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METABOLISM OF L-GLYCERALDEHYDE 3-PHOSPHATE IN ESCHERICHIA COLI

City University of New York

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METABOLISM OF L-GLYCERALDEHYDE 3-PHOSPHATE IN *ESCHERICHIA COLI*.

by

METARAMBA K.G.S. KALYANANDA

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.

1985

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

April 30, 1985
Date

Burton E. Tropp
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April 19, 1985
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Abstract

METABOLISM OF L-GLYCERALDEHYDE 3-PHOSPHATE IN *ESCHERICHIA COLI*.

by

Metaramba K.G.S. Kalyananda

Adviser: Professor Burton E. Tropp

E. oli is able to incorporate L-glyceraldehyde and L-glyceraldehyde 3-phosphate into phospholipids. L-[3-^{3}H]Glyceraldehyde was synthesized and the purity and the chemical identity of the product were checked by paper chromatography. L-[3-^{3}H]Glyceraldehyde 3-phosphate was synthesized from L-[3-^{3}H]glyceraldehyde in a reaction catalyzed by glycerokinase. *E. coli* extract contains a new enzyme activity which catalyzes an NADPH dependent reduction of L-glyceraldehyde 3-phosphate into *sn*-glycerol 3-phosphate. This enzyme, L-GAP reductase, was purified approximately 110 fold by ammonium sulfate precipitation followed by DEAE-Sephadex A-50 column chromatography. A procedure, specifically suitable for assaying the reductase activity in the crude extract, was developed. A more convenient spectrophotometric assay method was employed for the purified enzyme. The purified enzyme is free of NADPH dependent *sn*-glycerol 3-phosphate synthase, and contained only a trace of triosephosphate isomerase activity. Neither D-glyceraldehyde 3-phosphate nor dihydroxyacetone phosphate could act as a substrate.

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However when present in ten times the concentration of the normal substrate, L-glyceraldehyde 3-phosphate, both these compounds inhibited the enzyme activity by about 50%. The apparent K_M values for L-glyceraldehyde 3-phosphate and NADPH are 28 and 35 μM respectively. A glyceraldehyde 3-phosphate analog, DL-3-hydroxy-4-oxobutyl-1-phosphonate was also found to be a substrate with an apparent K_M of 280 μM. Three other carbonyl compounds, methylglyoxal, L-glyceraldehyde and D-glyceraldehyde are very poor substrates with apparent K_M values of 14, 28 and 100 mM respectively. At moderate concentrations sulfhydryl group inhibitors had no effect on the enzyme activity. At 100 μM concentration Zn^{+2} inhibited the enzyme activity by about 30% while Mn^{+2} elevated the activity by about the same margin. Mg^{+2}, Ca^{+2} and Fe^{+2} were without effect at this concentration. L-Glyceraldehyde 3-phosphate is known to be bactericidal at 1.25 mM concentration and the D-enantiomer is without effect. Furthermore, methylglyoxal is known to be bactericidal at or above 0.5 mM concentration. Strains of E. coli resistant to 1 mM methylglyoxal were isolated. The cell extract prepared from the mutant possessed increased capacity to transform methylglyoxal into D-lactate via a glutathione dependent reaction. These mutants were less sensitive to to 2.5 mM DL-GAP suggesting that conversion of L-glyceraldehyde 3-phosphate into methylglyoxal may at least partly be responsible for the bactericidal activity of L-GAP.

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To my parents

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1.Introduction

1.1. L-Glyceraldehyde 3-phosphate, a bactericidal agent.

Metabolism of D-glyceraldehyde 3-phosphate (D-GAP) in bacteria has been well documented (Wood, 1966 ; Fraenkel & Vinopal 1973 ; Axelrod, 1967). As a glycolytic intermediate this compound plays an important role in passing structural carbon and chemical energy from fructose 1,6-diphosphate to phosphoglyceric acid 3-phosphate. Similarly, during gluconeogenesis, carbon and energy are channeled through D-GAP in the reverse direction. As shown in Fig.1, D-GAP can also serve as a precursor for glycerol 3-phosphate (G3P) in lipid biosynthesis. Furthermore, this compound is also implicated in the pentose phosphate pathway which is mainly responsible for the biosynthesis of five carbon and seven carbon sugars found as structural units of nucleic acids and lipopolysaccarides, respectively.

L-Glyceraldehyde 3-phosphate (L-GAP) on the other hand is not a normal metabolite in living organisms and the available evidence (Tang et al, 1977) places this compound among antimetabolites. Tang et al (1977) discovered that 2.5 mM DL-GAP is bactericidal to E. coli. Based upon several lines of evidence, they concluded that the L-isomer of the racemic mixture is the active form. Present studies (Sec.3) revealed that L-GAP can serve as a

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precursor for phospholipids in E. coli. In this context, the present studies were undertaken to elucidate the pathway(s) that lead to biosynthesis of phospholipids from L-GAP and also to investigate the mechanism by which L-GAP inhibits cell growth.

Antimetabolic activity of L-glyceraldehyde was first reported by Mendel in 1929. He discovered that 1mM DL-glyceraldehyde almost completely inhibits the anaerobic formation of lactic acid from glucose in rat sarcoma. Since then inhibition of glycolysis by DL-glyceraldehyde has been reported in brain (Holmes 1937; Asford 1934), chicken embryo (Nowinski, 1939) and other tissues (Needham, 1937). Research carried out in the next two decades successfully established the site of action of L-glyceraldehyde as hexokinase (Stickland, 1941; Rudney, 1949). Lardy et al (Lardy et al, 1950) reported that L-sorbose 1-phosphate, formed as a result of fructose 1,6-diphosphate aldolase catalyzed condensation of L-glyceraldehyde with cellular dihydroxyacetone phosphate, is the true inhibitor of hexokinase. Fenselau and Long (Fenselau & Long, 1976) have reported that phosphorylated sugars such as DL-GAP, D-erythrose 4-P, and D-ribose 5-P are more potent growth inhibitors (all at less than 1 mM) of 3T3 mouse fibroblast than their corresponding aldehydes. There is an apparent discrepancy between these data and the Lardy's hypothesis. However the

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possibility exists that the potency difference could be a result of some preferential transport of phosphorylated products into the 3T3 cells.

Observations made on the effect of L-GAP on bacterial growth (Tang et al, 1977), cannot be explained by the mechanism proposed by the Lardy et al., for animal tissue preparations. There are at least three lines of evidence which stand against the existence of a sorbose-1-phosphate mediated mechanism in bacteria. Primarily, Tang et al demonstrated that the phosphorylation is a prerequisite for the bactericidal activity of L-glyceraldehyde in E. coli. Growth of strain 8, a derivative of E. coli K-10, was inhibited by 2.5 mM L-glyceraldehyde, while strain 9 (glycerokinase negative, but otherwise isogenic to strain 8) continued to grow under those conditions. Consequently, the aldolase catalysed condensation product between the antimetabolite (L-GAP) and dihydroxyacetone phosphate would be L-sorbose 1,6-diphosphate and not L-sorbose 1-phosphate. However, the subsequent dephosphorylation of L-sorbose-1,6-diphosphate to yield the monophosphate is not precluded by the above arguments. Nonetheless this mode of action is impossible for two additional reasons; (i). L-Glyceraldehyde 3-phosphate blocks the growth of E. coli mutants that have a temperature sensitive FDP-aldolase under non-permissive temperatures, precluding the

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formation of L-sorbose 1,6-diphosphate and (ii) The carbon source used in the bacterial growth experiments was succinate and not glucose making hexokinase a highly improbable primary target (Tang et al 1977).

Bacterial glycerokinase, which will be discussed in detail elsewhere, can recognize and phosphorylate L-glyceraldehyde (Hayashi & Lin 1967). It is conceivable that in E. coli, L-GAP is an important intermediate in the metabolism of L-glyceraldehyde. In aqueous solutions, GAP exists as an equilibrium mixture of a hydrated form (a geminal diol) and the free aldehyde in a molar ratio of 29:1 (Trentham, 1969). The geminal diol form is structurally similar to G3P. The only difference between the two is that the former has a hydroxyl group on carbon 1 in place of the hydrogen found in the corresponding position of the latter. The striking similarity between the two compounds led Tang et al (Tang et al, 1977) to evaluate the action of DL-GAP on several enzymes involved in G3P metabolism in E. coli. The results of these studies will be reviewed in relation to the glycerol 3-phosphate metabolism.

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1.2. L-Glyceraldehyde 3-phosphate and enzymes of G3P metabolism.

Glycerol 3-phosphate metabolism in E. coli has been reviewed in detail by Lin (Lin, 1976). Fig.2 depicts the metabolic pathways responsible for G3P dissimilation in E. coli. L-G3P can be taken up directly from the growth medium via an active transport system (Hayashi et al, 1964). The protein involved in the transport process, G3P permease, is coded by glpT locus, and mutants defective in this transport system have been isolated (Hayashi et al, 1964 ; Hayashi & Lin 1965). Glycerol 3-phosphate permease has been identified as a single gene product (Ludtke et al, 1982). Beside G3P permease two other transport systems in E. coli can recognize G3P. They are the transport activity conferred by ugp$^+$(Argast et al, 1978 ; Argast and Boos 1979) (ugp$^+$ is the derepressed state of the wild type ugp^o) and the hexosephosphate transport system determined by the uhp locus (Guth et al, 1980). The former (ugp$^+$) is dependent on the pho regulon (Schweizer et al, 1982 ; Schweizer & Boos, 1983) and constitutively synthesized in strains derepressed for alkaline phosphatase (Argast & Boos, 1980). The latter is specifically induced by extracellular glucose 6-phosphate (Kadner & Eidens, 1983). Strains constitutive for the hexose phosphate transport system have been employed in G3P transport studies (Guth et al, 1980). Despite the fact that ugp$^+$

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alone can channel G3P into the cell efficiently, the presence of active G3P permease is essential for the cell to utilize glycerol or G3P as the sole source of carbon and energy (Schweizeret al, 1982). One explanation provided for this difference is that G3P permease, being able to recognize and transport orthophosphate in and out of the cell, is capable of handling excess inorganic phosphate, produced during G3P metabolism, which would otherwise be toxic. The ugp transport system does not recognize P_i and has a narrow specificity. Both uhp (Guth et al, 1980) and G3P permease (Tang et al, 1977) possess relatively broader specificities and they recognize and transport L-GAP into the cell. Substances toxic to the cell such as arsenate (Bennett, R.L., 1973), 3,4-dihydroxybutyl- 1-phosphonate (DBP) (Shopsis, et al,1972, & 1974), sn-glycerol 3-phosphorothioate (Hammelburger, & Orr, 1983) and fosfomycin (Hendlin, et al, 1969) are also substrates for G3P permease.

Glycerol enters the cell via facilitated diffusion (Britten & McClure, 1962; Richey & Lin, 1972). Subsequent phosphorylation of this compound is responsible for its accumulation in the cell. The phosphorylation takes place at the expense of ATP and is catalyzed by glycerokinase. Magnesium is required for the activity and the K_M for Mg.ATP has two values one in the range of 80-100 µM and the other in the range of 400-500 µM. The enzyme is

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inhibited by fructose 1,6-diphosphate with a K_i of 0.5 mM (Thorner, & Paulus, 1973). The K_M for glycerol is 10 μM and a value of 1.2 μM has been obtained under slightly different assay conditions (Hayashi & Lin, 1965 ; Hayashi & Lin, 1967). The enzyme can also recognize and phosphorylate both L-glyceraldehyde and dihydroxyacetone (Hayashi & Lin, 1967) with K_M values 0.5 mM and 3 mM respectively. D-glyceraldehyde, on the other hand when presented to this enzyme, undergoes abortive phosphorylation, in which the net result is the hydrolysis of ATP into ADP and P_i . No D-GAP was detected among the products of such a reaction mixture (Hayashi & Lin, 1967). Improper orientation of the D-isomer in the active site was considered as a possible explanation for the above difference. One of the two G3P dehydrogenases, aerobic G3P dehydrogenase or anaerobic G3P dehydrogenase catalyzes the subsequent oxidation of G3P (Kistler & Lin 1971). Membrane bound aerobic G3P dehydrogenase is required for the aerobic oxidation of G3P and mutants lacking this enzyme cannot utilize either glycerol or G3P as carbon source. Furthermore, their growth on casein hydrolysate or succinate is inhibited by either compound (Koch et al 1964). However, this enzyme activity is not important for DL-GAP mediated inhibition. Addition of 2.5 mM DL-GAP inhibited the growth of both wild type (strain 7) and aerobic G3P dehydrogenase negative (strain 8)

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strains (Tang et al, 1977). The anaerobic enzyme has a slower sedimentation rate on sucrose gradient (Kistler et al, 1969) and requires added flavins for in vitro activity (Lin, 1976). Mutants missing this enzyme do not grow anaerobically on glycerol or G3P with fumarate as the electron acceptor. However, if nitrate is the electron acceptor the possession of one of the two dehydrogenases is sufficient for the anaerobic growth on glycerol or G3P.

Both glpF, the locus coding for glycerol facilitator and glpK, the locus coding for glycerokinase map at min 87 on the recalibrated chromosomal map. Evidently they share the same operon (Berman & Lin, 1971 ; Cozzarelli & Lin 1966). Mutations affecting the anaerobic G3P (glpA) and the locus glpT are closely linked (91% co-transduction) at min 47 (Kistler & Lin, 1971) and activities of the two gene products vary cordinately, suggesting that they belong to the same operon (Freedberg & Lin, 1973). However, the independent insertion of the bacteriophage Mu into glpA or glpT has revealed that they indeed belong to two different operons (Miki et al, 1979). The structural gene for glpD located at min 74 shows 98-99% cotransduction with, glpR, the structural gene for repressor protein. The expression of glpF,glpK ; glpT,glpA ; glpD and the recently discovered gene, glpQ, coding for a glycerophosphodiesterase (Larson et al, 1983), are under the repression of the glpR product (Lin, 1976). The

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repression can be relieved by glycerol or G3P. However, G3P is the true inducer for all glp operons and glycerol has the effect of an inducer by virtue of its being the precursor of G3P (Koch et al, 1964 ; Lin et al, 1962). Sensitivity to the repressor varies from operon to operon and glpD shows the highest affinity for the repressor (Freedberg & Lin, 1973). This biased shut down of glpD operon assures rapid accumulation of the inducer, especially when the exogenous glycerol or G3P level is low. Mutants of E. coli with deletions in the repressor region have been isolated and they show constitutive expression of all the glp genes (Cozzarelli et al , 1968 ; Schwartz 1966).

