 0.0032 units of enzyme as defined by Weiner and Heppel (1972); and the concentrations of sn-glycerol-3-phosphate and the phosphonic acid analogues were as indicated in

Table 3-1. The extinction coefficient of reduced MTT was taken to be 17 $mM^{-1}cm^{-1}$ at 570 nm (Kistler & Lin 1971). The spectrophotometric assays were performed at 25°C and initiated by the addition of enzyme. The change of absorbance in this as well as subsequent colorimetric assays was monitored using a Gilford model 240 spectrophotometer fitted with a constant temperature cuvette holder and a continuous recorder.

Assay of the sn-Glycerol-3-Phosphate:NAD(P) Oxidoreductase (EC 1.1.1.8): One hundred grams (wet weight) of E. coli B cells were suspended in 200 ml of 10 mM triethanolamine-HCl buffer, pH 7.5, containing 2 mM β-mercaptoethanol and the cells were disrupted by two passages through a French pressure cell at 15,000 psi. Cell debris was removed by centrifugation for 15 minutes at 8,000 x g in the Sorvall RC2B. The subsequent steps of purification followed were those of Kito and Pizer (1969). The enzyme was purified through the first DEAE-Sephadex column chromatography. High activity fractions were pooled and precipitated with 60% ammonium sulfate. The precipitate, collected by centrifugation, was dissolved in 0.05 M phosphate buffer, pH 7.5 which was then made 1 M in ammonium sulfate. The enzyme was stored frozen and just prior to use was desalted on a Sephadex G-25 column. The assays were performed at 30°C by the procedure of Kito and Pizer (1969). The reaction

mixture contained in a final volume of one ml: 50 μmoles triethanolamine-HCl buffer, pH 7.5; 10 μmoles dithiothreitol; and the indicated concentrations of NADPH and enzyme. The reactions were initiated by the addition of the specified concentration of dihydroxyacetone phosphate or 4-hydroxy-3-oxobutyl-1-phosphonate. When used, the inhibitor (DL-glycerol-3-phosphate or DL-3,4-dihydroxybutyl-1-phosphonate) was present at concentrations of 100 μM.

The Assay of Acyl-CoA:Glycerol-3-Phosphate Acyltransferase: E. coli strain 8 was cultured to the late logarithmic phase of growth, harvested, and washed once with 0.01 M phosphate buffer, pH 7.0 Approximately 2 g of cells (wet weight) were suspended in 20 ml of 0.01 M phosphate buffer, pH 7.5 and then ruptured by sonic oscillation with a Bronson model W140D sonifier at a setting of 60 watt. The cellular debris was removed by centrifugation at 5,000 x g for ten minutes. The supernatant was centrifuged at 30,000 x g for 30 minutes in the Sorvall RC2B. The pellet was washed once with 0.01 M phosphate buffer, pH 7.0 and resuspended in 5 ml of the same buffer. The suspension was stored frozen until required. The assay conditions were similar to those of van den Bosch and Vagelos (1970). The reaction mixture contained in a final volume of 0.35 ml: 30 μmoles tris-HCl, pH 8.5; 2.45 μmoles Mg^{++}; 33 nmoles palmityl-CoA; 54 nmoles DL-glycerol-3-phosphate; 0.1 μCi sn-[^{14}C]-glycerol-3-phosphate (80 mCi

per mmole); 150 μg BSA; 120 μg of the enzyme preparation; and the indicated concentration of 3,4-dihydroxybutyl-1-phosphonate or 2,3-dihydroxypropyl-1-phosphonate. In those assays where 3,4-dihydroxybutyl-1-phosphonate was tested as a substrate 840 nmoles of DL-3,4-dihydroxy-[3-^{3}H]-butyl-1-phsophonate (31 mCi per mmole) was added to the assay mixture in place of glycerol-3-phosphate. The assays were performed at 30°C and initiated by the addition of the palmityl-CoA. The reaction was monitored by placing 50 μl of the assay mixture on a Whatman no. 3MM filter paper disk. The disks were treated by the procedure of Goldfine (1969) as modified by van den Bosch and Vagelos (1970). In some cases the assays were performed by monitoring the release of coenzyme A by measuring its chemical interaction with DTNB according to the colorimetric procedure of Cronan, Ray, and Vagelos (1970). These assays provided the same type of results as those based upon the incorporation of radioactive label.

C. Results

The present studies are directed toward obtaining insights into the mode of action of 3,4-dihydroxybutyl-1-phosphonate on E. coli. For this reason only two of the three glycerol-3-phosphate dehydrogenase activities reported to be present in E. coli were studied. Since the anaerobic dehydrogenase does not appear to function in vivo in aerobically cultured cells (Kistler & Lin, 1971), it was not studied. 3,4-Dihydroxybutyl-1-phosphonate inhibits the growth of E. coli strains 7 and 8 (Shopsis et al., 1972). These two strains differ in one major respect. Strain 8 lacks an active catabolic membrane-bound glycerol-3-phosphate dehydrogenase whereas strain 7 synthesizes active enzyme constitutively (Hayashi et al., 1964). If strain 7 is able to catalyze the oxidation of the four-carbon phosphonic acid analogue the mode of action in strain 7 might differ from that in strain 8. Experiments with crude extracts of strain 7 indicated that neither 3,4-dihydroxybutyl-1-phosphonate nor 2,3-dihydroxypropyl-1-phosphonate could serve as a substrate for the catabolic dehydrogenase. To be certain that the phosphonates could not serve as a substrate the author was fortunate in obtaining through the generosity of Dr. L. Heppel, a sample of purified catabolic membrane-bound dehydrogenase. The results of experiments using this purified enzyme are reported in

Table 3-1. Since the experiments were performed with the dilithium salts of the analogues. it is important to demonstrate that the lithium ion concentrations used do not affect the enzymatic activity. Table 3-1 reveals this to be the case and also shows that neither 3,4-dihydroxybutyl-1-phosphonate nor 2,3-dihydroxypropyl-1-phosphonate is a substrate or inhibitor of the catabolic dehydrogenase.

The primary role of the anabolic glycerol-3-phosphate: NAD(P) oxidoreductase is to convert dihydroxyacetone phosphate to glycerol-3-phosphate which is in turn required for phosphoglyceride synthesis. For this reason we studied the enzymatic conversion of dihydroxyacetone phosphate and its phosphonic acid analogue, 4-hydroxy-3-oxobutyl-1-phosphonate, to their reduced forms. Fig. 3-1a is a Lineweaver-Burk plot (1934) of the reciprocal of the initial velocity (v) versus the reciprocal of the molar concentration of either dihydroxyacetone phosphate or 4-hydroxy-3-oxobutyl-1-phosphonate. The apparent Michaelis constants calculated from this plot are 278 μM for the natural substrate and 182 μM for the analogue. This compares to an apparent K_m of 170 μM reported by Kito and Pizer (1969) for the natural substrate. Dihydroxyacetone phosphate has an apparent V_{max} at least three times greater than that for 4-hydroxy-3-oxobutyl-1-phosphonate. Figure 3-1b is a Lineweaver-Burk plot (1934) of the reciprocal of the initial velocity (v)

versus the reciprocal of the molar concentration of NADPH. The apparent K_m for NADPH when dihydroxyacetone phosphate was reduced is 11 μM and when the analogue was reduced it is 43 μM. Kito and Pizer (1969) have reported that glycerol-3-phosphate is an inhibitor of the enzymatic reduction of dihydroxyacetone phosphate. Figure 3-2 presents confirmation of their findings and indicates that 3,4-dihydroxybutyl-1-phosphonate is also a competitive inhibitor of this reaction. Neither lithium ions nor 2,3-dihydroxypropyl-1-phosphonate has any effect upon this reaction. The apparent K_i for glycerol-3-phosphate is 19 μM and that for the four-carbon phosphonate is 42 μM indicating that the natural metabolite is a somewhat better inhibitor.

In vivo studies revealed that 3,4-dihydroxybutyl-1-phosphonate not only inhibited phospholipid synthesis but is also incorporated into a chloroform soluble fraction of E. coli strain 8 (Shopsis et al., 1974). The two most likely sites of action are the acylation of glycerol-3-phosphate and the formation of phosphatidylglycerol phosphate. Figure 3-3 reveals that 3,4-dihydroxybutyl-1-phosphonate does not inhibit the acylation of glycerol-3-phosphate while 2,3-dihydroxypropyl-1-phosphonate has only a very slight inhibitory effect. Colorimetric assays involving DTNB demonstrated that neither analogue was a substrate (date not shown). These results were confirmed in the ease of the four-carbon

analogue by using 3,4-dihydroxy{3-^{3}H}-butyl-1-phosphonate as the substrate.

Nunn (1972) has demonstrated that 3,4-dihydroxybutyl-1-phosphonate inhibited the formation of phosphatidylglycerol phosphate by the CDP-diglyceride dependent assay system described by Chang & Kennedy (1967). He found that D-3,4-dihydroxybutyl-1-phosphonate is both a competitive inhibitor (apparent K_i of 740 μM) and a substrate (apparent K_m of 450 μM) for the CDP-diglyceride : glycerol-3-phosphate phosphatidyl transferase[1]. The apparent K_i for D-2,3-dihydroxypropyl-1-phosphonate is at least five times higher than the K_i for the four-carbon analogue. Neither of the phosphonic acid analogues has an inhibitory effect upon the conversion of serine to lipid extractable material in the CDP-diglyceride:serine phosphatidyl transferase assay.

As described in the beginning of this section both the purified aerobic, membrane-bound catabolic glycerol-3-phosphate dehydrogenase and crude enzyme preparations containing this activity prepared from the membrane fraction of E. coli strain 7 have been studied to evaluate the

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1. The author wishes to thank Dr. W. Nunn for showing the unpublished results of experiments performed at the Department of Molecular Biophysics and Biochemistry, Yale University.

ability of the enzyme to recognize the phosphonic acid analogues. It is clear from talbe 3-1 that the phosphonate analogues were neither substrates nor inhibitors for the catabolic enzyme. However, analysis of the crude extracts of E. coli strain B revealed that although there was a considerably lower glycerol-3-phosphate dehydrogenase activity in these extracts than in those of strain 7, there was some enzymatic capacity to oxidize both 3,4-dihydroxybutyl-1-phosphonate and 2,3-dihydroxypropyl-1-phosphonate. It was subsequently found that the crude extracts from E. coli K-12 (a parent strain of strain 7) gave similar results to that for strain B. For this reason, the enzymes from E. coli strain 7, B, and K-12 were partially purified through the deoxycholate extraction step according to the procedure of Weiner and Heppel (1972) (see Materials and Methods in Chapter 4) and then compared. It was found that differential precipitation of protein by added ammonium sulfate caused a partial resolution of the activities for the phosphonic acid analogues and glycerol-3-phosphate. The results are similar in the preparations from both strain B and K-12. This indicates that there might be either two different enzymes or two isozymes present in these two strains. This assumption is partially supported by the finding that the activity towards the phosphonate analogues is inhibited by the presence of KCN, Triton

X-100, or both in the reaction mixtures. The activity towards glycerol-3-phosphate is not inhibited.

In the spectrophotometric assay procedure of Lin et al., (1962) the enzyme's activity is measured by PMS coupled reduction of MTT. KCN is added to block the passage of electrons through the cytochrome system and divert it into PMS mediated reaction. Triton X-100 releases the membrane-bound enzyme and prevents the precipitation of the reduced MTT during the course of enzymatic reaction. Table 3-2 compares enzyme activities from E. coli strain 7 and K-12 in the presence and absence of KCN. With Triton X-100 the results are the same as those for KCN (data not shown). Phosphonate analogues were not recognized by the catabolic glycerol-3-phosphate dehydrogenase from E. coli strain 7 but they were substrate for enzymes from E. coli K-12 and strain B (data for strain B is not given). When glycerol-3-phosphate is the sole substrate the enzyme activities in different fractions of strain 7 and K-12 are stimulated by KCN and Triton X-100. This is in agreement with the original observation by Lin et al., (1962). In contrast, when phosphonate analogues serve as substrate, the enzyme activity of E. coli K-12 disappears.

D. Discussion

The *in vitro* studies reported here help to clarify some of the questions raised by the *in vivo* investigations that were previously reported (Shopsis *et al.*, 1973; and 1974) and raise still other questions. The inability of the catabolic membrane-bound glycerol-3-phosphate dehydrogenase to catalyze the oxidation of 3,4-dihydroxybutyl-1-phosphonate (Table 3-1) explains why the four-carbon analogue is effective against *E. coli* strain 7 as well as strain 8 (Shopsis *et al.*, 1972). The initial reason for selecting strain 8 for study was to avoid complications arising from the oxidation of 3,4-dihydroxybutyl-1-phosphonate. This precaution appears to have been unnecessary. However, the observation that crude extracts of *E. coli* strains K-12 and B contain an enzymatic activity capable of oxidizing the three and four-carbon phosphonic acid analogues indicates that *in vivo* oxidations may be a genuine concern. It is not clear why this activity could not be detected in extracts of *E. coli* strain 7. The difference in the activities of the extracts may reflect the use of different strains or culture conditions, or it may be an artifact of the assay system. In the latter regard, it is important to note that the ability to oxidize the three- and four-carbon analogues was drastically reduced by the addition of KCN and Triton X-100 to the assay mixture. (see Table 3-2).

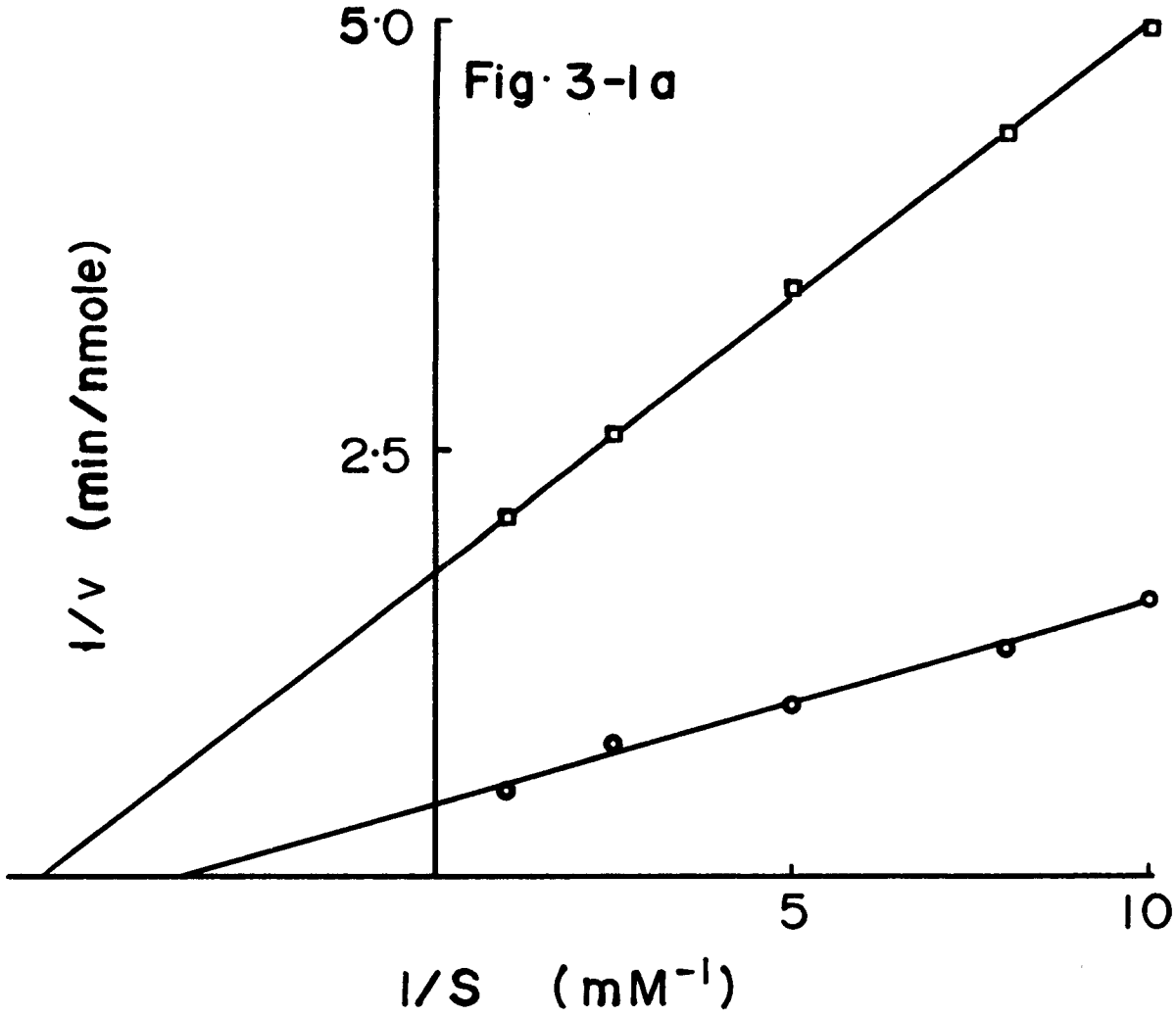
While 3,4-dihydroxybutyl-1-phosphonate causes a specific inhibition of phosphatidylglycerol synthesis it also causes a general inhibition of phosphoglyceride synthesis. That this inhibition is not due to interference with the acylation of glycerol-3-phosphate is revealed by Fig. 3-3 and the observation that the four-carbon phosphonic acid analogue is not a substrate for this reaction. The in vivo inhibition of phosphatidylethanolamine synthesis caused by 3,4-dihydroxybutyl-1-phosphonate does not appear to be due to the inhibition of CDP-diglyceride:serine phosphatidyl transferase. Furthermore, the four-carbon analogue does not appear to inhibit phosphatidylserine decarboxylase since phosphatidylserine does not accumulate in treated cells (Shopsis et al., 1974).