Beside the repressor mediated specific regulation, there are two other controls which modulate the expression of glp operons. They are catabolite repression and differential respiratory repression. The glpF, glpK operon is highly sensitive to catabolite repression and the sensitivity of glpD is an order of magnitude less than that of glpK. The glpA, glpT operon is moderately sensitive to this control. Existence of the third regulatory mechanism became evident when cAMP failed to lift the catabolite repression, of glpA, caused by glucose while that of glpK of the same cells was relieved (Freedberg & Lin, 1973). Recently, using glpA-lac fusion

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strains it has been shown that a protein coded by the locus fnr is a positive regulator of anaerobic G3P dehydrogenase (Kuritzkes, et al, 1984).

Two other enzymes important in dissimilation of G3P are G3P synthase and G3P acyltransferase. G3P synthase also known as the biosynthetic G3P dehydrogenase catalyzes the conversion of dihydroxyacetone phosphate (DHAP) into G3P. The enzyme can utilize either NADH, NADPH or nicotinamidehypoxanthine dinucleotide as the coenzyme (Edgar & Bell, 1980) and it catalyzes the reverse reaction if the intracellular G3P becomes excessively high. The enzyme is subjected to feedback inhibition by G3P and is also sensitive to inhibition by palmitoyl-coenzyme A (Edgar & Bell, 1979). A feedback resistant enzyme has been purified from strain 8 derivative BB-26-36 R2 (Edgar & Bell, 1978) and characterized in comparison to the wild type-enzyme (Edgar & Bell, 1980). The K_M for NADPH, DHAP and G3P are 10 μM, 170 μM and 210 μM, respectively (Kito & Pizer, 1968 ; Kito & Pizer, 1969 ; Spector & Pizer 1975). In the absence of exogenous glycerol or G3P , the enzyme activity is indispensable for the growth of E. coli. Mutants, deficient in G3P synthase, are auxotrophic for glycerol or G3P and the mutations (gpsA) map at 80 min. The enzyme is also susceptible to the G3P analog, 3,4-dihydroxybutyl 1-phosphonate (Cheng et al, 1975). However this enzyme is not the primary site of

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action of DL-GAP, because a strain deficient in this enzyme activity, CY 115 (Cronan & Bell, 1974), cultured in synthetic medium containing glycerol as the sole source of carbon was also susceptible to DL-GAP (Tang et al, 1977).

1.3. L-Glyceraldehyde 3-phosphate and enzymes of phospholipid biosynthesis.

Phospholipids are the main constituents of the inner and outer membranes of E. coli. Phosphatidylethanolamine(75-85%), phosphatidylglycerol (10-20%) and cardiolipin (5-15%) are the three major classes of phospholipids found in E. coli. The final portion of the pathway that leads to biosynthesis of phospholipids in E.coli is outlined in Fig.3. An excellent review on the enzymology, genetics and regulation of membrane phopholipid synthesis is available (Raetz, 1978). Only the two enzymes which utilize G3P as one of the substrates (Fig.3) will be discussed here. G3P acyltransferase, the first enzyme of the pathway, catalyzes acylation of sn-glycerol 3-phosphate at position one (Snider & Kennedy, 1977). Both acyl-acyl carrier protein and acyl-CoA can serve as the acyl donor for the reaction. When acyl-CoA is the donor, guanosine tetraphosphate (ppGpp) inhibits the enzyme activity

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(Lueking & Goldfine, 1975 ; Ray & Cronan, 1975). One class of mutants of this enzyme, designated by plsB, produces a kinetically different acyltransferase. The apparent K_M for G3P for the mutant enzyme is ten times larger than that for the wild type enzyme (Bell, 1974 ; Cronan & Bell, 1974 ; Pizer et al, 1974). The locus plsB has been identified as the structural gene for G3P acyltransferase (Larson et al, 1980). Consequently, the mutant strain is phenotypically G3P auxotropic. Recently it has been shown that in addition to the plsB, a second mutation, plsX, is also necessary for this phenotype (Larson et al, 1984). The loci plsB and plsX have been mapped near min 92 and min 24 respectively on the E. coli linkage map (Lightner et al, 1980 ; Larson et al, 1984).

DL- Glyceraldehyde 3-phosphate is a competitive inhibitor of G3P acyltransferase and the inhibition was seen when the enzyme was assayed by both a radioactive method (monitoring the incorporation of radioactive G3P into phospholipids) and a spectrophotometric method (monitoring the release of CoA by measuring its chemical interaction with DTNB). D-glyceraldehyde 3-phosphate was without effect on this enzyme (Tang et al, 1977). The apparent K_i for L-GAP is 1.8 mM. Furthermore, inhibition by DL-GAP was seen with both palmitoyl CoA and oleoyl CoA as substrates. The spectrophotometric assay revealed that DL-GAP is not a

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substrate for the acyltransferase. The competitive nature of the inhibition indicates that L-GAP is a substrate analog of L-G3P for the acyltransferase. Therefore, if G3P acyltransferase is the primary site of action of L-GAP, it is quite likely that the K_M mutants of this enzyme are resistant to L-GAP. However, the K_M mutant BB26-36 R2 (Bell & Cronan, 1975) grown in the presence of 0.4 mM sn -G3P was inhibited by 7.1 mM DL-GAP by more than 60% and caused cell death. The second enzyme in the pathway, acyl CoA:lysophosphatidate acyltransferase, is not sensitive to DL-GAP.

CDP-diglyceride:sn-glycerol 3-phosphate phosphatidyltransferase catalyzes the synthesis of phosphatidylglycerophosphate (Chang & Kennedy, 1967). DL-GAP is a competitive inhibitor of this enzyme (Tang et al, 1977) and D-GAP does not inhibit the activity. The apparent K_i for L-GAP is 2.7 mM. When E. coli strain 8 was treated with DL-GAP there was no differential inhibition of the accumulation of the two major classes of phospholipids, phosphatidylethanolamine and phosphatidylglycerol. Accumulation of cardiolipin was not noticeably affected.

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1.4. DL-Glyceraldehyde 3-phosphate and methylglyoxal metabolism.

Metabolism of methylglyoxal in microorganisms has recently been reviewed (Cooper, 1984). Uncontrolled catabolism of DHAP generating metabolites can lead to synthesis of the lethal product methylglyoxal. One classic example is unregulated catabolism of glycerol or G3P in E. coli. Glycerol dissimilation in E. coli is tightly regulated at the phosphorylation step by three interrelated mechanisms. They are FDP mediated inhibition of glycerokinase (Zwaig & Lin, 1966), self-catabolite repression of glycerokinase by glycerol (Koch et al, 1964) and repressor (glpR product) mediated specific repression of glycerokinase (Koch et al, 1964 ; Lin et al, 1962). When the cells are in a glycerol rich environment, excessive phosphorylation of glycerol is surmounted by the non-competitive inhibition of glycerokinase by the increasing intracellular levels of FDP (Fig.2). The FDP mediated inhibition and the self- catabolite repression together control the rate of production of G3P. G3P in turn modulates the expression of all glp operons through its interaction with the repressor. Should all three controls be lifted, the uncontrolled phosphorylation of glycerol leads to formation of methylglyoxal (Freedberg et al, 1971; Krymkiewicz, 1971). Methylglyoxal production

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under these growth conditions has been seen only in cells possessing a functional aerobic G3P dehydrogenase. Thus DHAP is implicated as an intermediate in the pathway (Fig.1). The formation of methylglyoxal from DHAP is catalyzed by methylglyoxal synthase. The enzyme has been found in a variety of bacteria including E. coli (Hooper & Cooper, 1971), P. Saccharophila (Lin, et al, 1969) and P. vulgaris (Tsai & Gracy, 1976). Apart from this lethal-catabolism of glycerol, many other conditions are known to evoke biosynthesis of methylglyoxal. Triose phosphate isomerase negative strains growing on glucose (Cooper, 1984), FDP-aldolase negative strains growing on glycerol (Cooper, 1975), and cells constitutive in gluconate metabolic enzyme when exposed to gluconate (Rekarte, 1973) are among them. Under all these circumstances methylglyoxal formation is a result of elevation of the intracellular DHAP pool. Methylglyoxal formation during catabolism of amino acids (Elliot, 1959; Green & Lewis 1968; Willetts & Turner, 1970) and acetol (Taylor et al, 1980) have also been reported. Furthermore, reports are also available on nonenzymatic formation of methylglyoxal from glyceraldehyde (Riddle, & Lorenz, 1968) and glyceraldehyde 3-phosphate (Penninkx, et al, 1983). Riddle and Lorenz (Riddle & Lorenz, 1968) reported the formation of methylglyoxal on incubation of L-glyceraldehyde at 40°C with polyvalent anions such as phosphates or TRIS. They

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observed second order kinetics for the methylglyoxal formation from DL-glyceraldehyde. A second order rate constant of $6x10^{-4}$ mM/h was estimated. In the presence of 20 mM P_i, as the DL-glyceraldehyde concentration varied from 0.56 to 11.11 mM the initial rate of formation of methylglyoxal varied from zero to 121 µM/h. For instance, at 1.11 mM DL-glyceraldehyde concentration, a 15 µM/h rate was observed. The nonenzymatic formation of methylglyoxal from GAP was the explanation given by Penninkx et al (Penninkx et al, 1983) for the methylglyoxal production in methylglyoxal synthase negative Saccharomyces cerevisiae . They have reported phosphate or imidazole buffer catalyzed nonenzymatic conversion of DL-GAP into methylglyoxal. Unlike DL-glyceraldehyde, the rate of conversion of DL-GAP is independent of the concentration of the buffer and follows first order kinetics with respect to DL-GAP. At 37° C, and in phosphate buffer (pH 7.0) the rate constant for the conversion is 0.272 h^{-1}.

The primary site of action of methylglyoxal has been identified as protein synthesis. In addition to protein synthesis, initiation, but not elongation of DNA synthesis is also inhibited. (Fraval & McBrien, 1980). It has been suggested that methylglyoxal inhibits protein synthesis through its interaction with the ribosome (Otsuke & Egyud, 1968). Operation of such a mechanism is

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also supported by the fact that methylglyoxal is known to react readily with 7-methyl guanosine (Kozarich & Deegan, 1979) and such modified nucleotides are found in 16S and 23S RNA (Branlant et al, 1981).

E. coli and many other bacteria are equipped with metabolic machinery that could detoxify methylglyoxal by converting it into D-lactate. The enzymes catalyzing this conversion are glyoxalase I and glyoxalase II acting in that order. The glyoxalase enzyme may be responsible for the observed dependence of extent of inhibition on the cell density (Cooper, 1984). In one experiment 0.25 mM methylglyoxal completely inhibited the growth of an E. coli culture containing $3x10^6$ cell/ml while 1 mM concentration was needed for a culture of $3x10^8$ cell/ml (Fraval & McBrien, 1980). Glyoxalase I utilizes glutathione as a cofactor. In a nonenzymatic reaction, glutathione forms a hemimercaptal with methylglyoxal and this adduct is the true substrate for glyoxalase I (Vander et al, 1972). Consecutive action of glyoxalase I and II on the adduct results in D-lactate. Mutants resistant to 1 mM methylglyoxal have been isolated (Cooper, 1975 ; Freedberg et al , 1971 ; Murata et al, 1980) and they show increased levels of glyoxalase I activity with little or no change in the activity of glyoxalase II (Cooper, 1975). The mutants growing in the presence of added methylglyoxal excreted glutathione into the medium and increased

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glyoxalase activity of these mutants is responsible for the excretion (Murata et al, 1980). According to Cooper (Cooper, 1984) the growth of the mutants was only slightly better than that of the wild type, during the initial period of methylglyoxal-treatment. Apparently, cells must detoxify the methylglyoxal , conceivably through the action of glyoxalase system, before they start to show a significant growth rate.

Studies described in this thesis demonstrate incorporation of tritium labeled L-glyceraldehyde and L-glyceraldehyde 3-phosphate into E. coli phospholipids. Evidence for the presence of a new enzyme activity which converts L-glyceraldehyde 3-phosphate into glycerol 3-phosphate is provided. This activity offers an explanation for the incorporation of the tritium labeled compounds into phospholipids. Purification and characterization of this new enzyme activity are described. The bactericidal activity of L-GAP is attributed, at least in part, to its initial conversion into methylglyoxal. In vitro and in vivo studies on which the above hypotheses are based upon are presented.

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2.Materials and Methods

2.1.1. Chemicals:

D-glucose, D-sodium glucuronate (grade II), D-glyceraldehyde grade II (aqueous solution) L-glyceraldehyde grade II (aqueous solution), DL-glyceraldehyde 3-phosphate (diethylacetal monobarium salt), sn-glycerol- 3-phosphate, dihydroxyacetone phosphate, methylglyoxal, ethyl methanesulfonate, adenosine triphosphate, β-nicotinamide adenine dinucleotide phosphate ($NADP^+$) and its reduced form (NADPH) type III (enzymatically reduced), type I (chemically reduced), B-nicotinamide adenine dinucleotide and its reduced form, glutathione oxidized form (grade III), sn - glycerol 3-phosphate dehydrogenase (rabbit muscle), triosephosphate isomerase (type III from rabbit muscle), alkaline phosphatase (type III from E. coli), glycerokinase from (E. coli), glutathione reductase (type III from yeast), N,N-bis -(2-hydroxyethyl) glycine (Bicine), tris-(hydroxymethyl)- aminomethane (TRIS), triethanolamine hydrochloride, triethanolamine, cacodylic acid, and casein hydrolysate were purchased from the Sigma Chemical Co., St. Louis, MO. Bactoagar, tryptone, nutrient broth (dehydrated), eugon agar, and eugon broth (dehydrated) were products of Difco Laboratories, Detroit, Michigan. Yeast extract was purchased from Fischer Scientific, Fair Lawn, N.J.. Diethylaminoethyl Sephadex

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(DEAE-Sephadex) A-50 was a product of Pharmacia Fine Chemicals Inc., Piscataway, N.J.. Dowex-50 was obtained from Bio Rad Laboratories Richmond CA. Diethylaminoethyl (DEAE) cellulose (DE-52) and Whatman 3 MM chromatography paper were purchased from Scientific Products, Edison, N.J.. Silica gel G, thin layer plates were obtained from Analabs Inc., North Haven, Conn. Membrane filters (type HAWP, pore size 0.45 μm were purchased from the Millipore Corp., Bedford Mass. [2-^{3}H]Glycerol was purchased from New England Nuclear Corp., Boston, MA. DL-3-Hydroxy-4-oxobutyl-1-phosphonic acid (diethylacetal dilithium salt), and DL-3,4-dihydroxybutyl-1-phosphonate were prepared as described by Goldstein et al, 1974), and were a generous gift of Prof. R. Engel. sn-[2-^{3}H]Glycerol-3-phosphate was prepared by $NaBH_4$ reduction of dihydroxyacetone followed by phosphorylation of the labeled glycerol with ATP in a reaction catalyzed by glycerokinase. [L-[3-^{3}H]Glyceraldehyde and L-[3-^{3}H]glyceraldehyde 3-phosphate were prepared as described below]. Radioactively labeled material was monitored in an Isocap 300 liquid scintillation counter. The toluene scintillation fluid, consisted of 14.7 g PPO and 0.74g POPOP in 3 Kg of toluene and the Patterson - Greene scintillation fluid was a mixture of toluene scintillation fluid and triton X-100 (2:1) (Patterson & Greene, 1965).