The fact that 3,4-dihydroxybutyl-1-phosphonate inhibits the conversion of dihydroxyacetone phosphate to glycerol-3-phosphate suggests that this might be an important control site. Thus cells cultured on medium containing a carbon source such as glucose or succinate which requires the conversion of dihydroxyacetone phosphate to glycerol-3-phosphate, might, in the presence of 3,4-dihydroxybutyl-1-phosphonate, have a limited intracellular supply of glycerol-3-phosphate available for phosphoglyceride synthesis. The anabolic glycerol-3-phosphate-NAD(P) oxidoreductase is probably not the primary site of action since E. coli cells cultured

in the presence of glycerol, and therefore presumably capable of forming glycerol-3-phosphate by an alternate pathway are still inhibited by 3,4-dihydroxybutyl-1-phosphonate (Shopsis et al., 1972)

At present we consider CDP-diglyceride:glycerol-3-phosphate phosphatidyl transferase to be the most likely site of action but remain open to other possibilities. The finding that 3,4-dihydroxybutyl-1-phosphonate is incorporated into a chloroform soluble material suggests the formation of a new phosphonolipid (Shopsis et al., 1974). This lipid, which is hydrolyzed by phospholipase C from Bacillus cereus (unpublished data R. Tyhach, R. Engel, & B. Tropp), is thought to be the phosphonic acid analogue of phosphatidylglycerol phosphate. The appearance of such a negatively charged phosphoglyceride in the membrane in place of phosphatidylglycerol might have a profound effect upon membrane structure and function. The in vitro studies confirm that 3,4-dihydroxybutyl-1-phosphonate is both a competitive inhibitor of CDP-diglyceride:glycerol-3-phosphate phosphatidyl transferase as well as a substrate for this enzyme.

Figure 3-1a : Substrate kinetics for sn-glycerol-3-phosphate : NAD(P) oxidoreductase. The assay conditions described in the Materials and Methods sections were used. The initial velocity, v, is the change in nmoles per minute of NADPH disappeared. NADPH was present at a final concentration of 200 μM and the enzyme was present at a protein concentration of 170 μg per ml. The concentration of dihydroxyacetone phosphate ●——○——○ or 4-hydroxy-3-oxobutyl-1-phosphonate ■——□——□ was varied.



Fig· 3-1a

Figure 3-1b : Coenzyme kinetics for sn-glycerol-3-phosphate : NAD(P) oxidoreductase. Performed as in Fig. 3-1a except that 3 mM dihydroxyacetone phosphate ●——●——● or 3 mM 4 hydroxy-3-oxobutyl-1-phosphonate ◘——◘——◘ was used and the concentration of NADPH was varied.

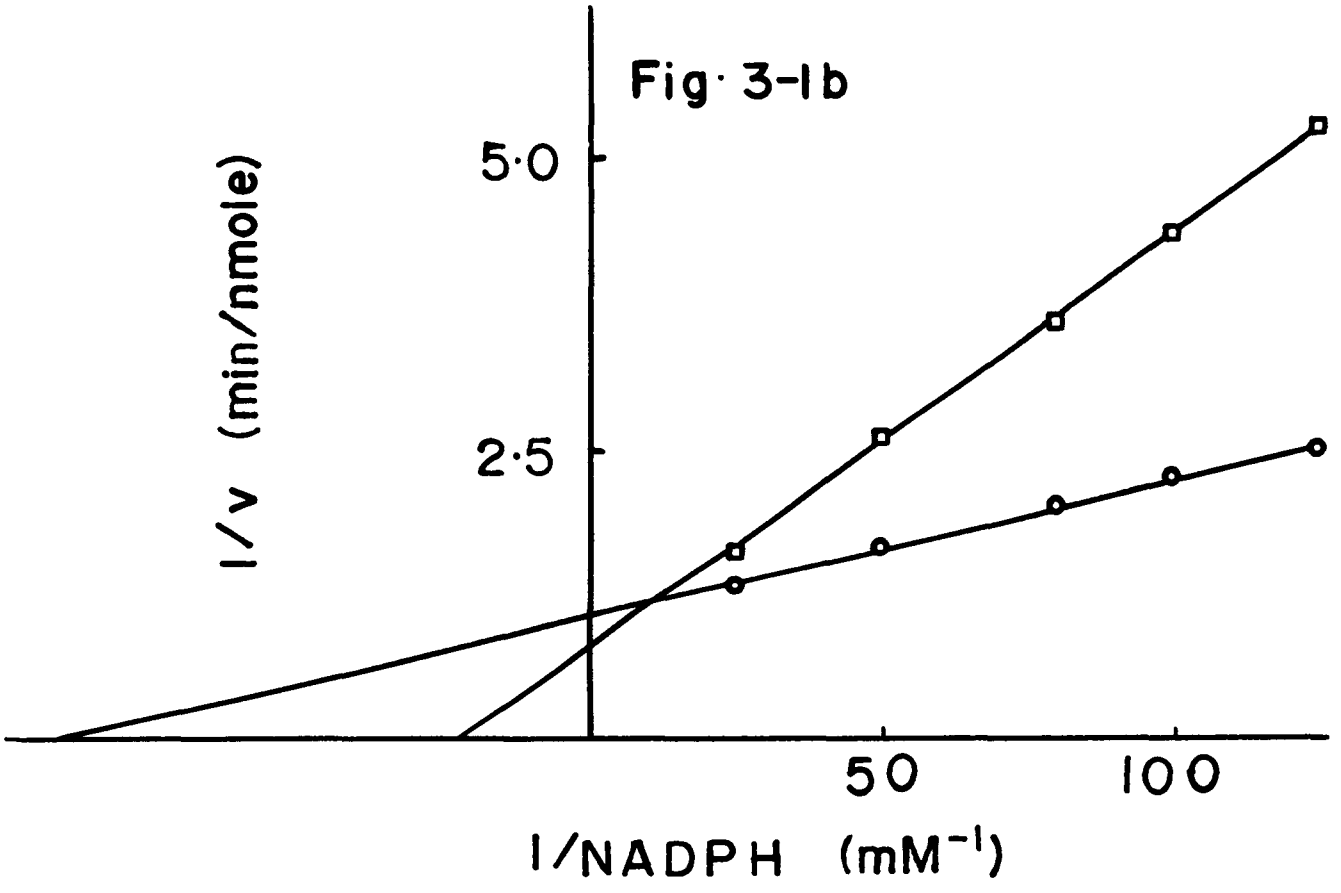

Fig· 3-1b
5·0
2·5
1/v (min/nmole)
50
100
1/NADPH (mM⁻¹)

Figure 3-2 : The determination of the apparent K_i of sn-glycerol-3-phosphate and of 3,4-dihydroxybutyl-1-phosphonate for sn-glycerol-3-phosphate:NAD(P) oxidoreductase. The reaction mixtures were 200 μM in NADPH and contained varying concentrations of dihydroxyacetone phosphate. Because the enzyme preparation used here was somewhat more active than that used in the experiments described in Figure 3-1 enzyme protein was present at a concentration of 56 μg per ml. The initial velocity, v, is the change in μmoles per minute of NADPH disappeared, ●——○——○——○ , no addition; ●——●——● , 100 μM DL-glycerol-3-phosphate; ◘——◘——◘ ,100 μM DL-3,4-dihydroxybutyl-1-phosphonate; ▲——▲——▲ , 100 μM DL-2,3-dihydroxypropyl-1-phosphonate.

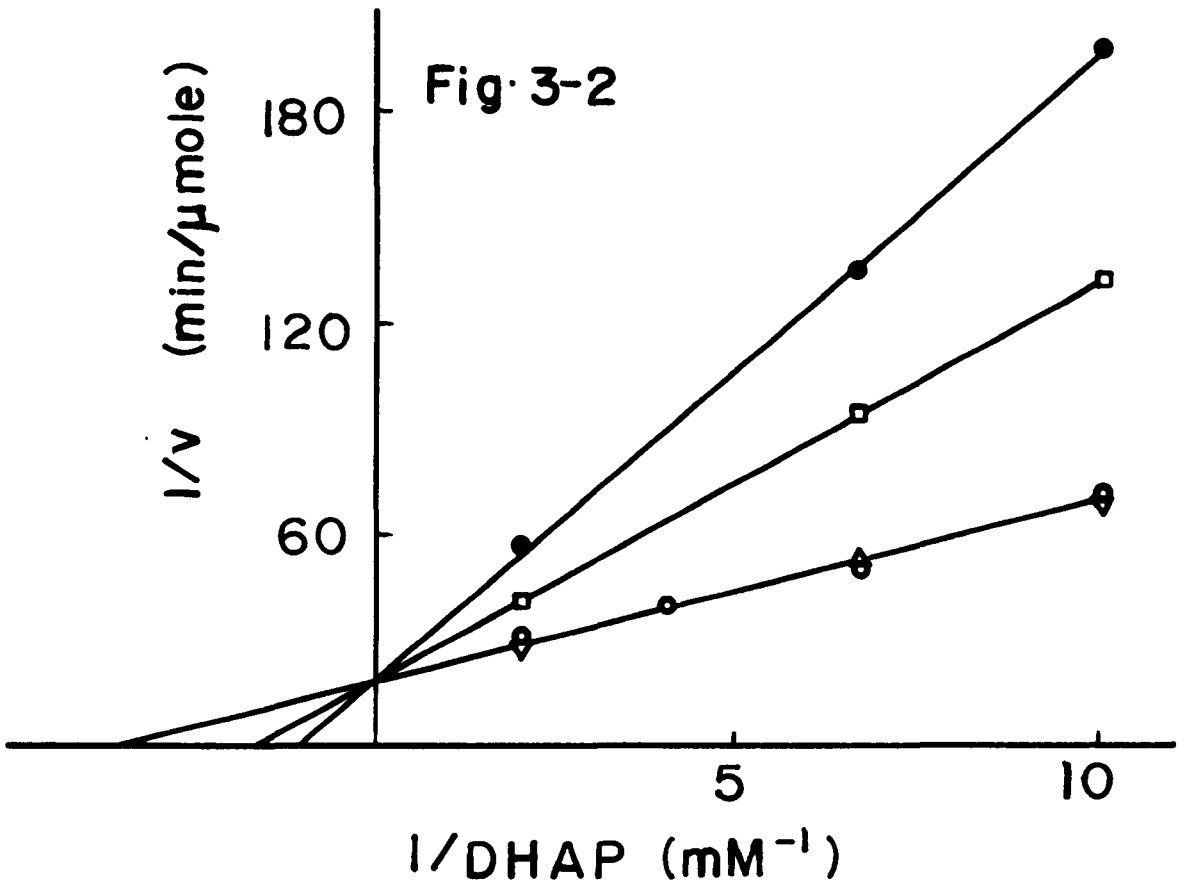

Fig·3-2
180
120
60
1/v (min/μmole)
5
10
1/DHAP (mM⁻¹)

Figure 3-3 : The effect of the phosphonic acid analogues and Li^+ on acyl-CoA:glycerol-3-phosphate acyltransferase. The reaction was performed as described in the Materials and Methods section. In addition to the normal components of the assay system the following were present: 7 mM Li^+ ●——●——● , 3.5 mM DL-3,4-dihydroxybutyl-1-phosphonate ▫——▫——▫, 3.5 mM DL-2,3-dihydroxypropyl-1-phosphonate △——△——△, or no addition ●——●——● .

Fig. 3-3

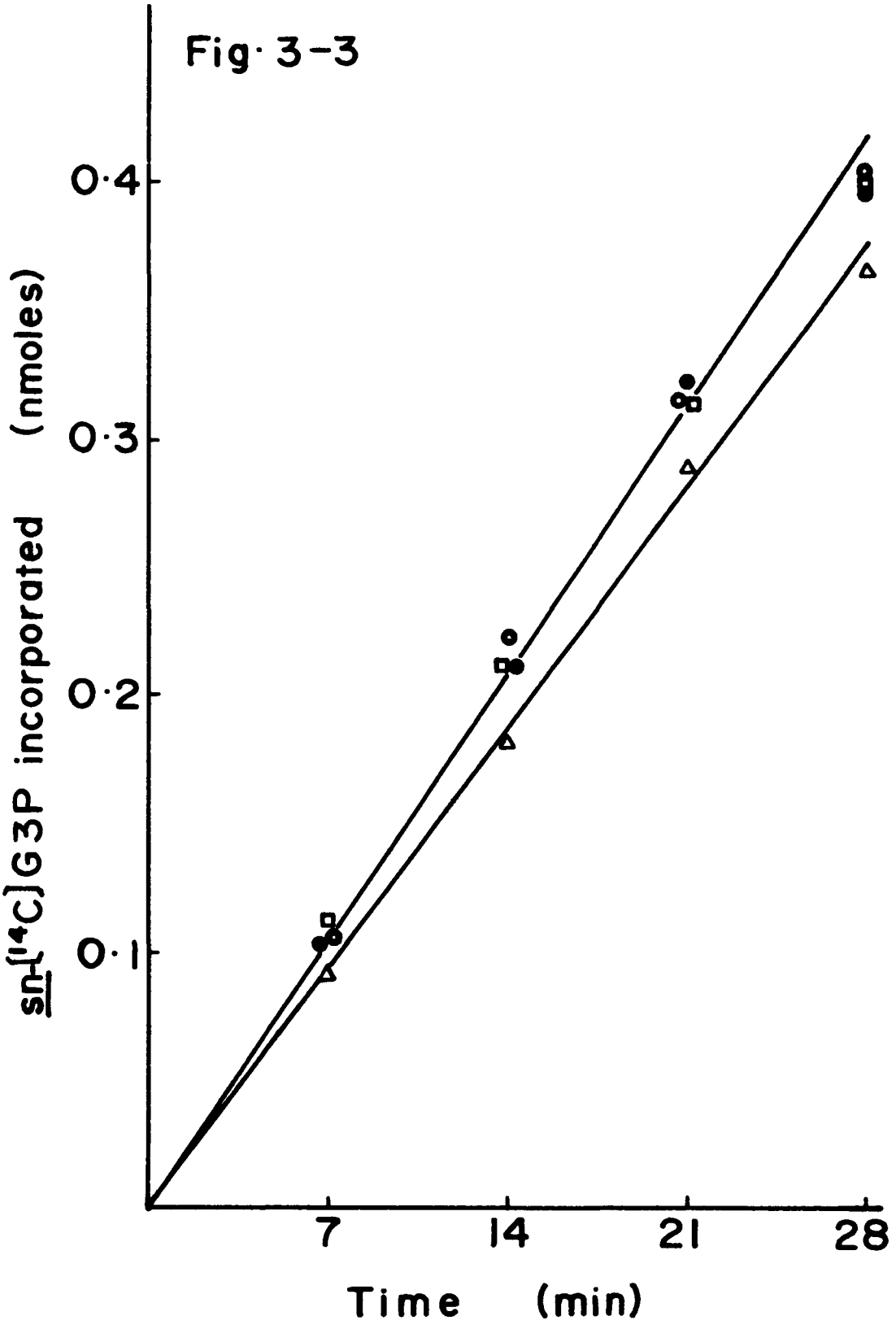


Table 3-1 : The assay of the membrane-bound and pyridine nucleotide-independent sn-glycerol-3-phosphate dehydrogenase was performed as described in the Materials and Methods sections. The final concentration of Li^+ and the racemic mixtures of glycerol-3-phosphate and the dilithium salts of 2,3-dihydroxy-propyl-1-phosphonate and 3,4-dihydroxybutyl-1-phosphonate were as indicated in the table.

TABLE 3-1

Effect of Substrate Analogues on the Catabolic Membrane-Bound Glycerol-3-Phosphate Dehydrogenase

CONCENTRATION (mM)				RATE
DL-Glycerol-3-phosphate	DL-2,3-Dihydroxy-propyl-1-phos-phonate	DL-3,4-Dihydroxy-butyl-1-phos-phonate	Li^+	nmoles per Minute
5	---	---	10	3.2
5	5	---	---	3.2
5	---	5	---	3.2
2.5	---	---	100	1.7
2.5	100	---	---	2.0
2.5	---	100	---	2.0
---	5	---	---	No Reaction
---	---	5	---	No Reaction

Table 3-2 : The assay of the membrane-bound and pyridine nucleotide-independent sn-glycerol-3-phosphate dehydrogenase from E. coli strain 7 or K-12 was the same as that described in Table 3-1 except that unless specified KCN was omitted from the reaction mixture.

Table 3-2

Effect of KCN on rates of MTT reduction by the catabolic dehydrogenase from *E. coli* strain 7 and K-12

	Rates (P moles/ml per mg protein)					
	Strain 7			K-12		
	Super-netant	Pellet	DOC. extract	Super-netant	Pellet	DOC extract
G3P*	200	2.8	193	3.0	--	47
G3P + KCN	--	17.4	263	--	6.7	85
DHBP**	0	0	0	10.1	60	412
DHBP + KCN	--	0	0	--	0	0
DHPP***	0	0	0	7.0	27	266
DHPP + KCN	--	0	0	--	0	0

* G3P: *sn*-Glycerol-3-phosphate

** DHBP: D-Dihydroxybutyl-1-phosphonate

*** DHPP: D-Dihydroxypropyl-1-phosphonate

CHAPTER 4

IN VITRO EFFECTS OF PHENETHYL ALCOHOL

A. Introduction

In the preceeding two chapters, we have described investigations concerning the effects of the phosphonic acid analogues of sn-glycerol-3-phosphate on the metabolism of the natural substrate. This chapter is devoted to a description of the effects of PEA, an autoantibiotic produced by the fungus Candida albicans (Lingappa et al., 1969), on the metabolism of sn-glycerol-3-phosphate. The role of PEA in biological systems has been reported rather extensively in the literature. PEA inhibits cell proliferation in microorganisms, plants and animals. Studies concerning the mechanism of action of PEA in vivo have been reported with respect to macromolecular synthesis and membrane structure and function. However, some of these reports conflict with one another. The following is a summary of various studies concerning different aspects of PEA action.

1. Effect on DNA mebabolism

PEA exerts a bacteriostatic effect on certain gram-negative microorganisms (Lilley & Bremer, 1953; Berrah & Konetzka, 1962). It appears to affect DNA, RNA, protein

and lipid synthesis in Escherichia coli (Berrah & Konetzka, 1962; Nunn & Tropp, 1969). PEA does not act by changing the physicochemical properties of isolated DNA (Rosenkranz et al., 1965). Lark & Lark demonstrated that PEA allows the completion of a chromosomal replication cycle but prevents the initiation of a new replication cycle (Lark & Lark, 1966). Furthermore, it was postulated that the synthesis of one protein required for the initiation of a new round of replication was sensitive to chloramphenicol while the synthesis of a second protein was sensitive to PEA (Lark & Lark, 1966). However, more recent studies of DNA replication have raised the question of the role of PEA as an inhibitor of a new round of replication in Bacillus subtilis (Zyskind & Pattet, 1972).

In Neurospora crassa (Lester, 1965) and in rat hepatoma cells (Plagemann, 1968) PEA causes a simultaneous inhibition of growth and of DNA, RNA, and protein synthesis. PEA also inhibits the replication of the DNA viruses T2 bacteriophage (Konetzka & Berrah, 1962), vaccinia virus, Herpes simplex virus and SV-40 (Weil et al., 1968). PEA does not specifically inhibit DNA synthesis of polyoma virus but exerts a more general effect apparently influencing several steps in the formation of new virus (Bowen et al., 1966). Bruchovsky et al.,(1967) concluded that PEA

causes the inhibition of DNA synthesis in mammalian cells as well as an interference with the onset of cell division. In related investigations Bostock (1970) reported that a concentration of 0.3% PEA inhibited the entry of Sacharomyces pombe into the S phase, whereas a concentration of 0.2% PEA moved the S phase to a different point in the cell cycle.

2. Effect on RNA

The effect of PEA on DNA synthesis has been seriously questioned, and RNA synthesis has been suggested as the primary site of action. In view of recent reports indicating that DNA synthesis may require an RNA primer (Wickner et al., 1973), these two viewpoints may be reconcilable. Sporulation and germination of Bacillus megaterium were inhibited by PEA at a concentration which did not inhibit DNA synthesis (Slepecky, 1963). Weil et al.,(1968) found no effect on the reproduction of Poliovirus type 1 (RNA virus) in tissue culture. However, RNA viruses of bacteria (QB and MS-2)(Nonoyama & Ikeda, 1964) and the animal mengovirus (Plagemenn, 1968), are inhibited by exposing the respective host cells to cytostatic concentrations of PEA. This suggested that the primary site of action may not necessarily be the inhibition of DNA synthesis.

A lower concentration of PEA is required to give 50%

inhibition of the incorporation of [^{32}P]-phosphate into the RNA than into the DNA of E. coli (Rosenkranz et al., 1965a). Rosenkranz et al. suggested that the synthesis of RNA was principal point of attack by PEA in E. coli (Rosenkranz et al., 1965b) on the basis of a preferential inhibition of the synthesis of alkaline phosphatase compared with that of total protein, and some measurements of "mRNA" of induced β-galactosidase. Kinetic studies in E. coli also confirmed that RNA synthesis rather than DNA synthesis is most sensitive to PEA. However, no evidence was provided concerning the relative sensitivity of the different classes of RNA to PEA (Prevost & Moses, 1966).

PEA causes complete inhibition of DNA and RNA synthesis, and cell division in cultures of Saccharomyces cerevisiae growing in a defined medium. However, in a complex medium RNA synthesis and cell division are inhibited to a lesser extent and a slight increase in DNA synthesis has been observed (Wehr et al., 1970).

3. Effect on protein synthesis and enzyme induction

Because PEA caused a rapid disappearance of polyribosomes (Brachousky & Till, 1967; Plagemann, 1968a,c,) with a concomitant increase in the number of single ribosomes and a reduced rate of protein synthesis in mouse L cells (Brachousky & Till, 1967) and rat hepatoma cells (Plagemann, 1968c), there has been a suggestion that PEA might interfere

with the reattachment of ribosomes to RNA while allowing polypeptide chains to go to completion. PEA also preferentially inhibits induced L-tryptophanase synthesis in growing cells of E. coli K-12 (λ) under conditions where this inhibition has no significant adverse effect on the growth of the organism. This effect seems to be exerted at the stage of enzyme synthesis since the activity and the stability of the preformed enzyme is unaffected (Mohan et al., 1969). PEA does not affect the induction of alkaline phosphatase in E. coli, but it does appear to prevent the conversion of inactive monomer subunits to active alkaline phosphatase dimers (Tribhuvan et al., 1970). These results force a partial reevaluation of the conclusion of Rosenkranz et al., (1965b) concerning enzyme induction described above. PEA appears to stimulate the induction of amylase (EC 3.2.1.1.) as well as of β-fructofuranosidase (EC 3.2.1.26) and glucose dehydrogenase (EC 1.1.99). The stimulation seems to be exerted at the translational level (Sinohara, 1970). Thus the role of PEA in protein synthesis appears to be quite complex and ambiguous.

4. Effect on energy production

In bacteria, PEA increased the oxygen consumption of E. coli and decreased that of Staphylococcus aureus and Pseudomonas aeruginosa (Allawala & Speiser, 1971). In

the yeast, Mucor rouxii, PEA stimulated alcoholic fermentation (Terenzi & Storck, 1968; 1969), CO_2 production (Terenzi & Storck, 1969) and caused a concomitant decrease of respiration (Terenzi & Storck, 1968). It also appears to act as an uncoupling agent (Terenzi & Storck 1968) and inhibits the oxidative phosphorylation activity of mitochondria (Terenzi & Storck, 1969) and stimulates the Crabtree effect (Terenzi & Storck, 1968). Wilkie and Maroudas (1969) showed a general toxicity of PEA and growth inhibition on non-fermentable substrates. There is a predominant tendency of inhibition on glycerol and occasional appearance of ρ^+ colonies on glucose, suggesting an attack on the respiratory system.

5. Effect on membrane

The presence of PEA in cultures of E. coli results in a markedly increased uptake of acriflavine, a compound to which healthy growing cells are impermeable, and an increased rate of efflux of cellular potassium under conditions which do not greatly alter the influx of potassium via the energy-dependent potassium pump (Silver & Wendt, 1967). Genetically both PEA resistance and acriflavine resistance are controlled by closely linked genes (Nakmura, 1967). PEA reversibly inhibits the transport of serine, uracil and thymidine by B. subtilis into the cellular pool and irreversibly inhibits the uptake of transforming DNA (Richardson et al., 1969; Urban

& Oyss, 1969). In Neurospora crassa PEA partially inhibits the uptake of glucose, but severely restricted the accumulation of L-leucine, L-tryptophan and α-aminoisobutyric acid in germinated conidia (Lester, 1965).

Treatment of Saccharomyces cerevisiae with 0.5% PEA causes increases in the cellular viscosity and Ca^{++} content 5-fold, while lower concentrations of PEA causes a reversible increase in cell membrane permeability towards fluorescein (Burns, 1971). PEA treatment of mammalian cells reduces the ability to accumulate uridine which is subsequently reflected in a decreased incorporation of uridine (Higgins et al., 1969).

As studied by coulokinetic techniques, PEA does seem to increase the porosity of cell membranes (Charnecki & Allen, 1969). Furthermore, treatment with concentrations of 0.5% PEA or greater concentrations which stop DNA replication immediately in E. coli, also appear to release 25 to 80% of the DNA from the membrane. No significant release was observed with 0.25% PEA (Masker & Eberle, 1972). Concentrations of PEA slightly above the bacteriostatic concentration lysed actively growing cells of B. subtilis, S. aureus (Zyskind & Pattet, 1971), and E. coli (Zyskind & Pattet, 1971; Woldringh, 1973). Stationary phase cultures of each of these organisms were much less susceptible to the lytic action of PEA (Zyskind & Pattet, 1971). PEA

treatment of either isolated lysosomes or whole cells released lysosomal enzymes (Higgins et al., 1969). Studying the inactivation of virus by PEA, Roickhel and Zeithenok, (1969) found that the action of PEA was apparently selectively directed to the structure of supercapsid membranes of the viruses.

6. Effect on lipid metabolism

A strain of E. coli K-12 containing env A has a higher palmitic acid content than its wild-type parent in the lipopolysaccharide fraction of the cell envelope. Furthermore there was a decreased content of phosphatidylglycerol and cis-vaccenic acid in the organic solvent extractable lipid fraction. Culturing cells in the presence of PEA resulted in fatty acid and the phospholipid composition more like that detected in the wild-type parent (Normark, 1971). In our laboratory, Nunn and Tropp (1972) observed that the uptake of labeled acetate into the phospholipid fraction of E. coli was more sensitive to inhibition by low concentrations of PEA than the uptake of labeled precursors into the macromolecules RNA, DNA, or protein. The distribution of labeled acetate incorporated into phospholipids was markedly affected by the presence of PEA. The uptake of acetate into phosphotidylethanolamine and phosphatidylglycerol was inhibited, whereas the uptake of acetate into the cardiolipin fraction appeared to be unaffected. Barbu

et al. (1970) also reported that PEA added to cultures of E. coli at a concentration of 0.2% caused an increase in the cardiolipin content and a decrease in both phosphatidylethanolamine and phosphatidylglycerol. These changes were reversed upon removal of the PEA. At a concentration of 0.4% PEA, which was bateriocidal, cardiolipin increased only slightly, but lysophosphatidylethanolamine increased to approximately 15% of the phospholipid fraction after 2 hours incubation compared to 1% for untreated cells. This is a sign of irreversible transformation causing the death of bacteria as examined with E. coli K-12.

Thus the effects of PEA, in vivo, are quite complex. The use of different species and experimental conditions causes further complications of interpretation. It appears quite possible that PEA exerts its effect at the level of membrane function and perhaps synthesis. In vitro studies might provide fresh insights into the mechanism of action of PEA. For this reason, the studies in this chapter are designed to investigate the effects of PEA in vitro on certain aspects of phospholipid metabolism in E. coli. The scope has been limited to enzymes involved in sn-glycerol-3-phosphate metabolism. Nunn previously demonstrated that PEA does not inhibit glycerol-3-phosphate:CDP-diglyceride phosphatidyl transferase or serine:CDP-diglyceride phosphatidyl transferase activities (Nunn, 1972).

B. Material and Methods

Chemicals: NADPH type II: 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT); phenazine methosulfate (PMS); bovine serum albumin (BSA); DL-glycerol-3-phosphate, disodium salt; coenzyme A grade I; DEAE-Sephadex A-50; Sephadex G-25; streptomycin sulfate; tris-(hydroxymethyl)-aminomethane (Tris); stearyl-CoA, pamitoleyl chloride and vaccenyl chloride were purchased from the Sigma Chemical Company, St. Louis, Mo.. Supelcosil silica gel 12A and the chromatographic standards phosphatidic acid and lysophosphatidic acid were purchased from Supelco, Inc., Bellefonte, Pa.. Palmityl chloride was prepared from palmitic acid by treatment with an excess of thionyl chloride. Palmityl-CoA was then prepared from the acid chloride according to the method of Seubert (1960). Palmitoleyl-CoA and vaccenly-CoA derivatives were synthesized with the same method as modified by Pieringer et al., (1967). The di-mono-cyclohexylamine salt of dihydroxyacetone phosphate dimethylketal was a gift of L. Gelbaum. Dihydroxyacetone phosphate was generated by the method of Ballou and MacDonald (1963) as described by the Sigma Chemical Company. Triethanolamine was a product of the Aldrich Chemical Co., Milwauke, Wisconsin. sn-[^{14}C]-Glycerol-3-phosphate (80 mC_i/mmol) was purchased from New England Nuclear Corp., Boston, Mass., and phenethyl alcohol (PEA) from Matheson Coleman and Bell Company, Rutherford, New Jersey.

Bacterial Strains: E. coli strains 7,8 and B have been described in the previous chapter.

Assay of acyl-CoA:Glycerol-3-phosphate Acyltransferase: Two sets of experiments were designed to study the incorporation of sn-[^{14}C]glycerol-3-phosphate into phosphatidic acid. (A) By the disk filter paper method: The procedures for culturing cells, preparing the enzyme from E. coli strain 8 and its subsequent assay were as described in the preceeding chapter. The reaction mixture contained 0.1 M Tris-HCl buffer pH 8.5; 6.25 mM Mg^{++}; 3.75 µg/ml BSA; and enzyme. The amount of acyl-CoA, sn-glycerol-3-phosphate, and PEA varied according to the conditions of the experiment. The assays were performed at 30°C and initiated by adding acyl-CoA. Fifty µl of the assay mixture were removed at the indicated times, placed on Whatman 3 MM filter disks and treated as described in the previous chapter. (B) By chloroform extraction and thin layer chromatography: The assay mixture was the same as in (A) but in a total volume of 0.8 ml. The reaction was terminated after 16 minutes by adding 0.8 ml 10% cold TCA, mixed well, and then kept cold. To this mixture, 0.6 ml of 2:1 methanol-chloroform, 2.0 ml chloroform, and 2.0 ml water were added sequentially. The mixture was agitated after each addition and the chloroform fraction was collected by the prodedure of Bligh and Dyer (1959) as modified by Ames (1968). The chloroform extracts

were then washed three times with 2 M KCl and once with water. These chloroform soluble extracts were dried under a stream of nitrogen gas and then resuspended in 0.1 ml of chloroform. Twenty µl of solution were spotted on thin layer chromatographic plates. The plates were prepared with an adjustable Desaga applicator set at 250 µ thickness. Thirty grams of Supelcosil silica gel 12A was mixed with 0.01 M aqueous Na_2CO_3 to form a basic support and then dried at room temperature for 20-25 minutes and stored in a cabinet without drying agent. Prior to application the plates were activated at 80°C for 45-60 minutes. The chromatography chamber was shielded on three sides with Whatman 3 MM paper wetted with the developing solvent 45-60 minutes before chromatography (Skipski et al., 1963). The solvent system was modified from that described by Hajra and Agranoff (1968) and contained chloroform-methanol-acetone-acetic acid-water (160:45:15:20:10) instead of (200:40:80:40:20) as originally described. The lysophosphatidic acid of the standard always migrated as a small compact region on the plate with an R_F 0.25 but the phosphatidic acid streaked with an R_F of approximately 0.65. The separation was therefore made by scraping the plate above and below a line corresponding to an R_F of 0.44. The radioactivity was measured by suspending the scrapings from the silica gel plate into one ml of 10% glacial acetic acid in absolute ethanol and 10 ml of toluene

scintillation fluid.