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2.1.2. Bacterial strains and culture conditions

Relevant properties and the source of reference for the strains used in this study are given in table 1.

Garen and Levinthal (GL) medium (Garen and Levinthal, 1960) consists of Trizma base, 120 mM; NaCl, 80.3 mM; KCl, 20.1 mM; NH_4Cl, 20 mM; $FeCl_3$, 1.94 µM; K_2HPO_4 0.6 mM; Na_2SO_4, 0.49 mM; $MgCl_2.6H_2O$, 1.02 mM; and $CaCl_2$ 198 µM: pH 7.0 for B. subtilis and 7.4 for E. coli. Bicine medium (Tang et al, 1977) consists of bicine.HCl, 100 mM; NaCl, 90.1 mM; KCl, 40 mM; NH_4Cl, 200 mM and other salts in concentrations required for Garen and Levinthal medium: pH 7.4. Phosphate minimal medium (Tanaka and Lin, 1967) consists of NaH_2PO_4, 34 mM; K_2HPO_4, 64 mM; $(NH_4)_2SO_4$ 20 mM; $MgSO_4$, 0.3 mM; $FeSO_4$, 1 µM; $ZnCl_2$, 1 µM; and $CaCl_2$ 10 µM; pH 7.0. The three media were used where indicated for bacterial cultures. The minimal media were supplemented with 0.2-0.5% carbon source. All media and carbon sources, except, sodium glucuronate were heat sterilized. Glucuronate was sterilized by membrane filtration. Casein hydrolysate (CH) medium (casein hydrolysate, 1%; NaCl, 0.5%), LB medium (tryptone, 1%; yeast extract 0.5% and NaCl, 1%) and nutrient broth were among the rich media employed for culturing bacteria. Amino acids were added as required to give a 50 µg/ml concentration.

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Solid media were prepared by adding 1.5% agar to the liquid media. Cell growth was monitored at 660 nm on a Klett Summerson colorimeter. Stock cultures were maintained on slants or in stabs made using appropriate rich media. Unless otherwise stated bacteria were cultured at 37 ^{o}C on a controlled environment incubator shaker operating at 200 rpm..

2.2. Methods

2.2.1. Synthesis of L-[3-^{3}H]glyceraldehyde from L-mannose

L- Mannose (40 mg) was dissolved in 0.5 ml of distilled water and treated with 2.7 mg of tritiated sodium borohydride (350 mCi/mmol). The mixture was incubated at room temperature for two days. Ten microliters of glacial acetic acid were added, the mixture was allowed to stand at room temperature for 2 h and then 0.7 ml water were added to the product. The resultant solution was applied to a Dowex-50 (H^{+} form) microcolumn (1x0.5 cm) and the column was washed with 5 ml distilled water. The effluent, which is free of Na^{+} , was lypholized, redissolved in 1 ml of water and applied to a Dowex-1X8 (OH^{-}) microcolumn (1x0.5 cm). Again, the column was washed with 5 ml of distilled water and the effluent which is free of both Na^{+} and BO_3^{-} , was lypholized. L-[1-^{3}H]Mannitol, thus obtained, was converted into 1:2, 5:6, diacetal derivative by incubating at 37 ^{o} C

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for about 2 hr with 320 ul of anhydrous acetone/$ZnCl_2$ mixture (13.5 ml,acetone + 2.70 g $ZnCl_2$). Anhydrous acetone was prepared by distillation over P_2O_5 The product was treated with 1 ml of 33% K_2CO_3 and then extracted several times with 2 ml portions of dry ether. Etherial fractions were combined and dried under a stream of nitrogen. The diacetal derivative of L-[^{3}H]mannitol thus obtained was oxidized with 0.5 ml of 7.4% cold solution of $NaIO_4$ at 0° C for 10 min. The product was passed through a 3 x 0.5 cm microcolumn composed of Dowex 50 (H^+) at the bottom and Dowex 1 x 8 (OH^-) on the top. The column was washed with 5.5 ml distilled water. The effluent was treated with Dowex 50 x 4 on a shaker at 37° C for 30 min, centrifuged and the supernatant was stored in a freezer (-20° C) in small vials. This preparation of L-[3-^{3}H] glyceraldehyde has a specific activity of 43.8 mCi/mmol.

2.2.2. Preparation of L-[3-^{3}H]glyceraldehyde 3-phosphate

L-[3-^{3}H]Glyceraldehyde 3-phosphate was prepared by phosphorylation of L-[3-^{3}H]glyceraldehyde with ATP in a reaction catalyzed by *E. coli* glycerokinase. The reaction mixture contained 2 µmol L-[3-^{3}H]glyceraldehyde (43.8 mCi/mmol), 8.5 µmol triethanolamine.HCl, 0.88 µmol $MgCl_2$, 50 µg gelatin, 55 µg glycerokinase (2.75 units), 55 nmol β-mercaptoethanol and 2.3 umol ATP in a 420 µl volume at pH 7.0. The mixture was incubated at 27° C for

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one hour and applied to a DEAE-cellulose column (25x30 mm). The column was eluted stepwise with; 7 ml of distilled water and then with 7 ml of 30 mM NH_4HCO_3. The effluent was collected in 0.5 ml fractions. Samples (10 µl) from each fraction were counted for radioactivity in Patterson-Greene scintillation fluid. Fractions containing high activity were combined and treated with Dowex 50, (H^+) for 2 min. The product stored in a freezer. Purity of the product was checked by chromatography, on Whatman 3 MM paper in ethyl acetate : formic acid : water (5:2:1) solvent system (R_f=0.08).

2.2.3. Extraction and analysis of phospholipids

Phospholipids were extracted from cells cultured to 15-20 Klett by a modified procedure of Bligh and Dyer (Bligh and Dyer, 1959). To 2 ml of cell culture, were sequentially added 7.5 ml of methanol:chloroform (2:1) mixture, 2 ml of chloroform and 2 ml of water. The mixture was vortexed after each addition. (In the event of extraction of radiolabeled phospholipids, unlabeled cells amounting to about ten times the cells present in the 2 ml sample were added as a carrier). The mixture was centrifuged and the chloroform layer along with the interface was separated out from the aqueous phase. The chloroform phase was washed twice with 2 ml portions of 2 M KCl and twice with 2 ml of distilled water. The final

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extract was brought to a single phase by adding some methanol. The product was filtered through glass wool to remove protein at the interface. Total phospholipids (if labeled) were determined by drying 25% of the extract under a nitrogen stream and then counting in toluene scintillation fluid.

The lipid composition was determined by thin layer chromatography, on Sil-G sheets that were preheated at 100° C for 45 min. A chloroform : methanol : water (65:25:3) solvent system was used. Phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and cardiolipin (CL) chromatographed at 0.27, 0.40 and 0.74 R_f values respectively, in this solvent system. The chromatogram was developed in an iodine tank. Areas stained by iodine were located and individual chromatograms were cut parallel to the solvent front approximately into 1 cm wide strips (the iodine stained areas were cut so as to get the entire area on a single strip). The strips were counted in toluene scintillation fluid.

2.2.3.b Mild alkaline hydrolysis of phospholipids

The labeled phospholipid extract (from about 2 ml cells) was dried under a nitrogen stream. The residue was dissolved in 0.5 ml of chloroform : methanol (1:4) mixture. The resultant solution was treated with 50 µl of 1.2 M, NaOH in 50% methanol and incubated at 37 °C for

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10 min. The hydrolysate was neutralized with 75 µl of 1 M, acetic acid. One mililiter of chloroform : methanol (9:1) was added followed by 0.5 ml of isobutanol and 1 ml of distilled water. The mixture was vortexed, centrifuged in a bench-top centrifuge and the aqueous phase was removed without disturbing the interface. The chloroform layer was washed once with 0.5 ml of distilled water and the aqueous fraction was added to the already separated aqueous layer. The combined aqueous fraction was washed once with 0.5 ml of chloroform and the the washing was combined with the chloroform fraction. Half the total volume from both aqueous and chloroform fractions were dried in vials and counted in Patterson-Greene and Toluene scintillation fluids, respectively. The CPM value was converted into nanomoles, using the counting efficiencies listed below. The efficiencies were determined by counting known amounts of [^{3}H]G3P and [^{3}H]lauric acid under conditions given below.

composition	scintillation fluid	efficiency
[^{3}H]Lauric acid	toluene	40%
[^{3}H]Lauric acid + phospholipids*	toluene	38.4%
[^{3}H]Lauric acid + lipid hydrolysate* (chloroform phase)	toluene	37%
[^{3}H]G3P (10 µl, aqueous solution)	PG	32%
[^{3}H]G3P + 0.5 ml lipid hydrolysate* (aqueous phase)	PG	27%

PG = Patterson-Greene

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*Lipid or lipid hydrolysate present corresponds to that from 1 ml cells, at 20 klett.

2.2.4. Assay for L-glyceraldehyde 3-phosphate reductase

Three different assay methods were employed to quantitate the L-glyceraldehyde 3-phosphate reductase activity. The method employed in a particular experiment depended on the degree of purity of the enzyme preparation and the convenience of the method. A solvent extraction method, described below, was developed specifically to work with the crude enzyme preparations. The spectrophotometric method (sec,2.2.4.c) is suitable only for the purified enzyme. A chromatographic method was basically employed for characterization of the reaction (L-GAP ----> G3P) products.

(a). Chromatographic method

The reaction mixture for the assay contained 14-25 nmol, L-[3-^{3}H]glyceraldehyde 3-phosphate (37-43.8 mCi/mmol); 360 nmol NADPH (Sigma type III); 1.4 µmol bicine (pH 7.6)and the cell extract (8-230 µg) protein in a total volume of 132 µl. The reaction mixture was incubated at 27°C in a (7.5x1.0 cm) test tube. Samples of 30 µl, withdrawn at zero time and at regular time intervals thereafter, were pipetted into tubes containing

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10 ul of 88% formic acid . Tubes were incubated at 100° C in a boiling water bath for 2 min. The products were chromatographed on Whatman 3 MM chromatography paper using an ethyl acetate : formic acid : water (5:2:1) solvent system. The chromatogram was scanned on a radioscanner and the area corresponding to L-glyceraldehyde 3-phosphate (R_f = 0.08) and sn L-G3P (R_f = 0.28) were cut out and counted in Patterson-Greene scintillation fluid.

(b). Solvent extraction method

In this method, L[3-^{3}H]glyceraldehyde- 3-phosphate was employed as the substrate. The reaction mixture for the assay is the same as that described in sec.2.2.4.a. At zero time and at regular time intervals thereafter, 10 µl samples were withdrawn and pipetted into screw-cap test tubes (10x1 cm) containing 30 µl, 2,4-dinitrophenylhydrazine reagent (10 mg of 2,4-dinitrophenylhydrazine and 5-6 µl, conc. H_2SO_4 dissolved in 2 ml of methanol). Water (5 µl) was added to each tube and the tubes were incubated with their caps on, at 80±2° C for 10 min in a water bath. Caps were removed and the incubation was continued for another 20 min at which time all the solvent had evaporated. The product was redissolved in 100 µl of 10% sodium carbonate and to the resultant solution, was sequentially added 10 µl of 50 mM DL-G3P and 0.9 ml of distilled water. The mixture was

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sequentially extracted with 1.5 ml chloroform, 0.5 ml of liquified phenol (88%) and then 2 x 1.0 ml chloroform. A portion (200 µl) of the aqueous phase was transferred into a scintillation vial containing 0.5 ml of distilled water in 5 ml of Patterson-Greene scintillation fluid and counted in a Isocap 300 scintillation counter. The mole equivalent of the CPM value was calculate using the following conversion factors.

Efficiency of counting = 30%

%G3P recovery in the extraction = 62%

(c). Spectrophotometric method

The assay mixture contained 50 µmol, triethanolamine hydrochloride; 150 nmol, NADPH; 1.6 µmol, DL-glyceraldehyde 3-phosphate and enzyme in a total volume of 1 ml at pH 6.8. The absorption change at 340 nm was monitored at 30°C in a Gilford model 250 spectrophotometer fitted with a multi-sample absobance recorder.

The amount of NADPH utilized was calculated from the optical density data, using the molar absorption coefficient of NADPH as at 340 nm as 6.22×10^{3} cm^{-1} M^{-1}. The stoichiometry of the reaction between NADPH and L-GAP was considered 1:1 and the initial rate of the reaction was calculated.

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2.2.5. Assay for G3P synthase

Glycerol 3-phosphate synthase (biosynthetic G3P dehydrogenase) activity was assayed by the method of Spector and Pizer (Spector and Pizer, 1975). The assay mixture contained 50 µmol, triethanolamine.HCl; 100 nmol, NADPH; 0.92 nmol, DHAP and enzyme in a total volume of 1 ml. Absorption was recorded on a Gilford model 250 Spectrophotometer.