Assay of the sn-Glycerol-3-phosphate:NAD(P) oxidoreductase (EC 1.1.1.8): The anabolic enzyme was purified from E. coli strain B by the procedure of Kito and Pizer (1969) up to the first DEAE-Sephadex column chromatography. Dihydroxyacetone phosphate was used as a sole substrate and PEA as inhibitor. The other conditions were the same as described in the previous chapter.

Assay of the Membrane-Bound and Pyridine Nucleotide-Independent sn-Glycerol-3-phosphate Dehydrogenase: The catabolic glycerol-3-phosphate dehydrogenase was assayed with partially purified enzyme from E. coli strain 7. Two grams of washed cells were suspended in 20 ml of 0.1 M phosphate buffer, pH 7.5; and sonicated for two minutes on a Bronson Model W140D sonifier at a setting of 60 watts. The suspension was centrifuged at 3000 x g for 15 minutes in a Sorvall RC2B centrifuge. The supernatant was removed and recentrifuged at 4,000 x g for 20 minutes. The glycerol-3-phosphate dehydrogenase was present mainly in the pellet. The enzyme activity was then extracted from the pellet by suspending in 3 ml 0.25 M NaCl solution containing 10% glycerol and 0.1% sodium deoxycholate. After standing for four hours, the solution was centrifuged again for 30 minutes at 40,000 x g. The purification procedure described above is the same as the one described by Weiner and Heppel (1972) except that Brij 50 was omitted. The

spectrophotometric assays were performed at 30°C by the procedure of Kistler and Lin. (1971) in which the enzyme activity was measured by PMS coupled reduction of MTT. The reaction mixture of 1 ml contained 100 µmoles potassium phosphate buffer pH 7.5; 33 µg MTT; 200 µg PMS; and enzyme. The reaction was initiated by adding glycerol-3-phosphate. The progress of the reaction was followed by the rate of increase in absorbance of MTT at 570 nm and extinction coefficient was taken as 17 $mM^{-1}cm^{-1}$.

C. Results

Nunn and Tropp (1972) assumed the diverse effects of PEA might be due to the ability of the compound to (i) prevent normal protein synthesis, (ii) alter some aspect of phospholipid metabolism, or (iii) interact with the membrane or some vital membrane component. They studied the first two possibilities and concluded that PEA does not primarily affect protein synthesis. Phospholipid synthesis was more sensitive to inhibition by low concentrations of PEA than was RNA, DNA or protein synthesis. In addition, the distribution of labeled acetate incorporated into phospholipids was markedly affected by the presence of PEA. Barbu et al.(1970) reported similar results concerning phospholipid distribution. However, it is not clear at which stage of phospholipid synthesis PEA exerts its effect. This problem is the subject of this chapter.

Fig. 4-1a indicates the effect of incubation time on the relative incorportation of sn-glycerol-3-phosphate into trichloroacetic acid insoluble material on filter paper disks in the presence of various acyl-CoAs and the transferase derived from E. coli strain 8. Among the four acyl-CoAs tested palmityl-CoA appeared to be the most efficent acylating agent. Vaccenyl-CoA and stearyl-CoA are about equally effective. While palmitoleyl-CoA was

the least effective.

Fig. 4-1b shows that the presence of PEA did not change the pattern of incorporation of sn-glycerol-3-phosphate in the presence of various acyl-CoAs. However, in the presence of PEA, vaccenyl-CoA was about 15% less effective than stearyl-CoA. Fig. 4-1c to 4-1f, replots from Fig. 4-1a and 1b, shows the effects of PEA on various acyl-CoAs. These replots clearly demonstrate the inhibitory effect of PEA on each of the acyl-CoAs.

The studies described above do not distinguish between the formation of phosphatidic acid and of lysophosphatidic acid. This was examined by scaling up the assay volumes as described in the Materials and Methods section and analyzing the products by thin layer chromatography. When authentic standards of phosphatidic acid and lysophosphatidic acid were chromatographed, the R_F values were 0.65 and 0.25, respectively. The spot for authentic phosphatidic acid streaked out to a 2.5 times greater extent than the one for authentic lysophosphatidic acid. In view of the streaking, the plate was divided into four parts: (i) from the origin to an R_F of 0.08 (ii) from an R_F 0.08 to 0.44 (iii) from an R_F 0.44 to 0.90, and (iv) from an R_F 0.90 to the solvent front. It is assumed that fraction (ii) represents lysophosphatidic acid and fraction (iii) represents phosphatidic acid. However, the streaking of standard phosphatidic acid somewhat limits the validity of this assumption. The results

of scraping the silica gel within the boundaries described and counting as described in the Materials and Methods section is presented in Table 4-1. The ratio of phosphatidic acid to lysophosphatidic acid, when palmityl-CoA is the acyl donor, is 2.4. In the presence of PEA the ratio went down to 1.4. If palmitoleyl-CoA was used the ratio was 4.7; and in the presence of PEA it was changed only slightly to a ratio of 4.2. The ratio for vaccenyl-CoA were 10.0 and 7.1 in the absence and presence of PEA respectively. This hints at the possibility that the inhibition of palmityl-CoA is exerted mainly at the second stage of acylation, that is; in the conversion of lysophosphatidic acid to phosphatidic acid; while the stage of inhibition for palmitoleyl-CoA and vaccenyl-CoA as substrates is even less clear.

Fig. 4-2 is a plot of the percent of initial activity remaining in the presence of various amounts of PEA, for different acyl-CoAs as measured by the incorporation of radioactive sn-glycerol-3-phosphate. The enzyme activity decreases continuously as the concentration of PEA increases. The concentrations of PEA used are in the range of those that severely inhibit phospholipid synthesis in vivo (Nunn & Tropp, 1972).

Since the extent of sn-glycerol-3-phosphate incorporated is acyl-CoA dependent, kinetic studies might provide informa-

tion concerning the mode of action of PEA. Fig. 4-3a is a Lineweaver-Burk plot of the reciprocal of the initial velocity versus the reciprocal of the millimolar concentrations of palmityl-CoA at a constant concentration of sn-glycerol-3-phosphate. The results indicate that the apparent K_m for palmityl-CoA decreases and are accompanied by a decrease of V_{max} in the presence of PEA. Such results are typical for uncompetitive inhibition. When a similar experiment was performed with palmitoleyl-CoA instead of palmityl-CoA, the curves appeared to be concave upward (Fig. 4-3b) which may be indicative of substrate inhibition. Note that PEA also has an inhibitory effect on palmitoleyl-CoA mediated conversion of glycerol-3-phosphate to trichloroacetic acid insoluble materials. Both curves extrapolated from low substrate concentrations intercepted at the X axis. This might be expected for noncompetitive inhibition. A variation of vaccenyl-CoA concentrations as shown in Fig. 4-3c provided similar results to those obtained for palmitoleyl-CoA as concerns the upward concave shape of the curve. However, the two extrapolated lines intercepted each other at Y axis. There in no change of V_{max} but K_m decreased. This is the type of curve that might be expected for competitive inhibition. Thus the mechanism of PEA inhibition for the three acyl-CoAs tested varies and no consistent picture emerges. When the reciprocal of the concentrations

of sn-glycerol-3-phosphate at constant concentration of the various acyl-CoAs is plotted against the reciprocal of the initial velocity, the Lineweaver-Burk plots shown in Fig. 4-4a,b, & c are similar no matter which acyl-CoA was used.

From the results presented above, it is clear that acyl-CoA:glycerol-3-phosphate acyltransferase is a site of PEA action. No other site in the biosynthetic sequence for phospholipids beyond this step appears to be affected by PEA.

In the previous chapter, we have shown that 3.4-dihydroxybutyl-1-phosphonate as well as glycerol-3-phosphate inhibits the conversion of dihydroxyacetone phosphate to glycerol-3-phosphate competitively. It was decided to test the anabolic glycerol-3-phosphate dehydrogenase which catalyzes this reation. The results are presented in Fig. 4-5. PEA inhibits this conversion noncompetitively with respect to dihydroxyacetone phosphate. On the other hand, the catabolic enzyme, membrane-bound sn-glycerol-3-phosphate dehydrogenase, was not inhibited by PEA at concentrations up to 0.3%. At higher concentrations where PEA is bactericidal, it inhibits the catabolic enzyme as has been reported for some other enzymes (Barbu et al., 1970).

D. Discussion

PEA inhibits the biosynthesis of phospholipids as evidenced by decreased amounts of phosphatidylethanolamine and phosphatidylglycerol *in vivo* as reported by Barbu *et al.*, (1970) and by Nunn and Tropp (1972). *In vitro* studies should be able to comfirm their results and to locate the steps where PEA interferes with one or more of the enzymes involved in the biosynthesis of phosphoglycerides. Steiner and Lester (1970) reported CDP-diglyceride may be a precursor, either directly or indirectly, of all the major phospholipids in *S. cerevisiae*. A similar conclusion is generally accepted for *E. coli* and other procaryotes. The regulation of the metabolism of this intermediate is therefore of particular importance. Nunn (1972) has demonstrated that 3,4-dihydroxybutyl-1-phosphonate, the analogue of glycerol-3-phosphate, is a competitive inhibitor for the CDP-diglyceride:glycerol-3-phosphate phosphatidyl transferase. This phosphonate has no inhibitory effect upon the conversion of serine to lipid extractable material in the CDP-diglyceride:serine phosphatidyl transferase assay. These *in vitro* studies confirmed the *in vivo* observations concerning the inhibition of phosphatidylglycerol synthesis (see previous chapter). Nunn (1972) also demonstrated that PEA fails to inhibit the formation of phosphatidylglycerol and phosphatidylethanolamine in similar experiments to those performed with the phosphonate. These

results indicate that the action of PEA is probably different from that of the phosphonate. They also help to limit the number of steps in phospholipid synthesis that may be sensitive to PEA. The data presented in this chapter are directed towards determining the site(s) of inhibition.

It is generally thought that there are two acylthioester transferases involved in the biosynthesis of phosphatidic acid in E. coli; one catalyzes the formation of lysophosphoatidic acid from sn-glycerol-3-phosphate and acylthioester; the other the formation of phosphatidic acid from lysophosphatidic acid and another molecule of acylthioester (Pieringer et al., 1967). It is not clear whether acyl-CoA or acyl-acyl carrier protein (acyl-ACP) is the true acylating agent although the latter appears to be the more likely (Ailhaud & Vagelos, 1966). In E. coli, the saturated fatty acid consist primarily of palmitic acid with some myristic acid and only trace amounts of stearic and lauric acids. Palmitic acid comprises about half of the total fatty acids of the cell, and it is found esterified almost exclusively to position 1 of the glycerol backbone of the phospholipids. The other saturated fatty acids are distributed between both positions 1 and 2 (Cronan & Vagelos 1972). The unsaturated fatty acids found in E. coli are all monoenes of the cis-configuration. The hexadecenoic acid has been identified as palmitoleic acid, while the octadecenoic acid has been

shown by several techniques to consist solely of cis-vaccenic acid (Cronan & Vagelos, 1972). Both palmitoleic and cis-vaccenic acids are found esterified predominantly to position 2 of the sn-glycerol-3-phosphate backbone of the phospholipids (Cronan & Vagelos 1972). The recent report by Okuyama and Wakil (1973) indicating that 1-acyl-glycerol-3-phosphate is the true intermediate in phosphatidic acid formation may explain the comparatively higher incorporation of glycerol-3-phosphate when palmityl-CoA serves as the acyl doner (Fig. 4-1)

Since the labeled phospholipids analyzed by thin layer chromatography are not present in sufficient amounts to be stained by iodine vapor, all results refer to comparisons with authentic standards of phosphatidic and lysophosphatidic acid. When palmityl-CoA was the acyl doner, van den Bosch and Vagelos (1970) have reported the ratio of phosphatidic acid to lysophosphatidic acid as 5.4 compared to the value presented here of 2.4. This discrepancy could be due to several differences in the assay systems used. In their system, the incubation was at 35°C for 30 minutes with E. coli strain K-12. When palmityl-ACP was used as acyl-donor, they reported the ratio was 0.07. In fact, the acyl transferase is more selective toward acyl-ACP than to acyl-CoA (van den Bosch & Vagelos 1970). The relative amount of phosphatidic acid and lysophosphatidic acid synthesized

in vitro might be pH and time dependent. In addition, it should be mentioned that if acyl-ACP is the true acyl donor, the results presented here may be approximations of the physiological system. Interpretation of the kinetic results are quite difficult because two enzyme activities are being measured, acyl-CoA:glycerol-3-phosphate acyltransferase and acyl-CoA:1-acyl-glycerol-3-phosphate acyltransferase. The inhibition caused by high concentrations of palmitoleyl-CoA and vaccenyl-CoA further complicates kinetic analysis.

Another sensitive site for metabolic regulation in phospholipid biosynthesis is the formation of *sn*-glycerol-3-phosphate. Glycerol-3-phosphate is known as one of the basic, indispensable building blocks of phospholipid biosynthesis. In succinate or glucose supplemented cultures glycerol-3-phosphate originates from glycolysis. Reduction of dihydroxyacetone phosphate, a key intermediate in glycolysis, yields *sn*-glycerol-3-phosphate and this provides a link between carbohydrate and lipid metabolism. Another source of glycerol-3-phosphate is the asymmetric phosphorylation of the apparently symmetrical molecule glycerol, calalyzed by the enzyme glycerol kinase. The kinase pathway becomes important only if microorganisms are growing in a medium containing glycerol as the sole carbon source. The conversion of dihydroxyacetone phosphate to glycerol-3-phosphate is catalyzed by the anabolic glycerol-3-phosphate

dehydrogenase. This reaction strongly favors the formation of glycerol-3-phosphate. It is competitively inhibited by sn-glycerol-3-phosphate, one of its own products in the reaction and its analogue 3,4-dihydroxybutyl-1-phosphonate (see chapter 3). PEA inhibits this reaction noncompetitively. This inhibition may be physiologically important in evaluating the effect of PEA on phospholipid biosynthesis in vivo. The catabolic membrane-bound dehydrogenase is much less sensitive.

In summary, both PEA and 3,4-dihydroxybutyl-1-phosphonate inhibit the anabolic glycerol-3-phosphate dehydrogenase. The inhibition may be physiologically important in the regulation of phospholipid biosynthesis. The distribution of precursors incorporated into the phospholipid fraction of E. coli is affected differently by PEA and the phosphonate. The in vitro studies presented indicate that PEA affects the acylation of glycerol-3-phosphate. The phosphonic acid analogue does not affect this step. However, the phosphonic acid analogue affects the in vitro formation of phosphatidylglycerol, a step which is not inhibited by PEA. The in vitro studies presented here are consistent with earlier in vivo data and help to further our understanding of the mechanism of action of the two inhibitors of phosphoglyceride metabolism.

Fig. 4-1a : The effect of incubation time on the relative incorporation of sn-glycerol-3-phosphate into trichloroacetic acid insoluble material on filter paper disks in the presence of various acyl-CoAs by glycerol-3-phosphate acyltransferase from E. coli strain 8. The assay mixture contains Tris-HCl buffer pH 8.5, 0.1M; Mg^{++}, 6.25 mM; BSA 75 µg; sn-glycerol-3-phosphate 97 µM (0.1 μC_i); acyl-CoA 125 µM and enzyme 0.7 mg in a total of 0.4 ml. The reaction was initialed by adding Acyl-CoA. Fifty µl of reaction mixture were then removed at the indicated times and counted. ◘——◘——◘ represents palmityl-CoA, Δ——Δ——Δ palmitoleyl-CoA, O——O——O stearyl-CoA, and ✚——✚——✚ vaccenyl-CoA present in the reaction mixtures.

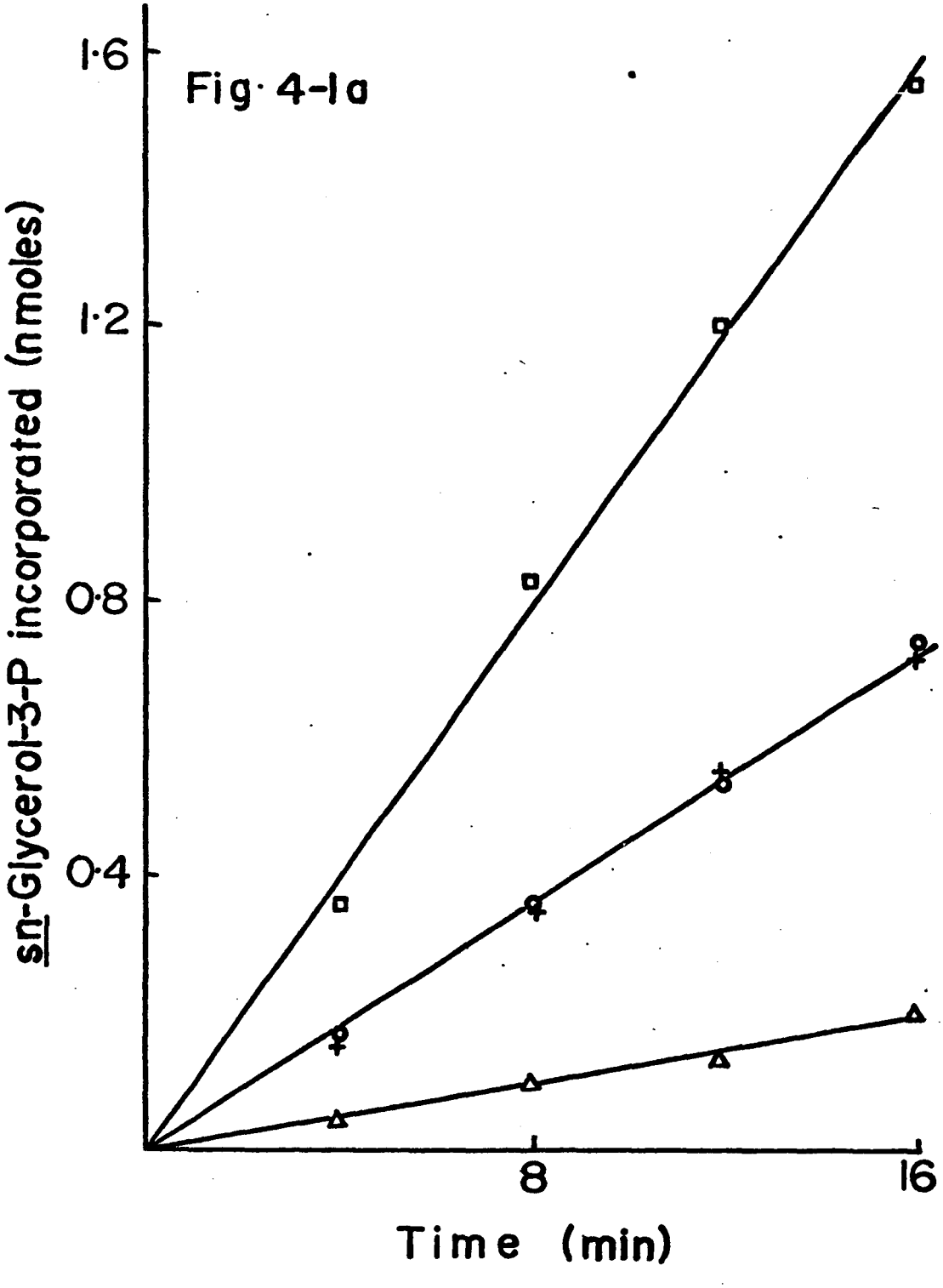
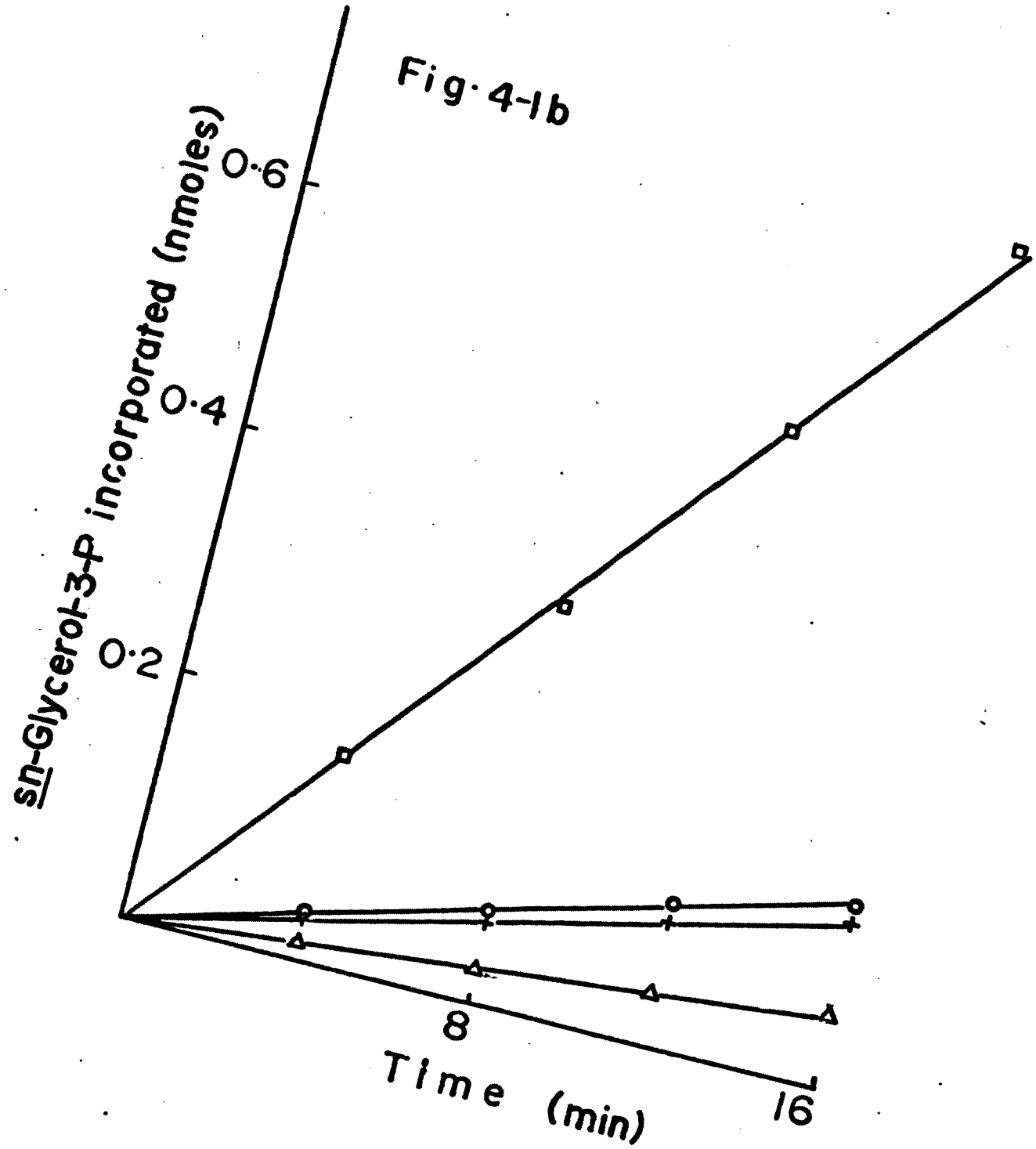

Fig· 4-1a
1·6
1·2
0·8
0·4
sn-Glycerol-3-P incorporated (nmoles)
8
16
Time (min)

Fig. 4-1b : The effect of PEA on the relative incorporation of labeled sn-glycerol-3-phosphate in the presence of various acyl-CoAs. The assay system was the same as described in Fig. 4-1a except 0.2% (v/v) of PEA was included. □——□——□ represents patmityl-CoA, △——△——△ palmitoleyl-CoA, ○——○——○ stearyl-CoA, and +——+——+ vaccenyl-CoA present in the reaction mixtures.



Fig·4-1b

Fig. 4-1c : The effect of PEA on the incorporation of labeled sn-glycerol-3-phosphate in the presence of palmityl-CoA. □——□——□ represents no addition, ○——○——○ PEA added. It is reproduced from Fig. 4-1a and 4-1b.

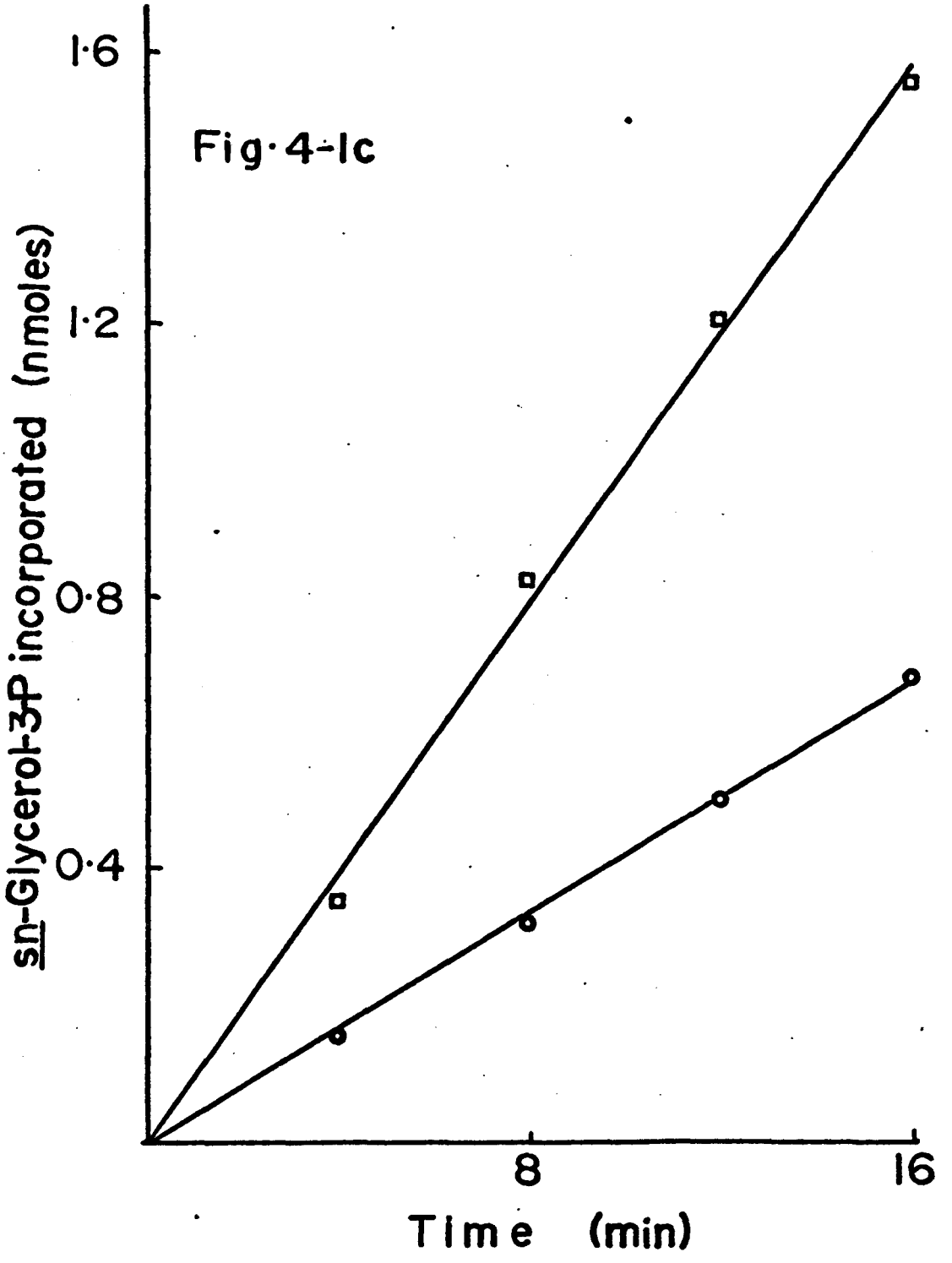
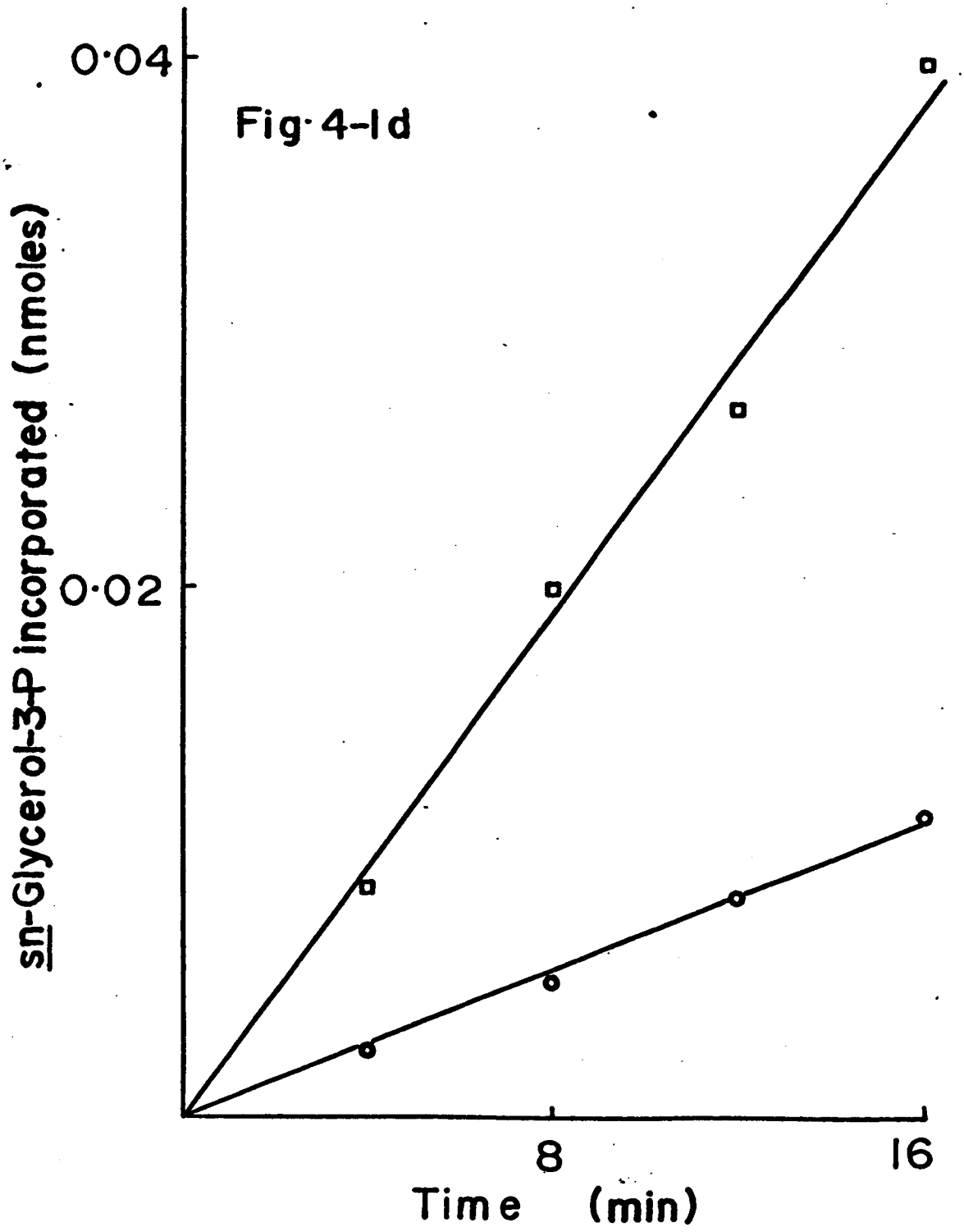

Fig·4-Ic
1·6
1·2
0·8
0·4
sn-Glycerol-3P incorporated (nmoles)
8
16
Time (min)

Fig. 4-1d : The effect of PEA on the incorporation of labeled sn-glycerol-3-phosphate in the presence of palmitoleyl-CoA. □——□——□ represents no addition, ●——●——● PEA added. It is reproduced from Fig. 4-1a and 4-1b.