Optical density change was used to calculate the initial rate of conversion of DHAP into G3P as described in sec. 2.2.4.c.

2.2.6. Assay for triosephosphate isomerase

The assay procedure is a slight modification of that described by Krietsch (Krietsch, 1975). The assay mixture contained 50 µmol, triethanolamine hydrochloride; 100 nmol, NADH; 0.7 unit (One unit of activity is defined as the amount of enzyme which converts 1 µmol of DHAP into G3P per min) of G3P dehydrogenase (rabbit muscle); 1.6 µmol, DL-glyceraldehyde 3-phosphate and enzyme in 1 ml volume at pH 7.5. The absorption at 340 nm was followed in a Gilford model-250 spectrophotometer.

Initial rate of isomerization was calculated from the optical density data, as described in sec.2.2.4.c.

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2.2.7. Preparation of cell extract

The temperature was maintained at 2-4° C throughout the entire process. Cells, grown to a desired Klett, were harvested by centrifugation at 5000 x g for 20 min in a refrigerated high speed centrifuge. The cells were washed once with buffer*(pH 7.6) and then resuspended in the same buffer to give a concentration of about 40-100 mg (wet weight) of cells/ml. The cells were distrupted by intermittant sonication with 10 sec bursts at out put setting 8 (about 45 watts) on a model-140, `Bronson' ultrasonic sonicator. Duration of sonication depended on the size of the cell suspension (2 sec/ml). The temperature of the extract was maintained at 0-4° C through out the sonication. An ethanol-dry ice bath was used to maintain the temperature, in the large scale experiment and an ice bath was used in other experiments. The crude extract was centrifuged at 5000xg for 20 min. The supernatant was centrifuged for another 30 min at 30,000xg. The pellet from the 30,000xg centrifugation (membrane fraction) was suspended in the original volume of the same buffer and stored in the freezer at -20° C. The supernatant (soluble proteins) was treated as described below.

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*Used 20 mM phosphate buffer in large scale (60 g cells) extraction and 20 mM bicine buffer in all other extractions.

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2.2.8. Ammonium sulfate precipitation

To the supernatant obtained in sec.2.2.7., was added, enzyme grade, solid $(NH_4)_2SO_4$, step-wise, to give 14, 27, 37, 47 and 57 saturations (details are outlined in sec.3.6.3.). Each addition was performed gradually over a period of about 1 hr at 2-4°C. At the end of each addition the insoluble protein was removed by centrifugation at 30,000xg. The fractionated proteins were redissolved in the buffer* (see footnote on page 31) and dialyzed three times against 1.8 l of buffer, over a period of 70 hr.

2.2.9. DEAE-Sephadex A-50 column chromatography

DEAE-Sephadex A-50 column chromatography was carried out on several different scales to meet the requirement of the sample size. Details pertaining to each experiment will be found in sec. 3. DEAE-Sephadex A-50 was soaked for 48 hr in 20 mM buffer* (see footnote on page 31) at pH 7.6. The column was packed under gravity and washed with about 2-3 bed volumes of the same buffer. A reservior and a mixing tank equipped for magnetic stirring were assembled for convex gradient elution. The column was equilibrated at 2-4 °C and loaded with the protein precipitated at 26-37% saturation of $(NH_4)_2SO_4$. The column

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was washed with about 0.5-1 bed volumes of the same buffer and eluted with 0.02-250 mM KCl gradient. Fractions were collected using an automated fraction collector.

2.2.10. Isolation of methylglyoxal-resistant mutants

Spontaneous mutants resistant to methylglyoxal were isolated by the procedure of Freedberg et al (Freedberg et al, 1971). Cells of E. coli were cultured to 20 Klett in phosphate minimal medium supplemented with 0.2% glucose. The culture was spread on glucose minimal plates supplemented with 1 mM methylglyoxal. Plates were incubated for about 40 hr at 37° C. Single cell colonies from these plates were isolated as possible methylglyoxal resistant mutants and transferred to LB-medium plates. The cells were checked for methylglyoxal resistance as well as for parental phenotypes using 0.2% glucose, 0.2% glucose + 1 mM methylglyoxal, 0.5% glucose + 60 µg/ml valine, 0.5% glucose + 48 µg/ml phosphonomycin and 0.5% G3P minimal plates. One methylglyoxal resistant mutant was subjected to further studies (sec. 3).

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2.2.11. Assay for the glyoxalase system

The assay method is same as that described by Freedberg et al (Freedberg et al, 1971). Cells growing exponentially in 125 ml of inorganic phosphate medium + 0.5% glucose at 37°C were harvested, washed once with 10 ml of 5 mM phosphate buffer (pH 6.6) and resuspended in 10 ml of the same buffer. The cell suspension was disrupted ultrasonically by six 10 sec pulses (at setting 8 on 'Bronson' ultrasonic sonicator) given over a period of 10 min. The extract was centrifuged at 5000xg for 20 min. The supernatant was centrifuged at 30,000xg for 30 min. The resultant supernatant was assayed for the activity of the glyoxalase system. The assay mixture contained 0.25 µmol, methylglyoxal; 3.3 µmol reduced glutathione; 50 µmol, K-phosphate buffer (pH 6.6); and 15-120 µl of cell extract in a total volume of 0.5 ml. The reaction mixture was incubated for 15 min at 30°C and the remaining methylglyoxal was estimated colorimetrically. The reaction mixture was transfered into test tubes containing 0.5 ml of 0.25% 2,4-dinitrophenylhydrazine in 2 M HCl. The mixture was permitted to stand for 15 min at room temperature after adding 0.4 ml of water. Finally 2 ml of 2.5 N NaOH was added to produce the characteristic violet-blue color of the methylglyoxal bis(2,4-dinitrophenylhydrazone). After one hour at the

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room temperature, optical densities were measured at 550 nm in a Gilford model-250 spectrophotometer.

2.2.12. Chromatography sprays

For DHAP, GAP, glyceraldehyde, and methylglyoxal : 2,4-Dinitrophenylhydrazine reagent (0.1% 2,4-Dinitrophenylhydrazine in 2 M HCl). A yellow spot.

For G3P and other phosphate esters: A reagent composed of 0.3% $FeCl_3.6H_2O$ and 7% sulfosalicylic acid in 80% ethanol. A white spot on purple back ground.

For mannitol: A reagent composed of 1% $KMNO_4$ and 2% Na_2CO_3 in water. After spray, dry at room temperature. An orange spot.

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3.Results

3.1. L-[3-^{3}H]glyceraldehyde as a probe

The effect of L-glyceraldehyde and its phosphorylated product, L-glyceraldehyde 3-phosphate on macromolecular synthesis (Tang et al, 1977) and on the G3P transport system (Hayashi et al, 1964) in E. coli have been documented. These studies indicated that L-GAP can enter the cell through the G3P transport system and is capable of interacting with a number of enzymes involved in phospholipid biosynthesis. However, the metabolic fate of these two non-physiological substances, in E. coli, was obscure. Non-availability of radiolabeled L-glyceraldehyde and L-GAP hampered such study. for this reason, synthesis of radiolabeled L-glyceraldehyde was undertaken.

3.1.1. Synthesis of L-[3-^{3}H]glyceraldehyde

L-[3-^{3}H]Glyceraldehyde was prepared* from L-mannitol as described in sec. 2.2.1.. The product contained [1-^{3}H]mannitol as an impurity.

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*This product was made available for the studies as a courtesy of Miss Grace Ilanjian, a former student in this laboratory.

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3.1.2. Purification of L-[3-^{3}H]glyceraldehyde

The crude product of L-[3-^{3}H]glyceraldehyde, as revealed by paper chromatography using an n-propanol : water : ethyl acetate (7:2:1) solvent system (Fig. 4), contained about 25% L-[1-^{3}H]mannitol. The R_f values of L-glyceraldehyde and L-mannitol in this solvent system are 0.53 and 0.29, respectively. L-Glyceraldehyde free of mannitol was obtained by eluting the radioactive material from the area corresponding to L-[3-^{3}H]glyceraldehyde on the chromatogram. The purified product was free of labeled mannitol (Fig. 5). A second solvent system (ethyl acetate : formic acid : water ; 7:2:1), was used to distinguish L-glyceraldehyde from glycerol. As shown in Fig. 6, the crude L-[3-^{3}H]glyceraldehyde preparation is free of [^{3}H]glycerol.

3.1.3. Evidence in support of the isomeric form of L-glyceraldehyde

Evidence 1

Hayashi and Lin (Hayashi & Lin, 1967) have reported that the L-, but not the D- enantiomer of glyceraldehyde could be phosphorylated by glycerokinase. Therefore the putative L-[^{3}H]glyceraldehyde was mixed with ATP in the presence of glycerokinase. The product of the reaction migrated as L-[^{3}H]GAP when chromatographed in

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the butanol : glacial acetic acid : water (2:1:1) solvent system (Fig. 7b). The product of the glycerokinase catalyzed reaction was also chromatographed in water : sec-butanol : t-butanol (48.4 : 43 : 8.6) solvent system, confirming the products identity (Fig. 8). The non-phosphorylated portion of the radioactive material appeared to consist of [^{3}H]mannitol (Fig. 9). This demonstrates complete conversion of L-glyceraldehyde into L-GAP during the reaction. Triosephosphate-isomerase converts D-glyceraldehyde 3-phosphate into dihydroxyacetone phosphate. The L-isomer is not a substrate for this enzyme . Formation of DHAP from D-GAP was visualized by coupling the triosephosphate-isomerase reaction with NADH mediated G3P-dehydrogenase activity. In such a coupled reaction, a decrease in absorbance at 340 nm indicates the formation of DHAP (Fig. 10). However, the phosphorylated product obtained in the glycerokinase reaction (sec. 3.2.1.), failed to produce any absorbance change confirming its chemical identity as L-GAP (Fig. 10).

3.2. Incorporation of labeled compounds into phospholipids

L-[^{3}H]Glyceraldehyde 3-phosphate was synthesized enzymatically from L-[^{3}H]glyceraldehyde and purified by column chromatography as described in sec.2.2.2. This product was hydrolysed with alkaline phophatase and passed

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through a DEAE-cellulose microcolumn to obtain L-[3-^{3}H] glyceraldehyde. Incorporation of these labeled compounds into phospholipids in E. coli was investigated. Surprisingly, both compounds were incorporated into E. coli phospholipids. Results of these studies are presented below.

3.2.1. Incorporation of L-[3-^{3}H]glyceraldehyde into E. coli-phospholipids

When cultures of E. coli strain 8 at 15 Klett were treated with L-[3-^{3}H]glyceraldehyde, L-[3-^{3}H]glyceraldehyde was incorporated into the phospholipid fraction of E. coli (Fig. 11), albeit of at a lower rate than [^{3}H]glycerol was incorporated into this fraction (Fig. 11). This, rather intriguing, observation formed the basis for the rest of the studies. Incorporation of both L-[3-^{3}H]glyceraldehyde and [2-^{3}H]glycerol were significantly lower in strain 9, a glycerokinase negative mutant of E. coli, (Fig. 11) suggesting that the phosphorylation of L-glyceraldehyde is an important step in the incorporation process. Furthermore, analysis of the labeled phospholipids, by thin layer chromatography, revealed that all the three major phospholipids, phosphatidylethanolamine, phosphatidylglycerol and cardiolipin are labeled (Fig. 12a & 12b). No novel phospholipids were detected in the lipid extract.

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Mild alkaline hydrolysis of phospholipids showed that (Table 2), in [^{3}H]glycerol treated strain 8 cells, about 98% of the tritium label is on the glycerol moiety of the phospholipid. The labeled phospholipids extracted from L-[^{3}H]glyceraldehyde treated strain 8 cells carry only about 76% of the activity on the glycerol moiety. Radioactivity of the lipids from either L-[3-^{3}H]glyceraldehyde or [2-^{3}H]glycerol treated strain 9 cells is distributed in a molar ratio of approximately 6:4 between the glycerol moiety and the rest of the molecule.

3.2.2. Incorporation of L-[3-^{3}H]glyceraldehyde 3-phosphate into phospholipids.

In a study, similar to the one described in sec.3.2.1., it was found that L-[3-^{3}H] glyceraldehyde 3-phosphate is incorporated into phospholipids of both strain 8 and 9 and there is no difference in the extent of incorporation in the two strains (Fig. 13). The rate of incorporation of G3P was somewhat greater than that of L-GAP. Thin layer chromatography of the phospholipid extract revealed that the radioactivity is associated mainly with the three major phospholipids, PE, PG and CL (Fig. 14a & 14b). Further analysis of the labeled phospholipids from G3P treated cells revealed that more than 98% of radioactivity is located on the G3P moiety.

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The comparable figure was about 85% for the L-[^{3}H]GAP treated cells (Table 2). There was no significant difference between strain 8 and 9 in this regard.

L-[3-^{3}H]Glyceraldehyde is also incorporated into Bacillus subtilis (Table 3). The incorporation of L-[3-^{3}H]GAP is insignificant.

3.3. A novel enzyme activity in E. coli cell extract

Several pathways may be envisioned for the incorporation of L-glyceraldehyde 3-phosphate into phospholipids. The simplest of these requires the conversion of L-glyceraldehyde 3-phosphate into sn-glycerol 3-phosphate. This may be accomplished by a reductase catalyzed conversion of L-GAP into G3P or an isomerase catalyzed conversion of L-glyceraldehyde 3-phosphate into dihydroxyacetone phosphate followed by glycerol 3-phosphate synthase catalyzed reduction into sn-glycerol 3-phosphate.

These two pathways may be summarized as follows.

Pathway 1; L-GAP ---(reductase)---> sn-G3P

Pathway 2; L-GAP ---(isomerase)---> DHAP ---(G3P-synthase)---> sn-G3P

If either pathway 1 or 2 exists, then either a reductase or an isomerase or both should be present in the

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cell extract of strain 8. Investigation of this possibility involves characterization of reaction products which in turn demands the availability of an analytical method that could resolve the products from the starting material. A chromatographic method was considered appropriate and a solvent system (ethyl acetate : formic acid : water, 5:2:1) which separates glyceraldehyde 3-phosphate from G3P, glycerol or DHAP was employed (Fig. 15).