Fig·4-Id

Fig. 4-1e : The effect of PEA on the incorporation of labeled sn-glycerol-3-phosphate in the presence of stearyl-CoA, □——□——□ represents no addition, ○——○——○ PEA added. It is reproduced from Fig. 4-1a and 4-1b.

Fig·4-1e

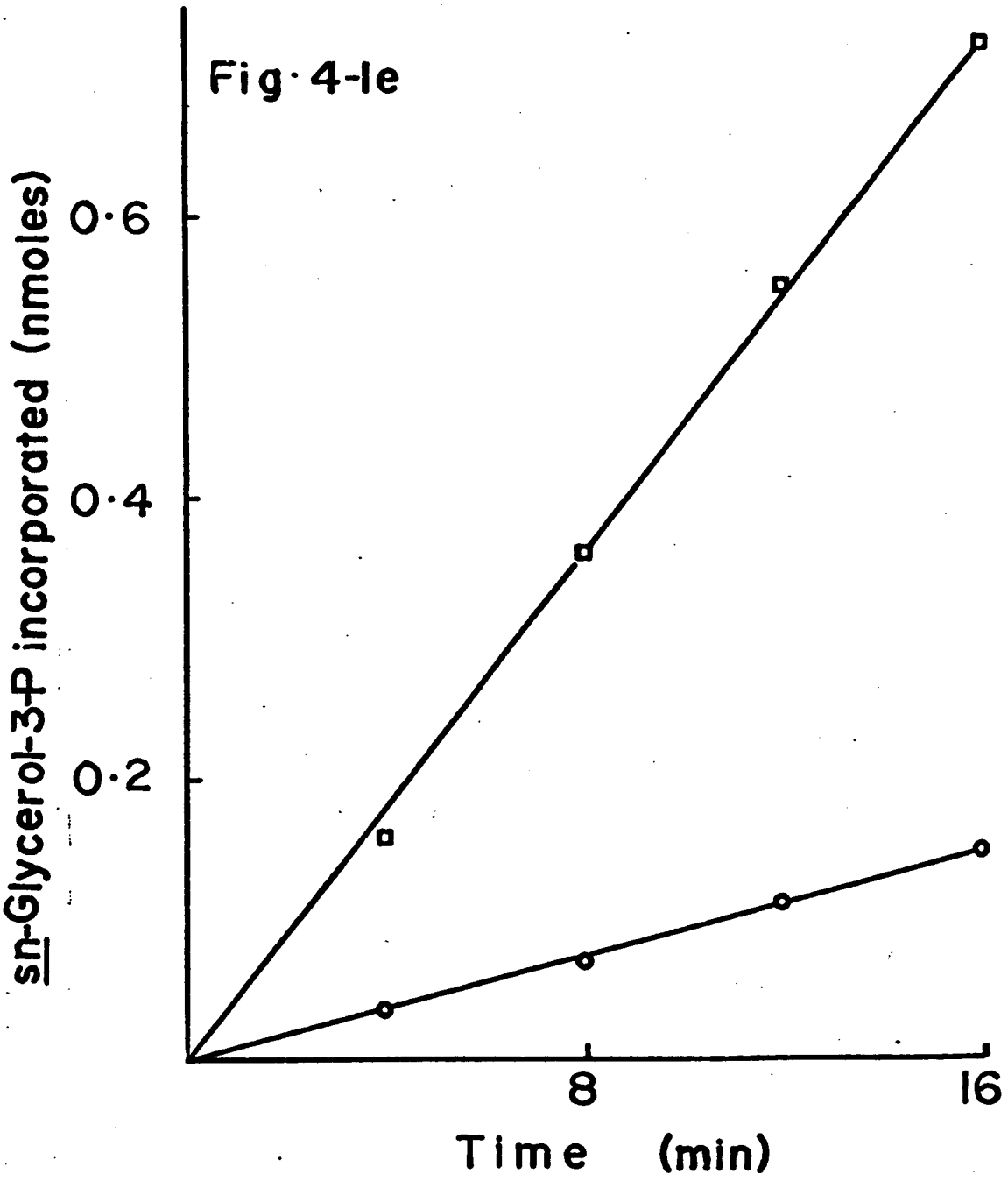


Fig. 4-1f : The effect of PEA on the incorporation of labeled sn-glycerol-3-phosphate in the presence of vaccenyl-CoA. □——□——□ represents no addition, ○——○——○ PEA added. It is reproduced from Fig. 4-1a and 4-1b.

Fig·4-If

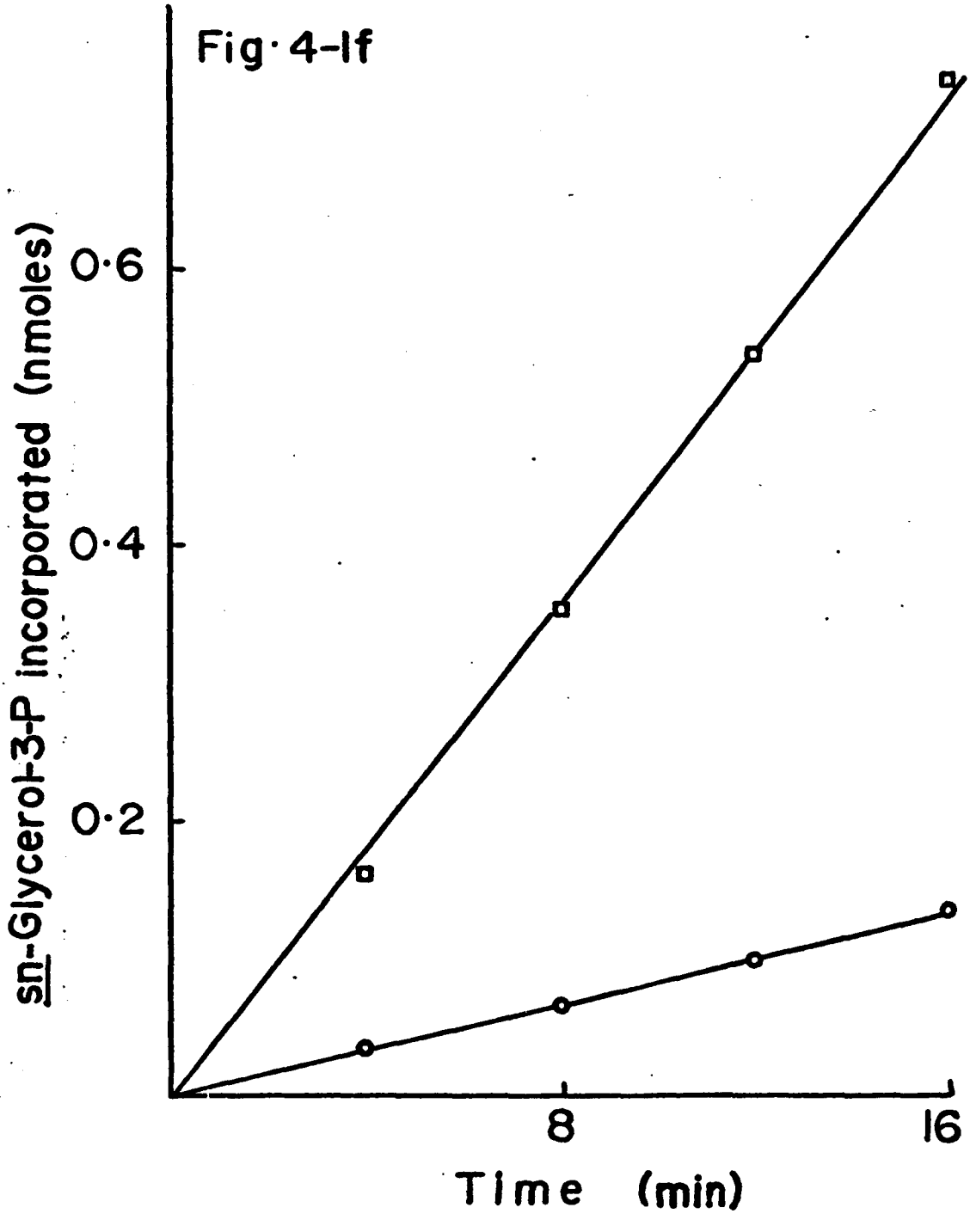
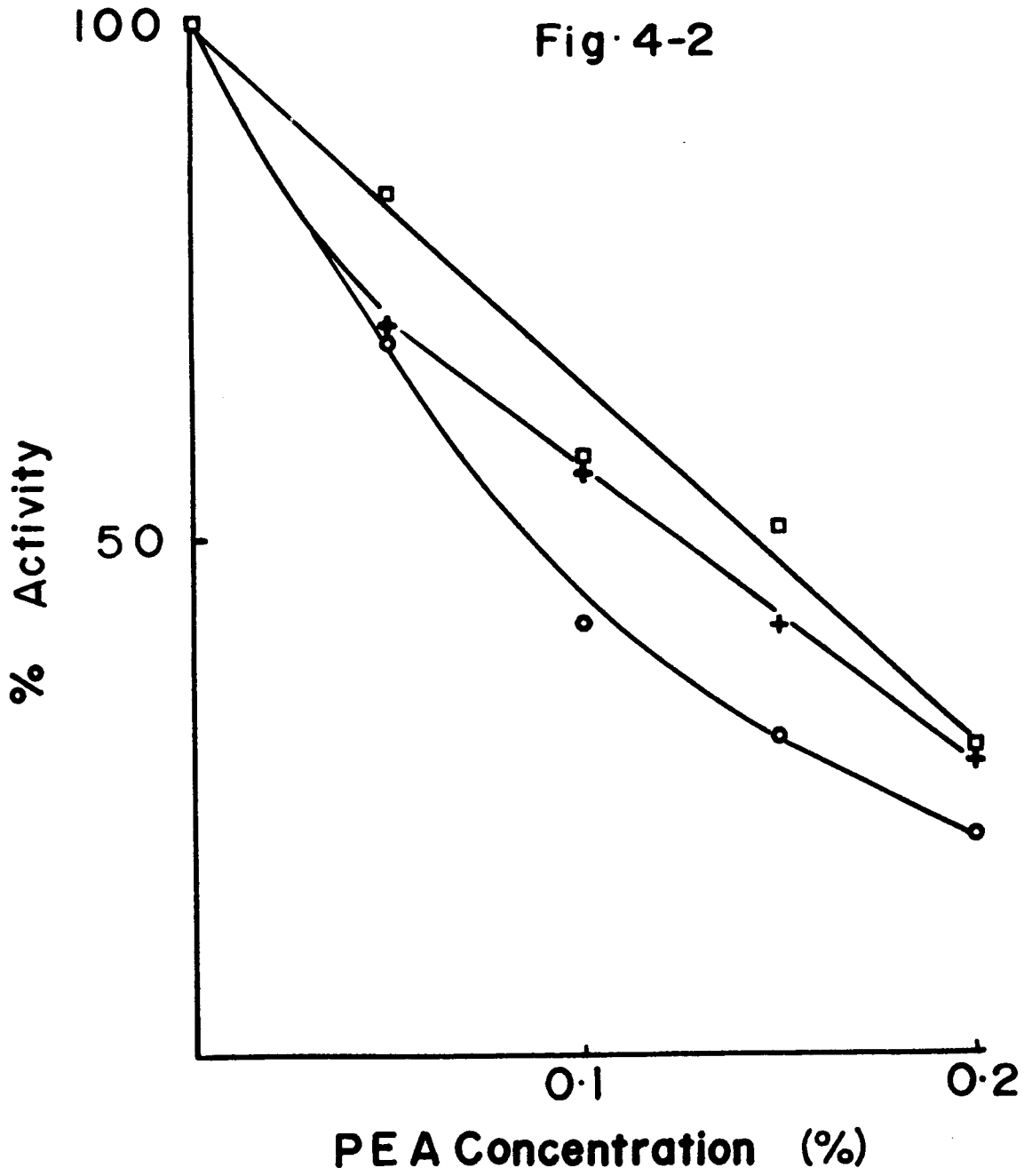


Fig. 4-2 : A plot of the percent of initial activity remaining in the presence of various amounts of PEA for different acyl-CoAs as measured by the incorporation of labeled sn-glycerol-3-phosphate. The reaction mixtures and conditions for incubation were described in Fig. 4-1 and in the Materials and Methods section except that the concentration of PEA varied in the presence of 50 nC_i labeled material and 100 µg of enzymes. The reaction was incubated at 30°C for 12 minutes. □——□——□ represents palmityl-CoA, +——+——+ palmitoleyl-CoA and ○——○——○ vaccenyl-CoA present in the reaction mixtures.



Fig·4-2

Fig. 4-3a: A Lineweaver-Burk plot of the reciprocal of the initial velocity versus the millimolar concentration of palmityl-CoA at a constant concentration of sn-glycerol-3-phosphate. The reaction mixture contains Tris-HCl buffer pH 8.5 0.1 M; Mg^{++}, 6.25 mM; BSA 76 µg; sn-glycerol-3-phosphate, 97 µM (50 nC_i) and enzyme 80 µg. The acyl-CoA concentrations were 75 µM, 37.5 µM, 25 µM and 17.5 µM. The final volume was 0.2 ml. The reaction was incubated at 30°C for 12 minutes. Fifty µl of reaction mixture was counted. □——□——□ represents the presence of PEA 0.15% (v/v) and ○——○——○ the absence of PEA in the reaction mixtures.