When L-[3-^{3}H]GAP was treated with the water soluble portion of the crude cell-extract plus NADPH, a radioactive product which co-chromatographed with G3P was formed (Fig. 16). Alkaline phosphatase catalyzed hydrolysis of this product yielded a compound which co-chromatographed with glycerol (Fig. 17). These results suggested that the product of the reaction, catalyzed by the cell extract is sn-[3-^{3}H]G3P, and this was confirmed by chromatographing the products in a different solvent system (Figs. 18 & 19). The product of the cell extract catalyzed reaction was also chromatographed in a third solvent system, n-butanol : water : n-propionic acid : n-propanol (80 : 74.6 : 57 : 35) to distinguish it from DHAP. The product cochromatographed with G3P at R_f = 0.27 while DHAP appeared at R_f = 0.20 in this solvent system. The presence of NADPH is essential for the enzyme activity (Fig. 20). NADH could not substitute for

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NADPH. The membrane bound portion of the cell extract was without effect (sec.3.4.3.b). Of the three different commercial preparations of NADPH tested, only Sigma type III and USB product could serve as the coenzyme. Failure of Sigma type I to act as a coenzyme in this reaction was investigated (sec.3.7.8.). The enzyme activity will hereafter be referred to as L-glyceraldehyde 3-phosphate reductase activity.

3.4. Development of an assay method for L-GAP reductase activity

In order to extend the studies on the L-GAP reductase activity, an alternate assay method had to be developed. Although the reaction under study requires, NADPH as a cofactor any method based upon change of absorption at 340 nm would have been meaningless with a crude enzyme preparation. Considering the high sensitivity associated with radioactivity counting techniques a method based upon quantitation of [3-^{3}H]G3P formed during the reaction was considered. In spite of its high sensitivity such a method demands complete separation of [^{3}H]G3P from unconverted [^{3}H]GAP. Separation of GAP from G3P could not be achieved by simple solvent extraction methods, because of the similar solubility properties of the two compounds in a variety of solvents. Attempts were made to convert L-[3-^{3}H]GAP into a water insoluble 2,4-dinitrophenyl-

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hydrazine derivative. At the same time different solvents and solvent combinations were tested for their ability to remove the 2,4 -dinitrophenylhydrazine derivative selectively, leaving [^{3}H]G3P in the aqueous phase. Liquified 88% phenol produced satisfactory results. Based on the results of numerous trials the assay method described in section 2.2.4.b, was established.

3.4.1. Limits of the assay method

During the initial 10 min incubation period, the GAP is converted into 2,4-DNP hydrazine derivative. However the subsequent incubation, in an open test tube is, evidently, essential for the complete (>95%) removal of GAP from the aqueous phase. Addition of Na_2CO_3, prior to extraction, converts unreacted 2,4 - DNP hydrazine.HSO_4 back into a water insoluble base. The removal of 2,4 DNP-hydrazine from the aqueous phase is important, because the presence of a large amount of this reagent in the final extract may interfere with (quench) the counting process. The initial chloroform extraction helps remove the bulk of 2,4-DNP hydrazine from the aqueous phase. The liquified phenol extraction along with the subsequent chloroform extractions removes 2,4-DNP hydrazine derivative of L-GAP almost completely from the aqueous phase. A calibration curve was obtained by subjecting varying, known amounts of [^{3}H]glycerol 3-phosphate to solvent extraction process

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(Fig. 21). A 62% G3P recovery was seen. Unlike the spectrophotometric assay method the applicability of the solvent extraction method is not restricted by the purity of the enzyme preparation.

Results produced by this method for a particular enzyme preparation were compared with those of chromatographic method. Two sets of data are in good agreement (Fig. 22).

3.4.2. Some characteristics of the reaction

(a). Rate of conversion of L-GAP into G3P is proportional to the enzyme concentration

A time course study on conversion of L-[3-^{3}H]GAP into [3-^{3}H]G3P was carried out at several different enzyme (cell extract) concentrations. Initial velocities at different enzyme concentrations were calculated. The graph of initial velocity against enzyme concentration produced a straight line (Fig. 23), a characteristic of an enzyme limited reaction.

(b). L-GAP reductase activity is not found in the membrane bound fraction of the cell extract

Both soluble and membrane bound fractions of the cell extract were assayed for L-GAP reductase activity. As seen in Fig. 24, only the soluble protein

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possessed the activity. Unless otherwise specified the term cell-extract hereafter refers to the soluble cell fraction .

(c). L-GAP reductase activity is independent of G3P synthase activity

In E. coli, sn- glycerol -3-phosphate is formed by the NADPH dependent reduction of dihydroxyactone phosphate by G3P synthase (Kito & Pizer, 1969). A 1000 fold purified G3P-synthase preparation showed activity with DL-glyceraldehyde 3-phosphate (Kito & Pizer, 1969). This behaviour was completely explained by the presence of triosephosphate isomerase in the G3P synthase preparation. However, L-glyceraldehyde 3-phosphate (free of D-isomer) has not been tested as a substrate for this enzyme. Therefore, the possibility exists that the L-GAP reductase activity observed in the cell extract was a result of broad specificity of G3P synthase. This possibility was investigated by employing strain BB.20, a G3P synthase negative strain of E. coli. Cell extract prepared from this strain was assayed for L- GAP reductase activity. Extracts of strain 8 were also assayed as a control. Extracts from strain BB-20 possessed a glyceraldehyde phosphate reductase activity comparable to that of strain 8 (Fig. 25) suggesting that the L-GAP reductase activity is independent of G3P-synthase activity.

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3.5. Some preliminary experiments to establish growth and purification procedures for enzyme isolation

Purification attempts were preceded by a set of preliminary experiments to determine the effect of the carbon source of the growth medium, culture age and storage conditions of cells and cell-extract on the enzyme activity. The information obtained was useful for establishing conditions for a large scale purification experiment.

3.5.1. Effect of carbon source on enzyme activity

Cell exracts of *E. coli* strain 8 grown in different media, casein hydrolysate, bicine + 0.5% potassium succinate, bicine + 0.5% glucose, were assayed for L-GAP reductase activity. The data presented in Fig. 26 indicate that cells cultured in glucose have relatively little activity compared to those cultured in K-succinate or in casein hydrolysate. Based on these experiments, casein hydrolysate medium was considered convenient for a large scale bacterial cultivation.

3.5.2. Effect of culture age on the enzyme activity

Cell extracts prepared from *E. coli* strain 8 cultured to several cell densities (70, 140 and 200 Klett), were assayed for L-GAP reductase activity.

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Culture age has some effect on the specific activity of the cell extract (Fig. 27). At turbidities of 70, 140 and 200 Klett units the specific activities of the cell extract were 2.9 , 5.1 and 5.4 nmol/min /mg protein respectively. Based on this data, use of late log phase cells for a large scale purification seemed appropiate.

3.5.3.Effect of freezing of cells on the enzyme activity

L- GAP reductase activity in extracts of cells, frozen at -20 ^{o} C was compared with that from freshly harvested cells. These results (Fig. 28) indicate that freezing for a short period of time has no effect on the activity. This permited accumulation of a large cell stock, by batchwise growth.

3.5.4. Effect of freezing and thawing of cell extract on the enzyme activity

Freezing and thawing did not change the activity of the cell extract significantly. When the cell extract was stored in a freezer at -20 ^{o} C for one day, the activity was decreased by only about 7% (Fig. 29). This suggested that the freezing of the cell extract is an acceptable means of temporary storage.

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3.5.5. Effect of KCl on the enzyme activity

Potassium chloride, up to a concentration of 125 mM, did not change the L-GAP reductase activity of the partially purified enzyme. This means potassium chloride is a suitable candidate for an elution in ion exchange column chromatography of this enzyme (data not shown).

3.5.6. Activity in cell extract from E. coli strain 7

Extracts of E. coli strain 7 grown in casein hydrolysate medium possessed L-GAP reductase activity. The activity of this preparation was found to be identical to that of similarly prepared strain 8 extract (Fig. 30). Hence, strain 7 provides, no additional advantage over strain 8, as a source of enzyme. Furthermore, when strain 7 was grown in glucose minimal medium very little activity was detected in the cell extract (Fig. 30). A similar observation was made with strain 8 (sec. 3.5.1). prepared from glucose

3.6. Purification of L-GAP reductase

Based on the results of preliminary experiments the following procedure was adapted for cultivation of bacteria. E. coli strain 8 was cultured in casein

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hydrolysate medium (500 ml, pH 7.4), in 2 l flasks at 200 rpm on a controlled environment incubator shaker. At 170 Klett units, cells were harvested by centrifugation at 5000 x g for 20 min. The cells were stored in a freezer maintained at -20°C. A total of about 90 g cells (wet weight) were accumulated. Cells from this stock were used in the following purification studies.

3.6.1. Experiment with 0.5 g cells

Wet cells (0.5 g) were extracted by the procedure described in sec.2.2.7. The 30,000 x g supernatant was brought to 57% saturation with solid enzyme grade ammonium sulfate. The ammonium sulfate addition was, done gradually over a period of 1 hr, while the solution was stirred consistently. Precipitated protein was separated by centrifuging at 30,000 x g for 30 min. and resuspended in the original volume of fresh buffer. This protein preparation and the 30,000 x g supernatant were dialyzed against 2 x 1.8 l of 20 mM bicine buffer (pH 7.6). Dialyzed enzyme was assayed for L-GAP reductase activity by the solvent extraction method. A sample of the crude supernatant was also assayed for the same activity. The total activity found in the crude supernatant was completely retained in the 57% precipitate. There was no activity in the 57% supernatant (Table 4).

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3.6.2. Experiments with 1 g of cells

The above results indicated that the enzyme could be precipitated by ammonium sulfate treatment without losing any activity. The following experiment was undertaken to obtain a narrower ammonium sulfate precipitate.

An extract from 1 g of cells was treated with solid ammonium sulfate according to the procedure described for 60 g of cells (pages 55 and 56). However, the quantities used in this experiment were proportionately lower. The fractionated protein samples were assayed by the solvent extraction method 2.2.4.b. The L-GAP reductase activity was mainly associated with protein precipitated between 26-37% ammonium sulfate saturation (Table 5). The specific activity of this fraction is two-fold greater than that of the original extract.

In another experiment with 1 g of cells, the 26-37% fraction was dialyzed, condensed and applied on a 17.5x0.7 cm DEAE-Sephadex A-50 column. The column was eluted with a 0-220 mM KCl gradient in 20 mM sodium phosphate buffer of pH 7.6. The activity was eluted as a single peak between 120-170 mM KCl concentration (Fig. 31). Fractions containing activity were combined dialyzed, and assayed for L-GAP reductase, G3P synthase and triosephosphate isomerase activities. As indicated in

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Table 6, approximately a three fold purification was obtained during the ammonium sulfate precipitation and another twelve fold purification during the column chromatography. The purification process increased the specific activity of G3P synthase by about 40% and reduced that of triosephosphate isomerase by about eight fold.

The increase in L-GAP reductase activity with relative to the G3P synthase activity in the purified enzyme makes the spectrophotometric method for assaying the L-GAP reductase activity feasible.

3.6.3. A Large scale purification

The enzyme purification experiments described above indicated that L-GAP reductase activity can be purified by ammonium sulphate precipitation followed by DEAE-Sephadex column chromatography. In a scaled up experiment, 60 g of wet weight cells were subjected to the following purification procedure at 2-4^{o} C. Cells were suspended in 600 ml of 20 mM sodium phosphate buffer (pH 7.6) and sonicated in three 200 ml batches, each batch for about 7 min by the procedure described in sec.2.2.7. After centrifugation, the extract was treated sequentially with 61.5, 60, 58.5, 58.5 and 58.5 g of enzyme grade ammonium sulfate to produce 14, 26, 37, 47, and 57% saturations respectively, in a step-wise procedure similar to the one outlined on pages 55 and 56. The fractionated

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proteins were resuspended separately in 30 ml quantities of 20 mM sodium phosphate buffer, pH 7.6 (Only 25 ml buffer was used for the 0-14% precipitate) and dialyzed against 3 x 1.8 L of the same buffer over a period of 70 hr. Samples were assayed for L-GAP reductase activity by the solvent extraction procedure. The Lowry procedure (Dawson, et al, 1978) was used to measure the protein concentration using BSA as standard. The dialyzed, 26-36% ammonium sulfate fraction (0.7 g) was applied on a 2.7x31 cm DEAE Sephadex A-50 column. The column was made by packing a Pharmacia Fine Chemical-glass column under gravity with the ion exchanger which had been soaked in 20 mM sodium phophate buffer (pH 7.6) for about 48 h. After the protein was placed on the column, the column was eluted with about 100 ml of 20 mM sodium phosphate buffer (pH 7.6) followed by a 0-250 mM convex gradient of KCl in 20 mM sodium phosphate , pH 7.6 In the convex gradient apparatus, the mixing chamber contained 350 ml of 20 mM sodium phosphate buffer (pH 7.6) and the reservior contained 500 mM KCl in 20 mM phosphate buffer(pH 7.6). Effluent was collected in 3.2 ml fractions on an automatic (ELDEX Universal) fraction collector. The fractions were assayed for L-GAP reductase activity by the spectrophotometric method (Fig. 32a). Protein content was determined by the Lowry procedure. Fractions, in the vicinity of L-GAP reductase activity peak, were also

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assayed for G3P synthase and triosephosphate isomerase activities. Activity profiles (Fig. 32a) indicated that the fractions 93-107 are free from G3P synthase and contain only traces of triosephosphate isomerase. These fractions, which form the reductase peak, were combined and dialyzed against 2x1.8 l 20 mM sodium ohosphate buffer(pH 7.6). Conductometric analysis, using a Beckman Model RC 16B2 conductivity bridge, revealed that the L-GAP reductase has been eluted between 135 to 180 mM KCl concentration (Fig. 32b). The dialyzed pool of column, fractions, here after be refered to as the purified enzyme, was characterized as described below. Over all purification process is summarized in Table 7.

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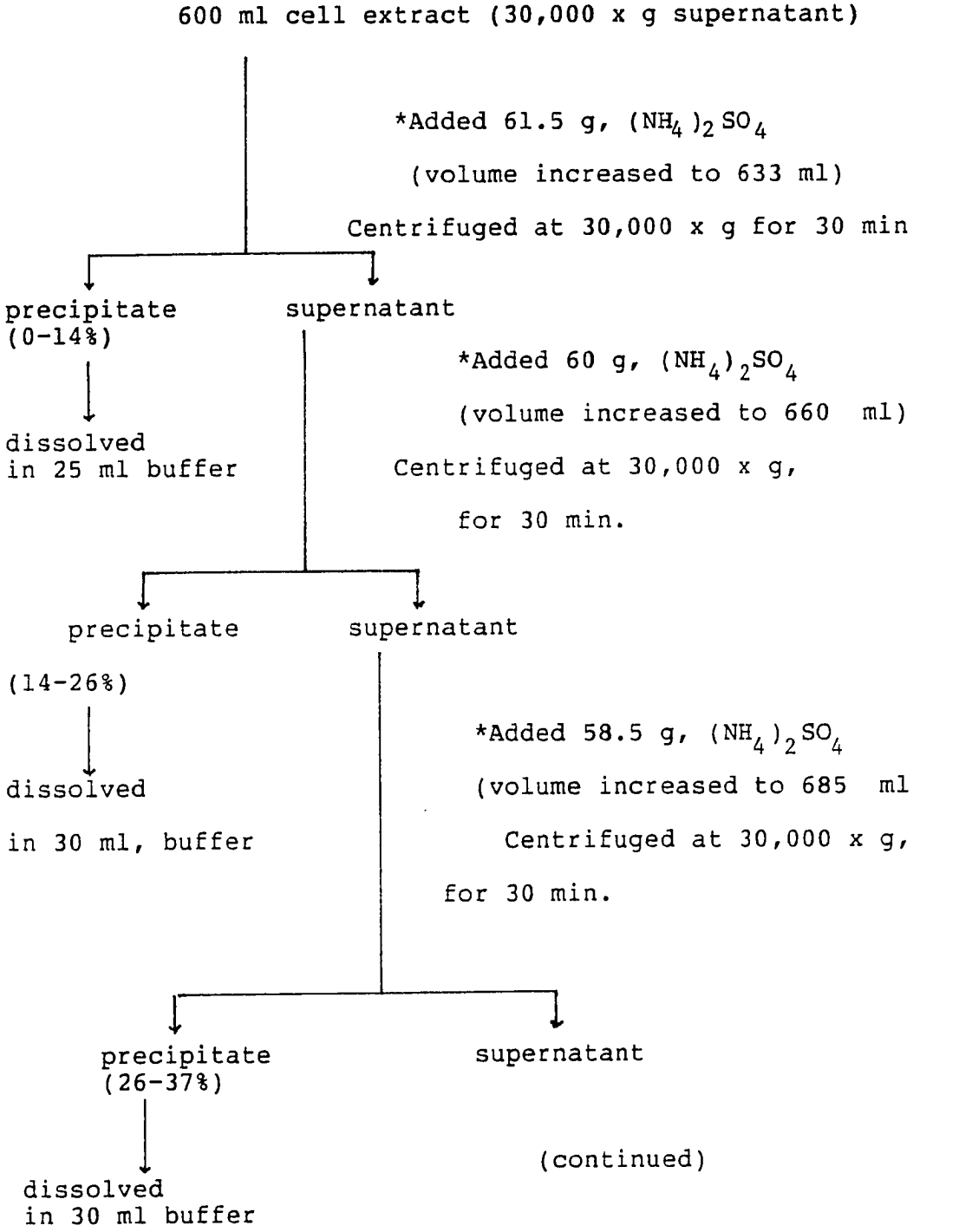
Fractionation of protein by ammoniumsulphate



*For the details of ammonium sulfate treatment, refer sec.2.2.8.

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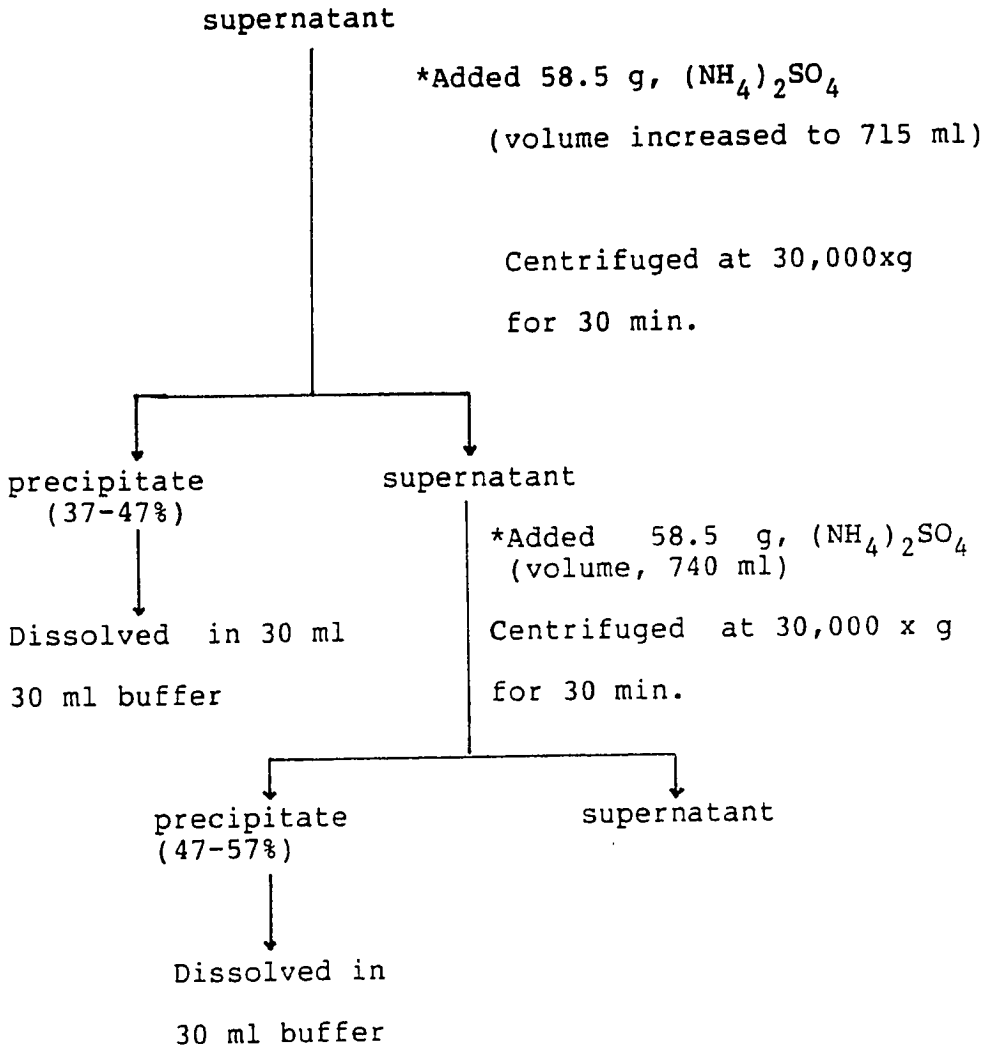
(continued from page 55)



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*For the details of ammonium sulfate treatment refer sec.2.2.8.

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3.7. Characterization of L-GAP reductase

The purified enzyme was characterized for pH optimum, inhibitor sensitivity, metal ion requirements recognition of substrate analogs and kinetic parameters.

3.7.1. The pH optimum

As evident from Fig. 33 the purified enzyme has a maximal activity at pH 6.6. Initial pH dependence studies were performed in cacodylate buffer (Fig. 33). In two other buffers, triethanolamine.HCl and phosphate, the optimum activity was also close to pH 6.6. However, the pH-activity profile was much broader in triethanolamine.HCl buffer than in cacodylate (Fig. 33).

It should be mentioned that prior to this experiment the enzyme assays were conducted at a pH (pH 7.6) different from the optimum value. However the enzyme still has about 60% of the maximal activity at this pH. Enzyme assays, described hereafter, were carried out at pH 6.7 in cacodylate or at pH 6.8 in triethanolamine.HCl buffer.

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3.7.2. Rate of conversion of L-GAP into G3P at different enzyme concentrations

In sec. 3.4.3.a, the dependence of rate of conversion of L-[3-^{3}H]GAP into L-[3-^{3}H]G3P, on the enzyme concentration was studied. However, at the time, the crude extract was the only available source of L-GAP reductase. The experiment was repeated with the purified enzyme measuring the rate of reaction as a function of enzyme concentration, spectrophotometrically. As shown in Fig. 34 the rate varied linearly with the enzyme concentration, confirming the enzymatic nature of the reaction.

3.7.3. Effect of sulfhydryl group inhibitors

Iodoacetate, N- ethylmaleimide (NEM) and p-chloromercurybenzoate (pCMB) inhibit enzymes by interfering with catalytically important enzyme sulfhydryl groups of the enzyme. Iodoacetate and NEM usually are used at 1 mM level while pCMB can be effective even at 10 µM concentration. When tested, none of the three reagents could inhibit the L-GAP reductase activity of the purified enzyme at their effective concentrations. However, at 2.5 mM concentration, iodoacetate exhibited about 20% inhibition (Table 8).

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3.7.4. Effect of metal ions

Five different metal chlorides, Mg^{+2}, Ca^{+2}, Zn^{+2} Fe^{+2} and Mn^{+2} were tested for their effect on L-GAP reductase activity in the purified enzyme. At 100 µm concentration Zn^{+2} decreased the activity by 38% while Mn^{+2} increased the activity by about 28% (Table 9). The other three ions were without effect at this concentration. Addition of EDTA to the assay mixture increased the activity by about 30% (Table 9). As was seen in section 3.5.5. KCl has no effect up to a concentration of 125 mM. This suggests that the effects observed with $ZnCl_2$ and $MnCl_2$ are due to the metal ions and not to the chloride ion.

3.7.5. Effect of substrate analogs

The effect of several carbonyl compounds and of G3P on the reductase activity of the purified enzyme was investigated. Conversion of L-[3-^{3}H]GAP into [3-^{3}H]G3P was studied in the presence of substrate analogs, in ten times the concentration of L-GAP. Next to DL-GAP, the greatest inhibition was caused by GAP analog rac-3-hydroxy-4-oxobutyl 1-phosphonate and by dihydroxyacetone phosphate (Table 10). They each produced approximately 50% inhibition in the rate of conversion when present at a concentration ten times that of the substrate. DL- G3P was

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without any effect. A minor degree of inhibition was observed with methylglyoxal, D-glyceraldehyde and L-glyceraldehyde.

3.7.6. Kinetic parameters

Apparent Michaelis-Menten constants for L-GAP and NADPH for the L-GAP reductase catalyzed reduction of L-GAP were determined (Fig. 35a & 35b). They are 25 and 35 μM, respectively (Table 11). Besides L-GAP, four other carbonyl compounds, rac-3-hydroxy-4-oxobutyl-1-phophonate, methylglyoxal, L-glyceraldehyde and D-glyceraldehyde were found to be substrates for this enzyme preparation (Fig. 36, 37, 38 & 39). Of the four carbonyls only the GAP analog rac-3-hydroxy-4-oxobutyl-1-phosphonate has a relatively low K_M (Table 11). This agrees with the inhibition data obtained in section 3.9.4. The K_M values for each of the other substrates were greater than 10 mM. It is not known whether these additional activities are associated with the reductase itself or represent one or more other proteins. Although, DHAP and D-GAP were inhibitors of the reductase they failed to act as substrates for this enzyme.

3.7.7.Reversibility of the L-GAP ---> G3P reaction.

The partially purified enzyme did not catalyze the conversion of G3P into GAP. The reversibility of the reaction was tested using both NAD^+ and $NADP^+$ as

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coenzymes. However, in the presence of 0.1 M glycine, the reverse reaction took place to a very limited extent. Again, in spite of a large excess of G3P present in the assay mixture, the reaction ceased in a few minutes.

3.7.8. NADPH, Sigma type I as a coenzyme for the reaction

As was already mentioned in sec. 3.3., NADPH type I purchased from Sigma Chemical Company, failed to act as a coenzyme for the L-GAP reductase catalyzed reduction of L-GAP. The difference between NADPH Sigma type I and type III is, that the former has been prepared through a chemical reduction process while the latter is formed as a result of an enzymatic reduction process. The possibility that NADPH Sigma type I is only a partially reduced form of $NADP^+$ was ruled out by comparing spectroscopic properties of this product with those of Sigma type III and USB products of NADPH (Table 12). As revealed by glutathione reduction assay (Table 12), the reducing capacity of NADPH type I was not as good as that of type III or the USB product. In an attempt to purify NADPH type I, it was absorbed onto a column of DEAE cellulose (DE -52), and eluted stepwise with distilled water and 30 mM NH_4HCO_3 in that order. The NH_4HCO_3 effluent containing most of the NADPH, was neutralized with dilute HCl. The NADPH type I purified by this method

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supported the reduction of L-GAP. This strongly suggests that an impurity present in type I NADPH interferes with the reduction. Furthermore, hydrosulfites are used for the reduction of NADP into NADPH (Lehninger, 1957; Personal communication with Sigma Chemical Co. MO.). In such chemical reductions SO_3^{-2} is formed as a by product. Hence, the sensitivity of L-GAP reductase to the SO_3^{-2} was tested using a partially purified enzyme preparation. The presence of 10 μM SO_3^{-2} inhibited the activity of the enzyme by 55% (Table 13). This may provide an explanation for the failure of NADPH type I to support the enzymatic conversion of L-GAP into G3P.

3.8. Bactericidal activity of L-glyceraldehyde 3-phosphate

At 1.25 mM concentration, L-glyceraldehyde -3-phosphate is bactericidal to E. coli (Tang et al, 1977). It has been reported that DL-GAP can be converted nonenzymatically into methylglyoxal(Penninkx et al, 1983). It is also known that methylglyoxal is bactericidal at 1 mM concentration (Egyud, 1967; Freedberg et al, 1971). Therefore, the formation of methylglyoxal from L-GAP may be responsible for the observed toxicity of the latter. This possibility was investigated in the experiments described below.

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3.8.1. Isolation of methylglyoxal resistant mutants

Spontaneous mutants, resistant to 1 mM methylglyoxal were isolated, according to the procedure (Freedberg et al, 1971) described in sec. 2.2.10. One such mutant KL-21, which has about two fold greater glyoxalase I activity when compared to strain 7, was further characterized for resistance to methylglyoxal and to L-GAP, in liquid growth media.