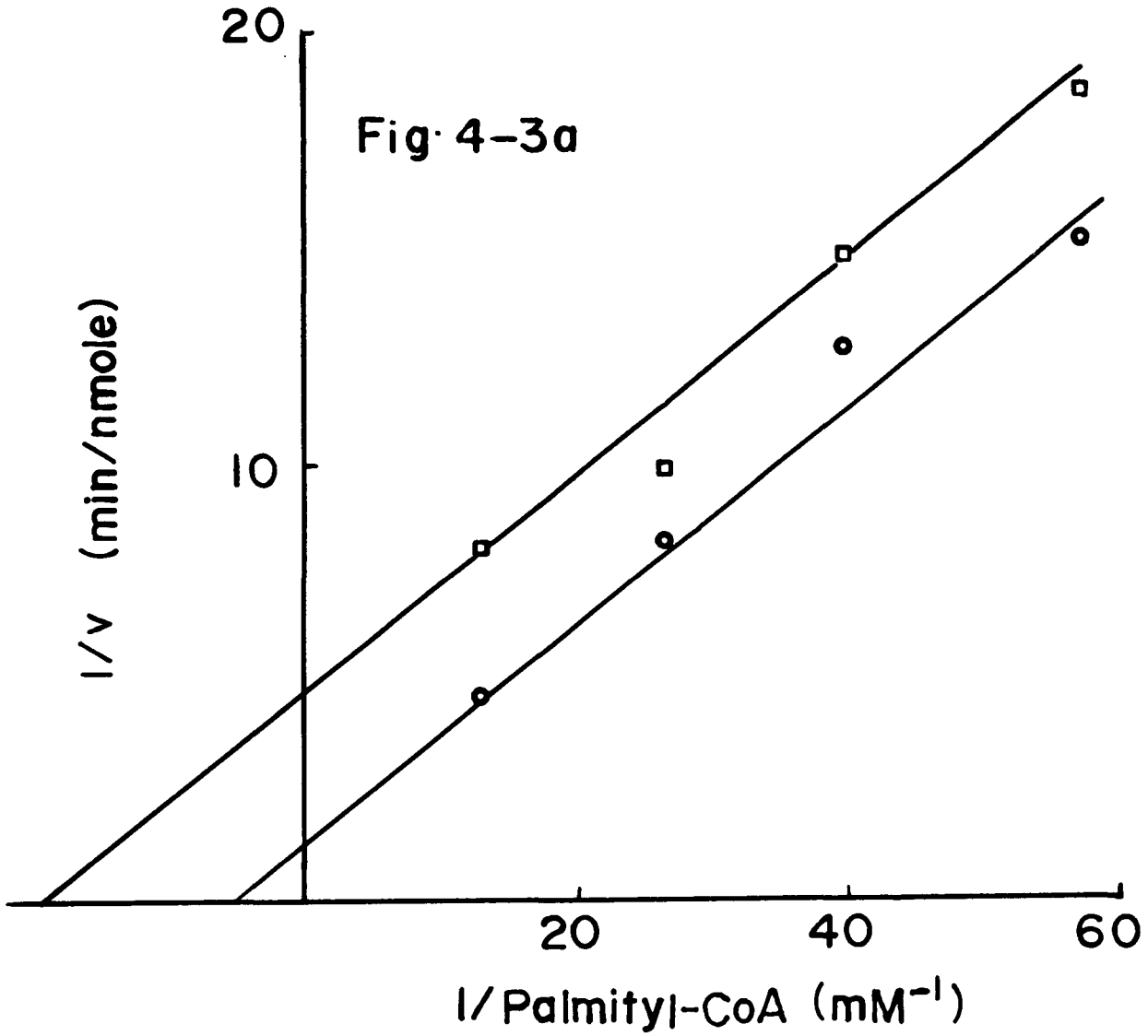
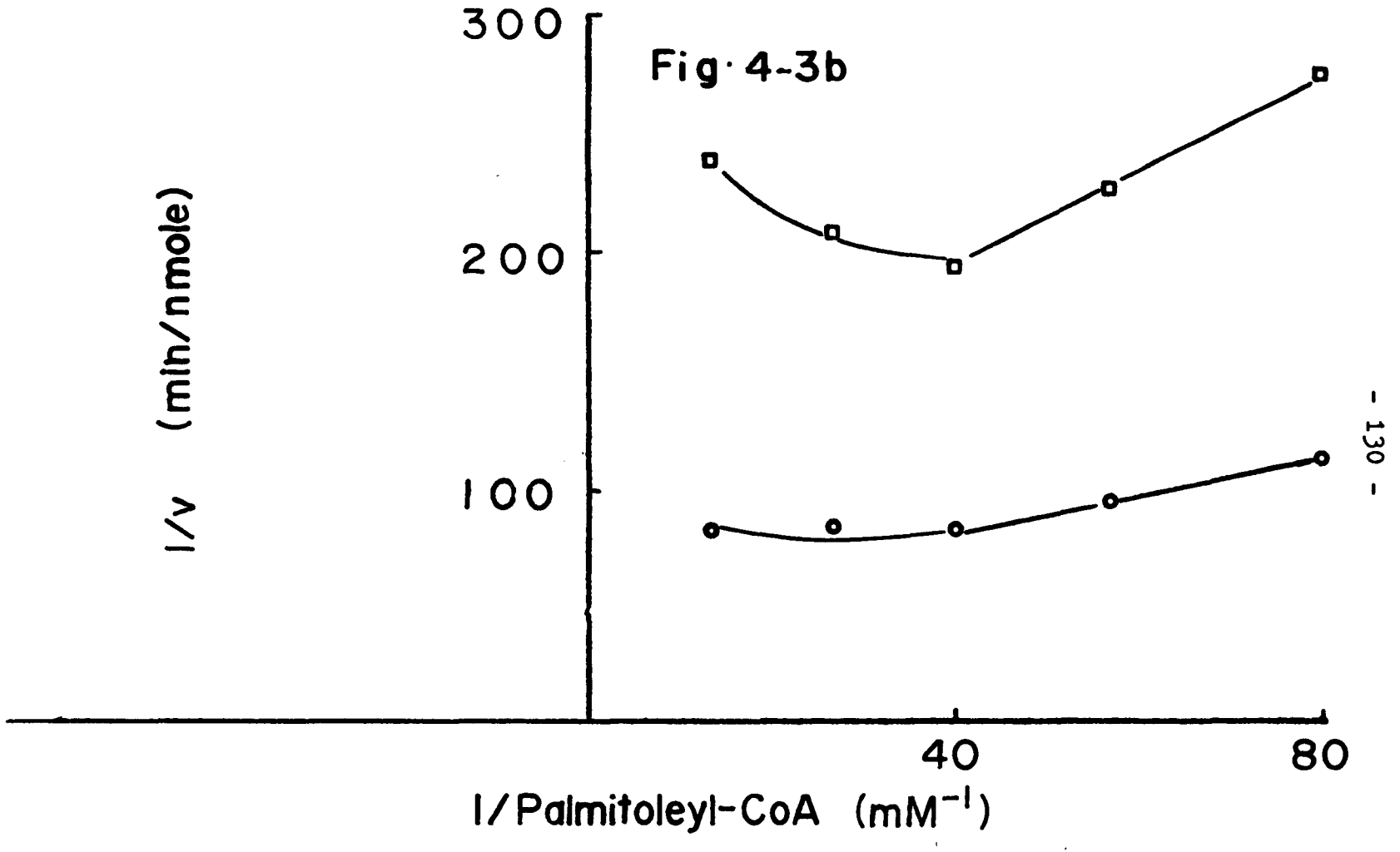


Fig. 4-3a

Fig. 4-3b : The Lineweaver-Burk plot of the reciprocal of the initial velocity versus the millimolar concentration of palmitoleyl-CoA at a constant concentration of sn-glycerol-3-phosphate. The reaction mixture contains Tris-HCl buffer pH 8.5 0.1 M; Mg^{++}, 6.25 mM; BSA 76 µg; sn-glycerol-3-phosphate, 97 µM (50 nC_i) and enzyme 80 µg. The acyl-CoA concentrations were 75 µM. 37.5 µM. 25 µM, 17.5 µM and 12.5 µM. The final volume was 0.2 ml. The reaction was incubated at 30°C for 12 minutes. Fifty µl of reaction mixture was counted. ◘——◘——◘ represents the presence of PEA 0.15% (v/v) and o——o——● the absence of PEA in the reaction mixtures.



Fig·4-3b

Fig. 4-3c: A Lineweaver-Burk plot of the reciprocal of the initial velocity versus the millimolar concentration of vaccenoyl-CoA at a constant concentration of sn-glycerol-3-phosphate. The reaction mixture contains Tris-HCl buffer pH 8.5 0.1 M; Mg^{++} 6.25 mM; BSA 76 µg; sn-glycerol-3-phosphate, 97 µM ($50nC_i$) and enzyme 80 µg. The acyl-CoA concentrations were 75 µM, 37.5 µM, 25 µM 17.5 µM and 12.5 µM. The final volumn was 0.2 ml. The reaction was incubated at 30°C for 12 minutes. Fifty µl of reaction mixture was counted. □——□——□ represents the presence of PEA 0.15% (v/v) and ○——○——○ the absence of PEA in the reaction mixture.

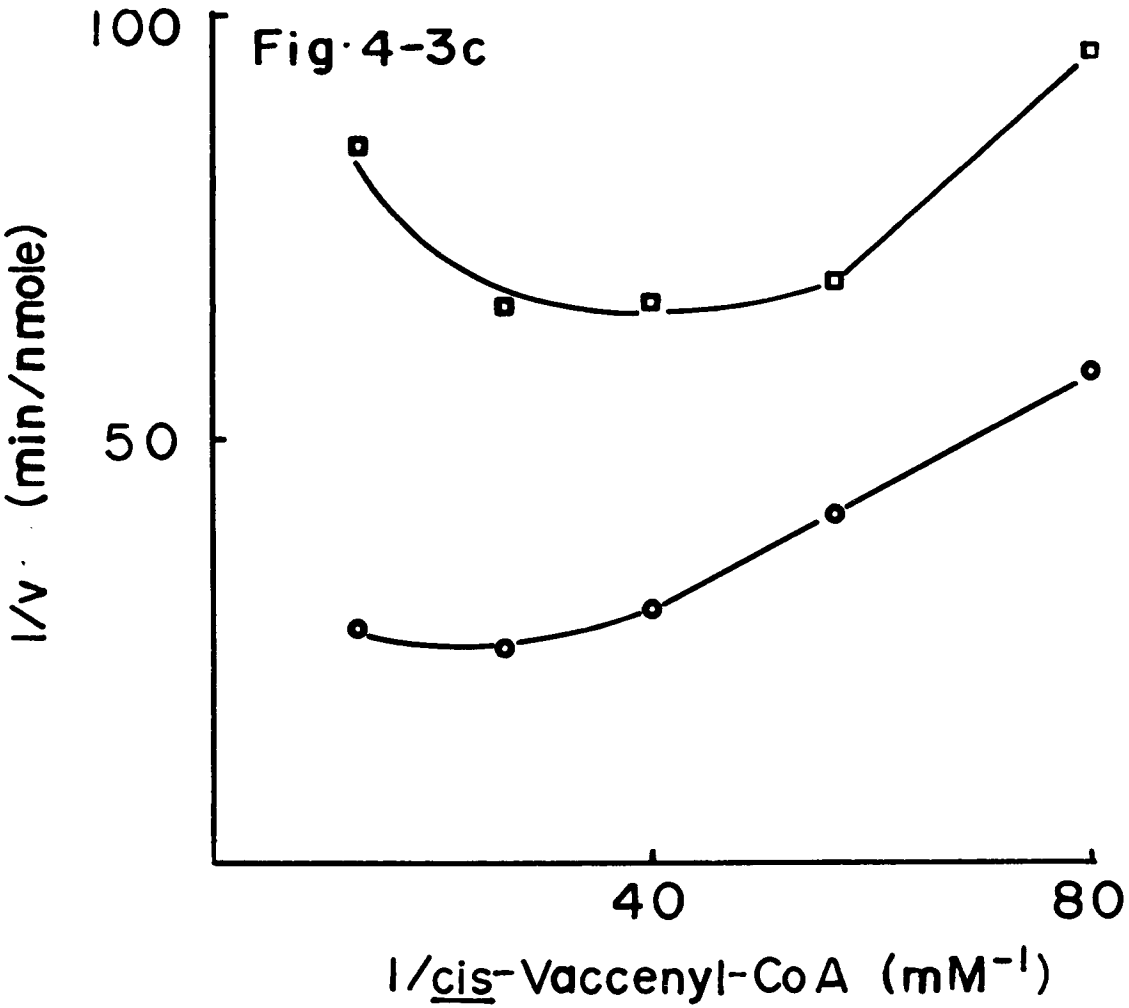
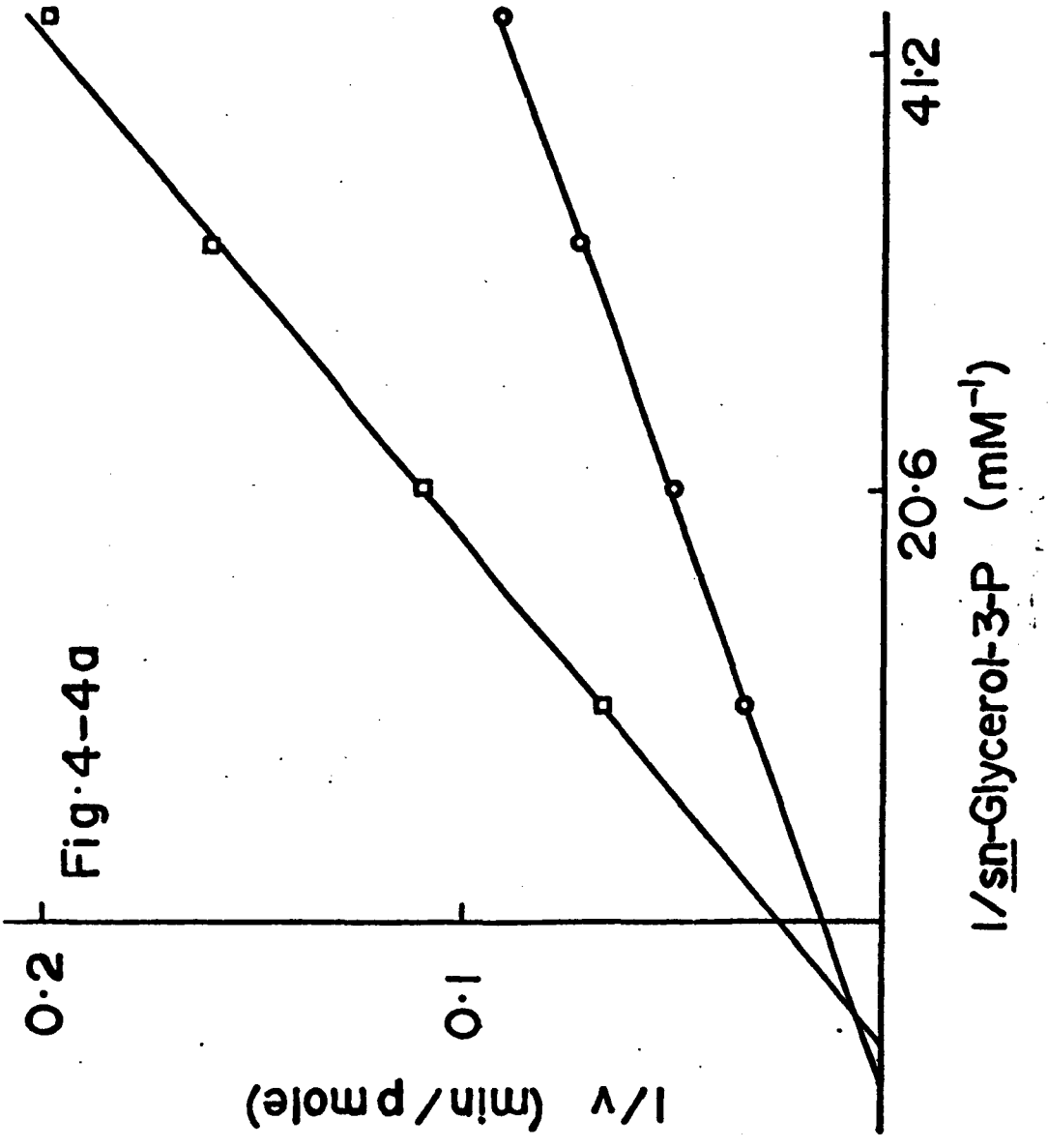

Fig·4-3c
100
50
1/v · (min/nmole)
40
80
1/cis-Vaccenyl-CoA (mM⁻¹)

Fig. 4-4a : A Lineweaver-Burk plot of the reciprocal of the initial velocity versus the millimolar concentration of sn-glycerol-3-phosphate at a constant concentration, 75 μM, of palmityl-CoA and enzyme 55 μg. The concentration of sn-glycerol-3-phosphate (2.85 mC_i/m mol) were 97.0 μM, 48.5 μM, 31.0μM and 23.3μM. ▫——▫——▫ represents in the presence of PEA 0.15% (v/v) and ○——○——○ in the absence of PEA in the reaction mixtures. The reaction was incubated at 30°C for 12 minutes.



Fig·4-4a

Fig. 4-4b : A Lineweaver-Burk plot of the reciprocal of the initial velocity versus the millimolar concentration of sn-glycerol-3-phosphate at a constant concentration, 75 μM, of palmitoleyl-CoA and enzyme 55 μg. The concentration of sn-glycerol-3-phosphate (2.85 mC_i/mmole) were 29.1 μM, 14.6 μM, 9.8 μM and 7.3 μM. □——□——□ represents in the presence of PEA 0.15% (v/v) and o——o——● in the absence of PEA in the reaction mixtures. The reaction was incubated at 30°C for 12 minutes.