3.8.2. Resistance of strain KL-21 to methylglyoxal

Cultures of strain KL-21 were resistant to 1 mM methylglyoxal. When cultured to 20 Klett units strain 7 and KL-21 were treated, with 1 mM methylglyoxal. Transitory growth inhibition was observed in strain KL-21 (Fig. 40). The wild type strain remained completely inhibited (Fig. 40). Furthermore, as determined after five hours from the drug treatment, the viability of strain KL-21 was about 1000 fold greater than that of strain 7 (Table 14).

3.8.3. Resistance of strain KL-21 to DL-GAP

DL-GAP, at 2.5 mM concentration, inhibited the growth of both strain 7 and KL-21 in liquid culture (Fig. 41). However, strain KL-21 overcame the inhibitory

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effect within 10 hours from the drug treatment. Strain 7 continued to be under growth inhibition, over the period of study. Furthermore, during the first six hours of GAP treatment, the extent of killing of strain 7 (wild type) was 8 fold greater than that of strain KL-21 (the mutant) (Table 15) Resistance of KL-21, to DL-GAP, suggests that the toxicity of DL-GAP to E. coli is at least in part a result of its conversion into methylglyoxal.

3.8.4. Effect of DL-GAP on E. coli KL-21 precultured in the presence of 1 mM methylglyoxal.

It is likely that E. coli KL-21 cultured in the presence of methylglyoxal may be less sensitive to both methylglyoxal and DL-GAP. This possibility was tested by transferring exponentially growing KL-21 from a medium containing 1 mM methylglyoxal to fresh media containing either 1 mM methylglyoxal or 2.5 mM DL-GAP. An initial lag period was seen in response to each drug (Fig. 42). These results indicate that the sensitivity to the drugs has not been reduced by the primary methylglyoxal treatment.

3.8.5. Nonenzymatic conversion of DL-GAP into methylglyoxal

Polyvalent anion catalyzed nonenzymatic conversion of DL-GAP into methylglyoxal has been reported by Penninkx et al (Penninkx et al, 1983). The reaction

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follows a first order kinetics with respect to DL-GAP and almost independent of the concentration of the polyvalent ion. A first order rate constant of 0.27 h^{-1} has been obtained for the conversion of DL-GAP into methylglyoxal in phosphate buffer at 37° C (Penninkx et al, 1983).

The possible interference from the nonenzymatic conversion of GAP on the interpretation of present data was investigated. The nonenzymatic conversion of DL-GAP into methylglyoxal was studied in the presence of bicine, triethanolamine hydrochloride or potassium phosphate buffer at 37° C. A first order rate constant of 0.22-0.23 h^{-1} was obtained with all three buffers (Table 16). When the same reaction was studied under the conditions comparable to that of the spectrophotometric assay, ie. at 30° C, the rate constant for the conversion is 5.6×10^{-2} h^{-1} (Table 16). This value is even lower in the presence of NADPH. The reason is not known. Consequently, as discussed in sec.4 the nonenzymatic process does not interfere with the interpretation of present data.

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4. Discussion

The presence of L-GAP reductase activity in the cell extract of E. coli suggests that the incorporation of L-[^{3}H]GAP into phospholipids may proceed via its initial conversion into L-glycerol 3-phosphate (Fig.43). The presence of more than 85% (Table 2) of the radioactivity on the back bone of phospholipid molecule and the absence of novel phospholipids in the radioactively labeled lipid extract (Fig.14) clearly indicates that the label on the lipid molecule is mainly associated with the G3P moiety. This is in agreement with a G3P mediated pathway for the incorporation of the tritium label of L-[3-^{3}H]GAP into phospholipids. Initial hydrolysis of L-GAP into L-glyceraldehyde could be ruled out because: 1) the strains employed for the studies (strains 8 & 9) are defective in alkaline phosphatase; 2) Strain 9, which has no glycerokinase activity, also incorporated L-[3-^{3}H]GAP into phospholipids. Incorporation of L-glyceraldehyde into phospholipids in strain 8 could be a result of its initial conversion into L-GAP, which is known to be catalyzed by glycerokinase. Moreover, the findings, that strain 9 is able to incorporate L-glyceraldehyde into phospholipids to some extent, but not glycerol, and that only 76% of the total activity was found in the backbone of the phospholipid of strain 8 (this value is as low as 67% in

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strain 9), suggest that in addition to the G3P mediated pathway there is at least one other route through which L-glyceraldehyde is incorporated into phospholipids. The mild alkaline -hydrolysis data indicate that this second pathway is responsible for carrying the tritium label from L-GAP to the fatty acid moieties of the phospholipids. The non-enzymatic conversion of L-glyceraldehyde into methylglyoxal (Riddle & Lorenz, 1967) and subsequent conversion of methylglyoxal into fatty acids via pyruvate (Fig.1) could be the second pathway (Fig.43) needed to explain the present data. Besides, L-glyceraldehyde 3-phosphate is known to be a substrate to several aldolases including L-fuculose 1-phosphate aldolase (Ghalambor & Heath, 1962). L-fuculose 1-phosphate aldolase can catalyze the condensation reaction between DHAP and L-glyceraldehyde to form L-tagatose 1-phosphate. Nevertheless in *E. coli* possessing functional glycerokinase (wild type) the relative importance of these latter pathways is less significant. The non-enzymatic conversion of L-GAP into methylglyoxal (Penninkx *et al*, 1983), is also of considerable importance in the interpretation of incorporation data. As proposed in Fig.43, the incorporation of L-[3-^{3}H]GAP into phospholipids may take place either via the G3P mediated enzymatic pathway or the methylglyoxal mediated pathway or through both. The

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relative importance of individual pathways in this process was not investigated. However, the methylglyoxal mediated non-enzymatic pathway is not likely to be a sound candidate for carrying the tritium label from GAP to the backbone of the phospholipid for the following reasons: 1) pyruvate, an intermediate of this pathway (Fig.43) can also channel the label to fatty acids (yet more than 85% of the label was found on the backbone of the phospholipid); 2) Because of the toxicity of methylglyoxal to E. coli, the utilization of methylglyoxal mediated pathway may not be the choice dictated by evolution. Nevertheless, this pathway may be responsible for the small amount (15%) of tritium label found on the fatty acid moiety of the lipid.

During the spectrophotometric assay of L-GAP reductase, the traces of triose phosphate isomerase present in the purified enzyme could convert the D-isomer of DL-GAP into DHAP. However, no interference on the assay is possible, because the purified enzyme is free from G3P synthase.

The effect of nonenzymatic conversion of GAP into methylglyoxal (Penninkx et al, 1983) on the interpretation of the results of enzyme (L-GAP reductase) assay should receive some attention. Nonenzymatic conversion of GAP into methylglyoxal was seen when 4.5 mM GAP was incubated at 37°C in several buffers such as bicine, phosphate and

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triethanolamine.HCl. The results are in close agreement with that of Penninkx (Penninkx et al, 1983). However, at 30° C and 0.9 mM DL-GAP (conditions closer to that of the spectrophotometric assay) the initial rate of nonenzymatic conversion in triethanolamine buffer (pH 7.0) is only about 0.83 μM/min. This was even lower in the presence of NADPH. This level of nonenzymatic conversion of DL-GAP into methylglyoxal does not interfere with the spectrophotometric assay of L-GAP reductase because: (i) the depletion of the GAP pool through nonenzymatic conversion can reach only about 7.4 μM during a maximum period of 5 min required for the assay, (ii) methylglyoxal so formed in the assay mixture can not serve as a substrate for the reductase due to its high K_M (K_M for methylglyoxal = 10 mM) and (iii) methylglyoxal at 6 μM concentration does not inhibit the L-GAP reductase activity.

The question of nonenzymatic route does not arise under the conditions, the two radioactive assays for the reductase were performed, because: (i) at 0.2 mM GAP and at 27° C the initial rate of nonenzymatic conversion is estimated to be lower than 0.25 μM/min (ii) as revealed by paper chromatography no methylglyoxal was detected in the assay mixture.

The failure of NADPH Sigma type I to serve as a coenzyme for the L-GAP reductase is probably the result

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of the presence of SO_3^{-2} as an impurity in the commercial product NADPH Sigma type I. This statement is based upon three lines of evidence: (i) SO_3^{-2} is known to be produced as a by product in the chemical reduction of $NADP^+$ to NADPH; (ii) NADPH Sigma type I showed activity when purified by DE 52 column chromatography (sec.3.7.8.) and (iii) SO_3^{-2} at 10 μM inhibits the L-GAP reductase activity by 50% (sec.3.7.8).

It should be mentioned that the purified enzyme, stored at -20° C, lost its activity slowly. The specific activity decreased by more than 90% during a period of about one year.

Beside the fact that the present studies clearly demonstrate the presence of L-GAP reductase activity in the cell extract, its in vivo significance is yet to be demonstrated. The only clue to its in vivo activity came from the incorporation studies (sec.3.2.). Even if the in vivo activity of L-GAP reductase is demonstrated, the normal physiological function of the enzyme is not yet known. It is doubtful that E. coli is equipped with an enzyme system merely to scavenge a chemical (L-GAP) which is hardly encountered by the cell in its natural enviornment. Isolation of mutants deprived of the reductase activity may be crucial for finding answers to both questions.

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5. Tables and Figures

Table 1. Bacterial strains, their properties, source and reference

Strain	Relevent properties	source & reference
E. coli		
7	*phoA8*, *glpR*^{c}*2* *tonA22*, T2 ^{r}, *rel-1*, λ .	E.E.C.Lin, Harvard Medical School, Hayashi *et al*,1967
8	strain 7, *glpD3*	J.Cronan,Jr., Yale University. Hayashi *et al*,1967.
9	strain 8, *glpk*	E.C.C.Lin, Harvard Medical School. Hayashi *et al*,1967
BB-20	starain 8, *gpsA20*	R.Bell, Duke University-Medical Center. Bell, 1974.
KL-21	strain 7, Meg^{r}	present study
B. sub.		
W-23, 1005	*met*	D.Dubnau, Public Health Research Inst., City of N.Y. Mindich, 1970.

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Table 2. Mild alkali-hydrolysis of radiolabeled phospholipids.

Labeled compound (nmol/ml cells)

Labeled compound	Strain 9			Strain 8		
	aqueous phase	$CHCl_3$ phase	% in aq.	aqueous phase	$CHCl_3$ phase	% in aq
Glycerol	0.08	0.06	57	4.28	0.09	98
L-gly	0.30	0.15	67	0.76	0.24	76
L-G3P	3.53	0.06	98	6.78	0.09	99
L-GAP	1.71	0.20	89	1.60	0.31	84

The remainder of the 60 min phospholipid, corresponding to 1.75 ml of cells (see legends to Fig.11 & 13), was subjected to mild alkaline hydrolysis (sec.2.2.3.b). The hydrolysate was extracted as described. The aqueous phase and the chloroform phase were dried and counted in Patterson Greene and toluene scintillation fluids respectively. Results have been normalized per 1 ml cells.

(*L-Glyceraldehyde)

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Table 3. Incorporation of L-[3-^{3}H]glyceraldehyde and L-[3-^{3}H]GAP into phospholipids of B.subtilis.

Labeled compound in phospholipids

(nmol/ml cells)

Time min	In GL + K-succinate				In casein hydrolysate	
	Glycerol	L-Gly	G3P	GAP	L-Gly	GAP
0	0.07	0.03	0.01	0.02	0.04	0.02
15	0.39	0.06	0.20	0.02	0.19	0.05
60	1.36	0.14	0.71	0.10	0.65	0.12

B. subtilis was cultured in either Garen and Levinthal +0.5% potassium succinate (pH 7.0) or caesein hydrolysate (pH 7.0) at 37^{o} C on a floor shaker. At 20 Klett units 2.5 ml of the culture was pipetted into 25 ml erlenmyer flasks containing 0.25 ml of tritiated, glycerol (8.3 µCi/µmol, 8.3 µCi/ml), L-glyceraldehyde (9.0 µCi/µmol, 9.0 µCi/ml), G3P (17.4 µCi/µmol, 9.1 µCi/ml) or L-GAP (18.2 µCi/µmol, 8.7 µCi/ml). Flasks were incubated in a water bath shaker operating at 200 rpm and 37^{o} C. Samples (0.5 ml) were withdrawn at zero, 15 and 60 min time points for Bligh and Dyer extraction.

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Table 4. L-GAP reductase activity in fractions obtained by ammonium sulfate treatment.

Sample	Total activity (nmol/min)
Crude supernatant	166
0-57% $(NH_4)_2 SO_4$ ppt.	163
Supernatant	nil

E. coli strain 8 cells (0.5 g wet weight) were extracted into 5 ml of 20 mM bicine buffer (pH 7.6). A portion of the extract (4 ml) was treated with 1.97 g of enzyme grade solid ammonium sulfate at 2-4 ^{o} C for 1 hr. The precipitate was separated by centrifugation at 30,000 x g in a refrigerated centrifuge. The pellet was redissolved in 2 ml of bicine buffer. Samples (1 ml) of the redissolved protein solution and the supernatant were dialyzed against 1.8 lit of the same buffer. The dialyzed products and the crude supernatant were assayed for the L-GAP reductase activity by the solvent extraction method. Activities shown above have been normalized to the total volume of the crude supernatant.

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Table 5. Activities in ammonium sulfate precipitated fractions

Sample	Volume (ml)	Protein (mg)	Activity (nmol/min)	Specific activity (unit/mg)
Cell extract	10.0	96	411	4.3
0-14% ppt.	4.9	25	44	1.7
14-26% ppt.	5.2	13	23	1.8
26-37% ppt.	5.6	26	257	9.9
37-47% ppt.	5.5	25	13	0.5
47-57% ppt.	5.8	14	24	1.7

E. coli strain 8 cells (1 g wet weight) were extracted in 20 mM bicine buffer (pH 7.6). The extract was treated with enzyme grade solid ammonium sulfate in a manner simillar to the one described on page 55 and 56. The resulting samples were assayed for L-GAP reductase activity and for the protein content by the solvent extraction and the Lowry`s methods respectively.