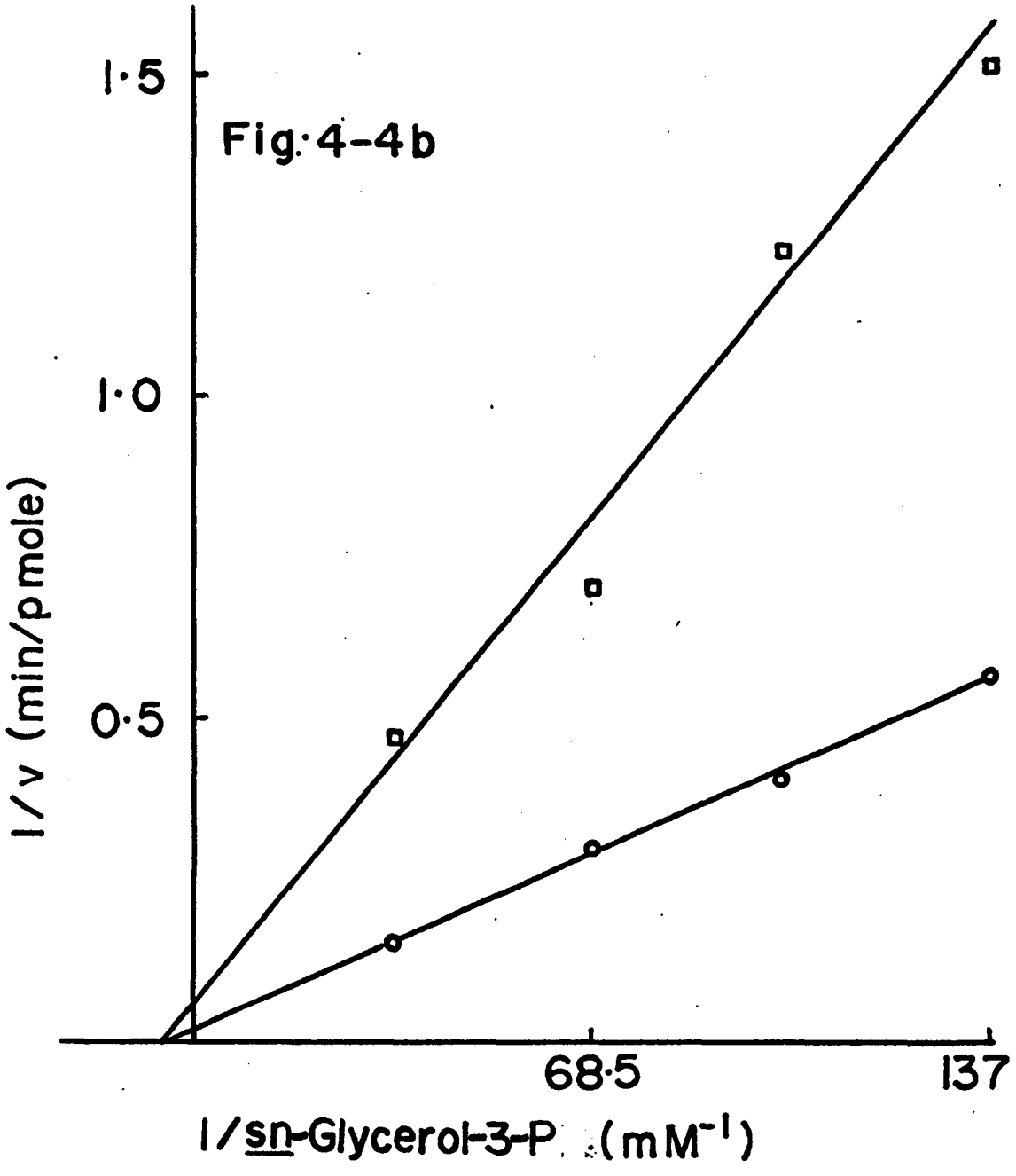


Fig. 4-4b

Fig. 4-4c : A Lineweaver-Burk plot of the reciprocal of the initial velocity versus the millimolar concentration of sn-glycerol-3-phosphate at a constant concentration, 75 μM, of vaccenyl-CoA and enzyme 55 μg. The concentration of sn-glycerol-3-phosphate (2.85 mC_i/ m mol) were 97.0 μM, 48.5 μM, 31.0μM and 23.3μM. □——□——□ represents in the presence of PEA 0.15% (v/v) and ○——○——○ in the absence of PEA in the reaction mixtures. The reaction was incubated at 30°C for 12 minutes.

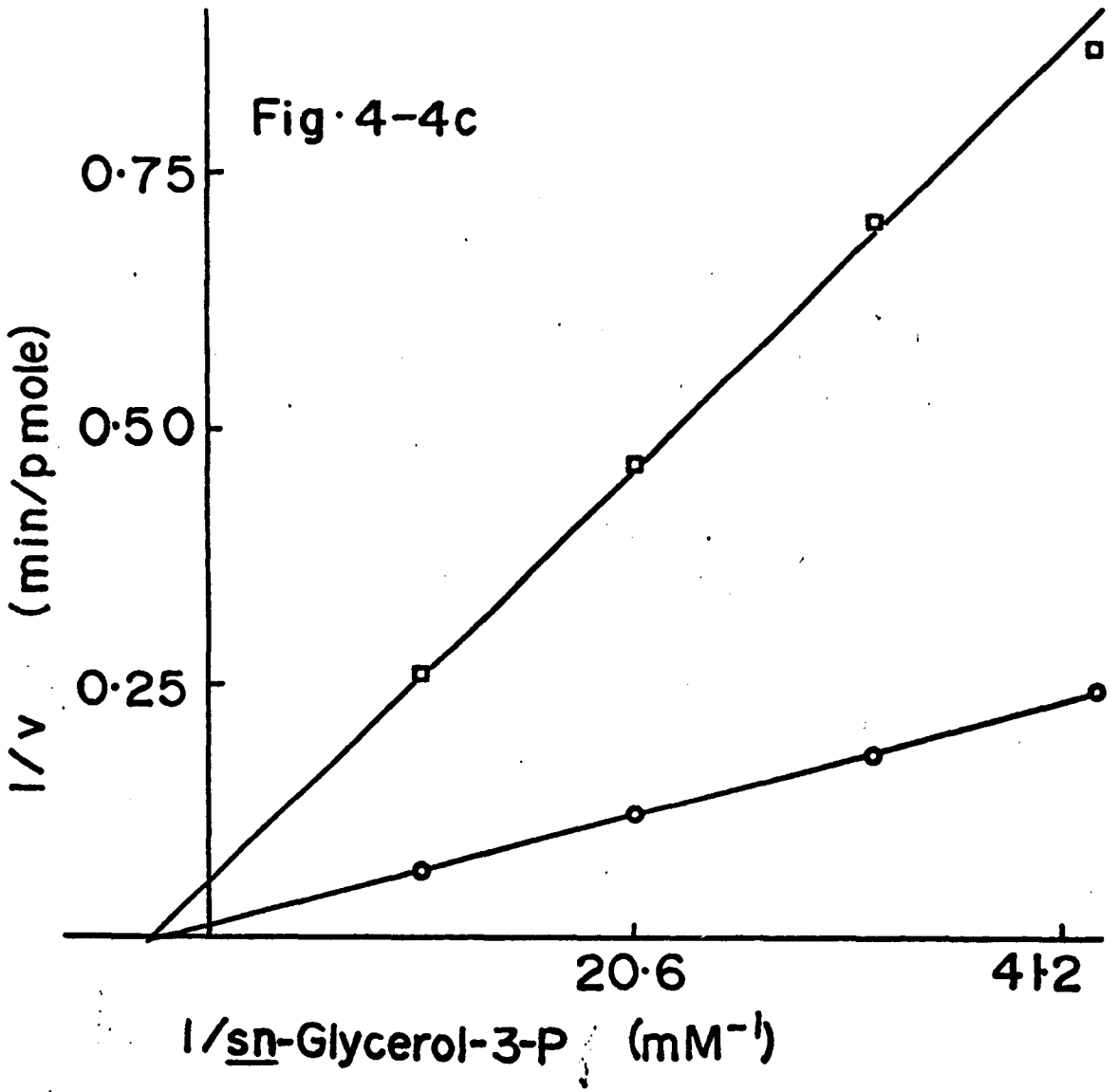
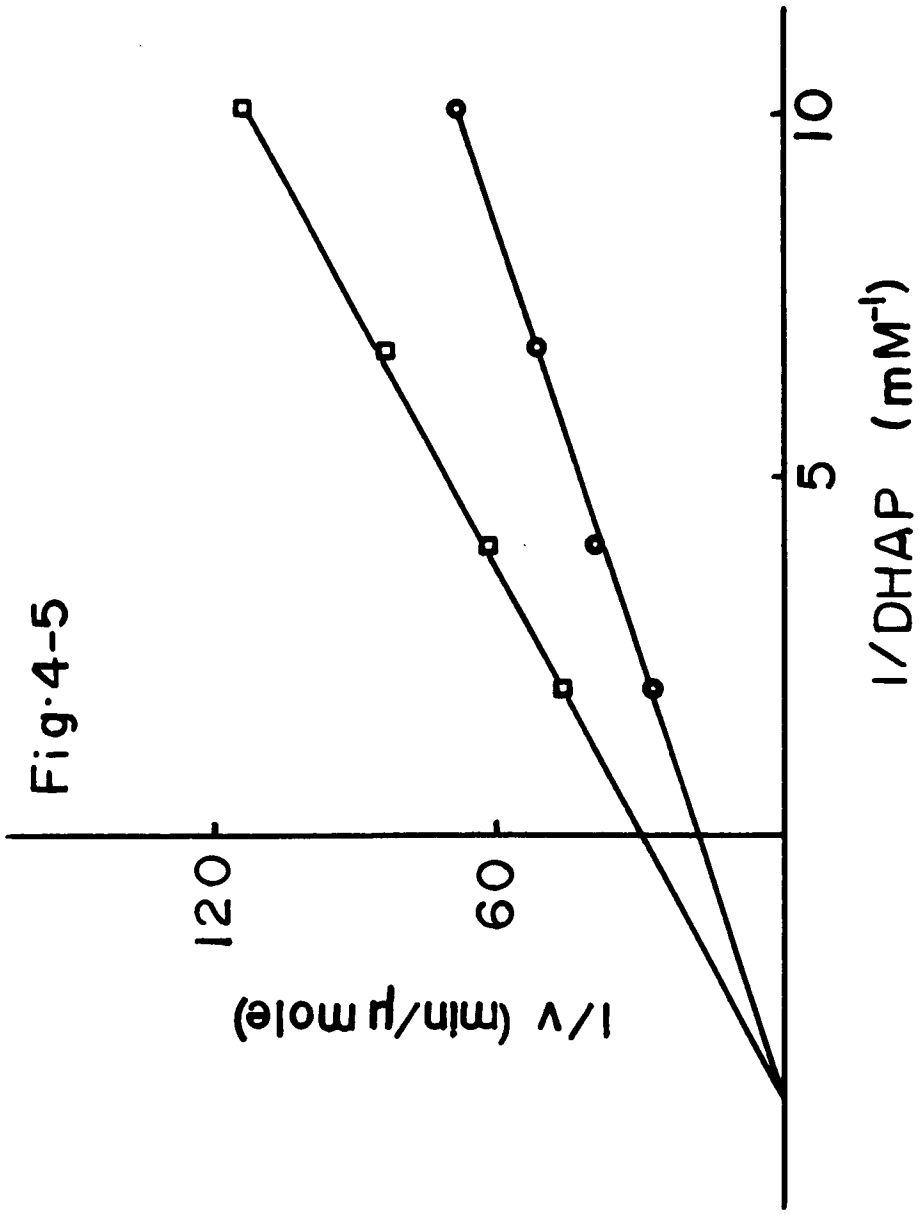
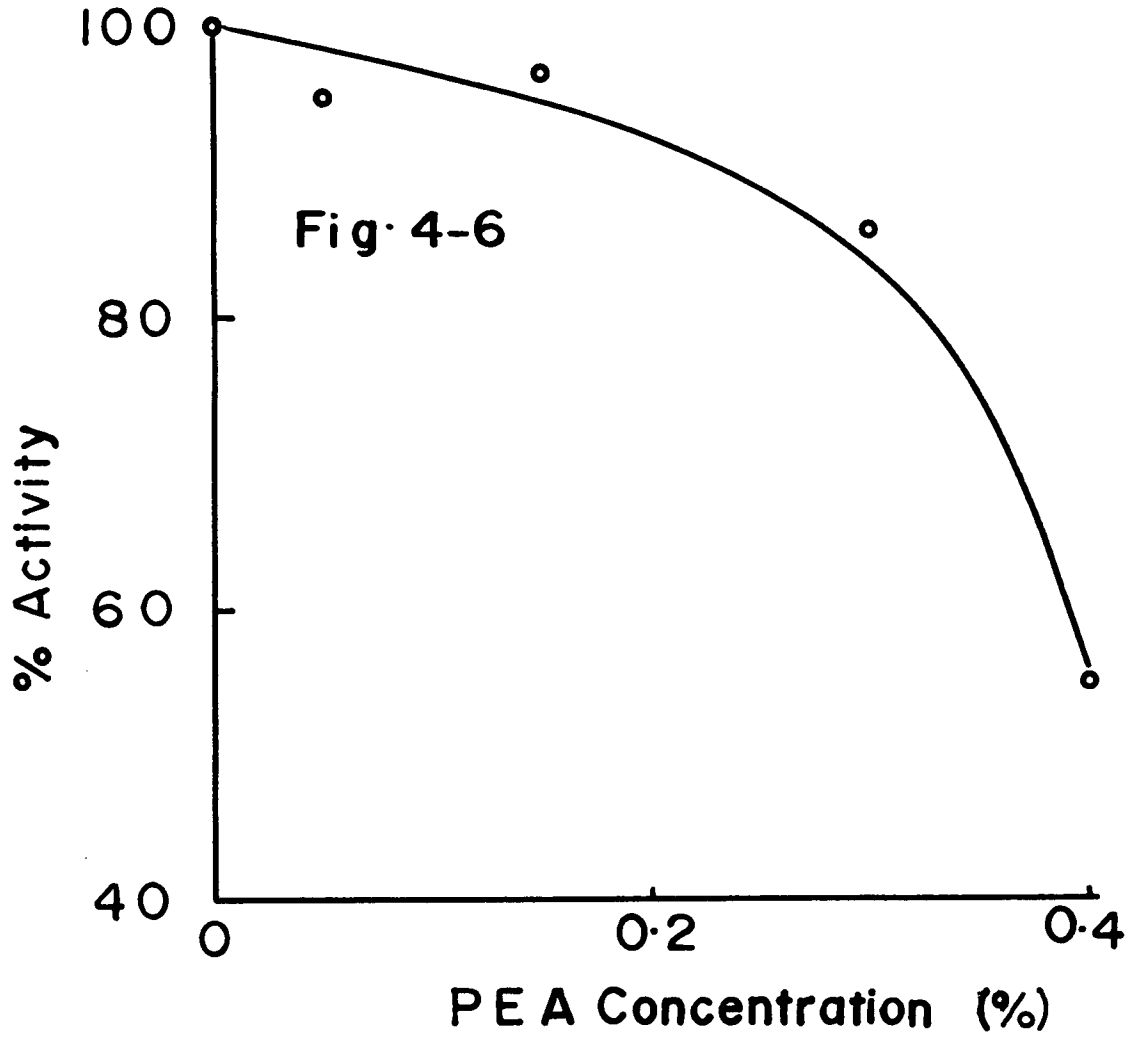

Fig·4-4c
0·75
0·50
0·25
1/v (min/p mole)
20·6
41·2
1/sn-Glycerol-3-P (mM⁻¹)

Fig. 4-5 : A Lineweaver-Burk plot of the initial velocity versus the millimolar concentration of dihydroxyacetone phosphate at a constant concentration of NADPH, 200 μM for the anabolic glycerol-3-phosphate dehydrogenase. The enzyme concentration was 170 μg in a total volume of 1 ml. The initial velocity, v, is the decrease in μ moles per minute of NADPH. ●——●——○ no addition, ▫——▫——▫ 0.15% PEA added.



Fig·4-5

Fig. 4-6 : A plot of the percent of initial activity remaining in the presence of various amount of PEA for catabolic sn-glycerol-3-phosphate dehydrogenase. The reaction mixture contains 100 μmoles potassium phosphate buffer pH 7.5; 33 μg MTT; 200 μg PMS; 6 μmoles KCN; 20 mM of DL-glycerol-3-phosphate and 16 μg of partially purified enzyme in a total of 1 ml. The extinction coefficient of reduced MTT was taken to be 17 $mM^{-1}cm^{-1}$ at 570 nm. The spectrophotometric assays were performed at 30°C and initiated by the addition of enzyme from E. coli strain 7.



Fig· 4-6

Table 4-1

Effects of PEA on the distribution of labeled glycerophosphate into the phosphatidic acids of E. coli strain 8 after 16 minutes of incubation. Detail of assay has described in Material and Methods section (Unit:CPm)

Fraction / Acyl CoA	Rf / PEA	i Origin 0--0.08	ii Lysophophatidic Acid 0.08--0.44	iii Phosphatidic Acid 0.44--0.90	iv Solvent Front 0.90--1.0	Patio of Phosphatidic Acid to Lyso-phosphatidic Acid
Palmityl-CoA	-	532	3568	8705	293	2.4
	+	323	3056	4444	250	1.4
Palmitoleyl-CoA	-	80	326	2527	31	4.7
	+	55	180	765	4	4.2
Vaccenyl-CoA	-	166	548	5645	259	10.0
	+	59	248	1756	58	7.1

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