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Table 6. Specific activities of three different enzymes at different stages of purification

Sample	L-GAP-reductase (nmol/min/mg)	Triosephosphate isomerase (umol/min/mg)	G3P-synthase (nmol/min/mg)
Cell extract	2.9	36.2	2.8
$(NH_4)_2 SO_4$ (26-37% ppt.)	10.1	2.2	4.0
DEAE-Sephadex A-50, pool*	121.7	4.5	4.7

*Fraction no.17 through 20 of the DEAE-Sephadex A-50 column chromatography (Fig.31) were combined together. L-GAP reductase activity was determined by the solvent extraction method (sec. 2.2.4.b). G3P synthase activities were determined by the methods described in sec.2.2.5. Triosephosphate isomerase activity was determined by the method 2.2.6, however, the absorption change at 366 nm was monitored. Molar absorption coefficient of NADPH at 366 nm ($3.44 \times 10^3 \ cm^{-1} M^{-1}$) was used to calculate the initial rate of isomerization.

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Table 7. Summary of the large scale purification process

Sample	Total volume (ml)	Total protein (mg)	Total activ. (unit)	Specific activity (unit/mg)	Purification (fold)	Yield %
Cell extract	600	3960	18878	4.8	1.0	100
$(NH_4)_2SO_4$ (26-37%) ppt.	16	672	8950	13.3	2.8	47
DEAE-Sephadex A-50 pool	40	16.2	8250	510	107	44

L-GAP reductase activity was measured by the solvent extraction method at pH 7.6. The protein content was determined by the Lowry's method.

One unit of activity is defined as the amount of enzyme that converts one nanomole of L-GAP into L-G3P per minute.

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Table 8. Effect of sulfhydryl reagents on L-GAP reductase activity.

-SH reagent	Concentration (mM)	Specific activity (nmol/min/mg prot.)	Inhibition %
Control	---	795 ± 37	0
Iodoacetate	0.5	762	4
Iodoacetate	5.0	631	21
NEM	2.5	763	4
pCMB	0.06	758	5

The purified enzyme (15 µl) was assayed by the spectrophotometric method, in the absence and the presence of indicated concentrations of -SH reagents. The assay mixture for the `control' contained 50 µmol sodium cacodylate (pH 6.7), 160 nmol NADPH, the purified enzyme (6 µg,protein) and 1.7 µmol DL-GAP in a total volume of 1 ml.

Each inhibitor was tested against an individual control. The data shown for the control is the average of individual controls. The specific activities determined in the presence of the inhibitor were normalized against the average control value.

The reagents were not tested against an enzyme which is known to be sensitive to them.

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Table 9. Effect of metal ions on the enzyme activity.

Concentration	100 µM		1mM	
Additive	Sp.activity (units/mg)	%control	Sp.activity (units/mg)	%control
Control	798 ± 89	100	798 ± 89	100
Zn^{+2}	494	62	311	39
Fe^{+2}	-	-	335	42
Mg^{+2}	806	101	870	109
Ca^{+2}	894	112	910	114
Mn^{+2}	1021	128	1524	191
EDTA	1021*	128*	1005	126

*At 2 mM EDTA.

The purified enzyme was assayed for L-GAP reductase activity in the absence (the control) and the presence of various divalent metal chlorides or EDTA. The metal chlorides were present in 100 µM or 1 mM concentrations, and EDTA was present in 1 mM and 2 mM concentrations. The spectrophotometric assay method was employed. The assay mixture for the `control' contained 50 µmol sodium cacodylate (pH 6.7), 160 nmol NADPH, the purified enzyme (6 µg,protein) and 1.7 µmol DL-GAP in a total volume of 1 ml. Individual activities were normalized against the average control value as described under Table 8.

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Table 10 . Inhibition by substrate analogs.

Inhibitor	$\frac{[\text{Inhibitor}]}{[\text{L-}(^3\text{H})\text{GAP}]}$	% Inhibition
Control	0	0
DL-G3P	20	0
Methyl glyoxal	10	2
D-Glyceraldehyde	10	4
L-Glyceraldehyde	10	7
D-GAP	10	44
DHAP	10	53
GAPA	10	55
DL-GAP	20	83

L-GAP reductase activity was assayed by the chromatographic method, in the absence and presence of inhibitors in indicated concentrations. Average of four to eight sets of experimental data are shown above.

The assay mixture for the `control' contained 620 nmol, bicine (pH 7.6); 162 nmol, NADPH; enzyme (4 µg protein) and 10.4 nmol L-[3-^{3}H]GAP in a total volume of 61 µl. In addition to the above, the test samples contained 100 nmol of inhibitor. Samples were analysed chromatographically as described in sec.2.2.4.a, at zero and 10 min time points.

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Table 11 Apparent Michaelis Menten constants

Substrate	K_m (μM)	K_M (NADPH) (uM)
L-GAP	28	35
GAPA	280	6.2
Methylglyoxal	1.4×10^4	10
L-glyceraldehyde	2.8×10^4	3.8
D-glyceraldehyde	1.0×10^5	52

The apparent K_M values were calculated from Fig.35 through Fig.39.

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Table 12 Reducing capacity of NADPH Sigma type I in comparison to Sigma type III and the USB products

Source of NADPH	* O.D. at 340 / O.D. at 260	** Initial rate of glutathione reduction nmol/min
Sigma I	0.397	8.0
Sigma III	0.439	12.1
USB	0.396	10.7

*Optical densities were measured at 340 and 260 nm on a Gilford-250 spectrophotometer.

** Optical density at 340 nm was measured, in a reaction containing 26.7 µmol, potassium phosphate(pH 7.6), 3.33 µmol glutathione, o.25 µmol, NADPH (one of the three types), and approximately 20 units of glutathione reductase, at zero, 5 and 10 min time points. Reaction rate was calculated using the molar absorption coefficient of NADPH.

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Table 13 Sensitivity of L-GAP reductase to the sulphite ion.

$[SO_3^{-2}]$ (μM)	% Inhibition
Control	0
5.0	41
9.8	54
18.8	70

A partially purified enzyme preparation was assayed for the L-GAP reductase activity by the spectrophotometric method, in the absence and the presence of indicated amounts of sodium sulphite. The assay mixture for the 'control' contained,50 μmol, triethanolamine hydrochloride (pH 6.8); 160 nmol, NADPH, partially purified enzyme (170 μg protein) and 124 nmol, DL-GAP in a total volume of 1 ml.

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Table 14. Viable cell count in methylglyoxal treated cultures.

Strain	Treatment	Cell-count (Cells/ml)		5 h / zero time
		Zero time	5 h after	
7	control	1.73×10^8	3.49×10^9	20.2
7	1 mM MeG	1.73×10^8	3.10×10^5	0.002
KL-21	control	1.79×10^8	3.56×10^9	19.9
KL-21	1 mM MeG	1.79×10^8	2.32×10^8	1.3

Appropiate dilutions of the cultures, immediately before the methylglyoxal treatment and five hours after the treatment (see Fig.40) were plated on LB plates and incubated at 37°C for about 18 h. The number of individual colonies were counted.

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Table 15. Viable cell counts of DL-GAP treated cultures

Strain	Treatment	Cell-count (cells/ml)		6 h / zero time
		zero time	6 h after	
7	control	1.45×10^8	2.94×10^9	20.3
7	2.5 mM GAP	1.45×10^8	1.80×10^7	0.1
KL-21	control	1.27×10^8	2.27×10^9	17.9
KL-21	2.5 mM GAP	1.27×10^8	9.50×10^7	0.8

Appropiate dilutions of the cultures, immediately before the DL-glyceraldehyde 3-phosphate treatment and six hours after the treatment (see Fig.41) were plated on LB plates and incubated at 37°C for about 18 h. The number of individual colonies were counted.

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Table 16 Nonenzymatic formation of methylglyoxal from DL-GAP

[DL-GAP] (mM)	[Other]	Buffer	Incubation temperatue	Initial rate (mM/h)
4.5		bicine	37° C	1.05
4.5		TEA.HCl	37°C	1.01
4.5		K-phosphate	37°C	1.03
0.9		TEA.HCl	30°C	0.05
0.9	160 µM NADPH	TEA.HCl	30°C	0.02
0.9	enzyme (4 µg prot.)	TEA.HCl	30°C	0.05

DL-GAP was incubated with 50 uM buffer (pH 7.0) at indicated temperature in test tubes. Indicated amounts of NADPH or purified enzyme was present in two of the test tubes. Methylglyoxal formed in the reaction mixture was determined by the method of Riddle and Lorenz (Riddle & Lorenz, 1968). Transfered 100 µl of the mixture into a tube containing 0.9 ml distilled water, and added 0.5 ml of 0.25% 2,4 dinitrophenylhydrazine in 2 M HCl. After 15 min 4 ml of 7% KOH in 60% ethanol were added to produce the characterestic violet-blue color.After 1 h at room temperature, optical densities at 540 nm were determined in 1 cm pyrex cuvettes, on

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Gilford -250 spectrophotometer. The optical density data along with the micromolar extinction coefficient of standard methylglyoxal hydrazone, 72.77 cm /μmol (Riddle & Lorenz, 1968) were used to calculate the initial rate of the reaction.

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Table 17. R_f values of several compounds relevant to the present study

Solvent system	Mannitol	glycerol	L-gly	DHA	G3P	GAP	DHAP
1	0.29	NI	0.53	0.55	NI	NI	NI
2	0.32	0.45	0.15	0.51	0.16	0.03	NI
3	0.41	NI	0.58	0.58	NI	0.31	NI
4	0.42	NI	NI	NI	NI	0.25	NI
5	0.44	0.53	0.25	NI	0.27	0.08	NI
6	NI	0.60	NI	NI	0.09	0.20	NI
7	NI	NI	NI	NI	0.27	NI	0.20

NI; Not included in chromatography

Solvent-
system 1; n-propanol : water : ethylacetate (7:2:1)

system 2; ethylacetate : formic acid : water (7:2:1)

system 3; n-butanol : acetic acid : water (2:1:1)

system 4; upper phase of, water : sce-butanol : t-butanol (48.4:43:8.6)

system 5; ethylacetate : formic acid: water (5:2:1)

system 6; t-butanol : methyl ethyl ketone : water : ammonium hydroxide (4:3:2:1)

system 7; n-butanol : water : n-propionic acid : n-propanol (80:74.1:57:35)

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FIG.1 GLYCOLYTIC PATHWAY, HEXOSE MONOPHOSPHATE SHUNT AND METHYLGLYOXAL BYPASS.

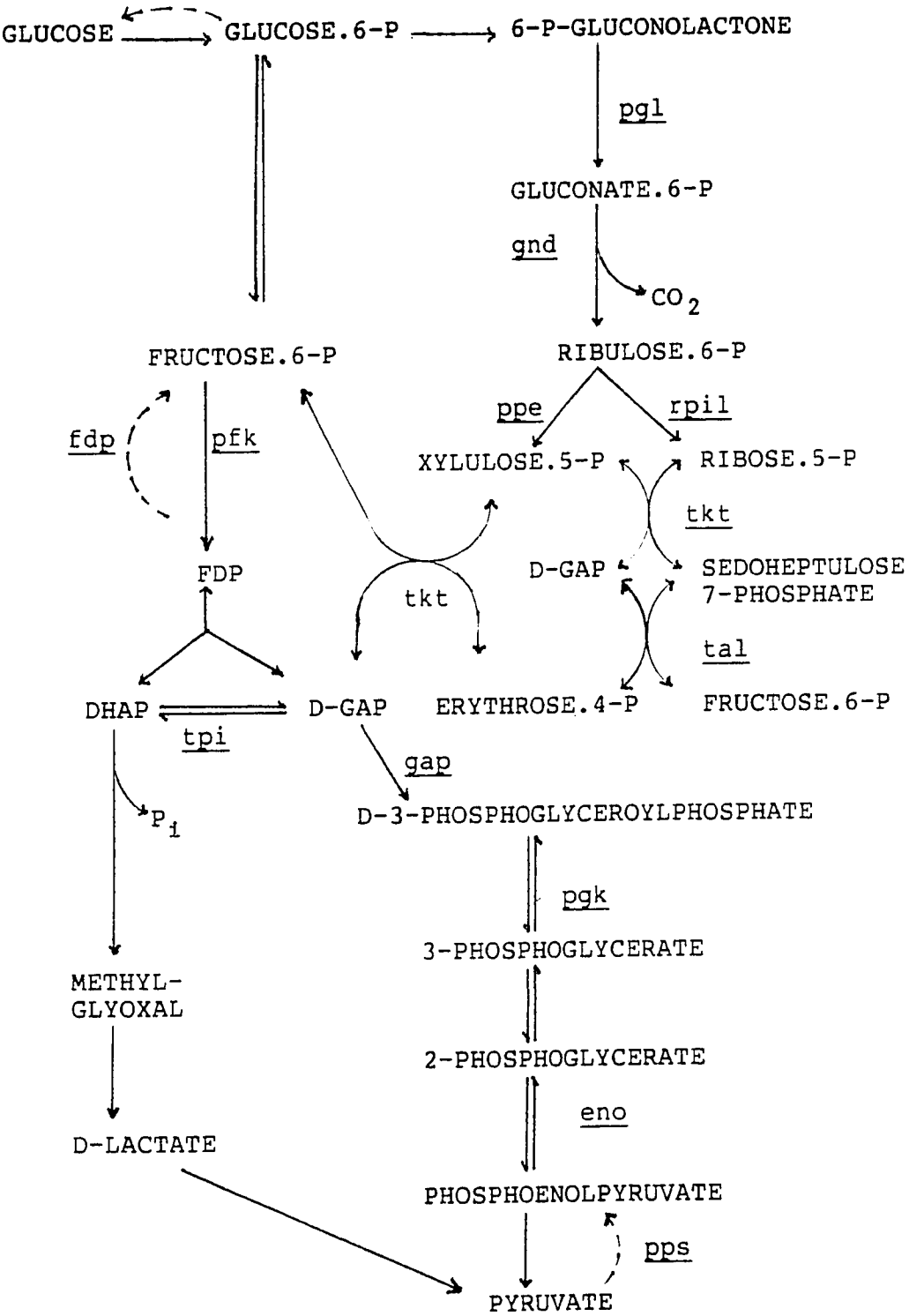

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FIG.2 METABOLISM OF L-GLYCEROL 3-PHOSPHATE IN E. COLI

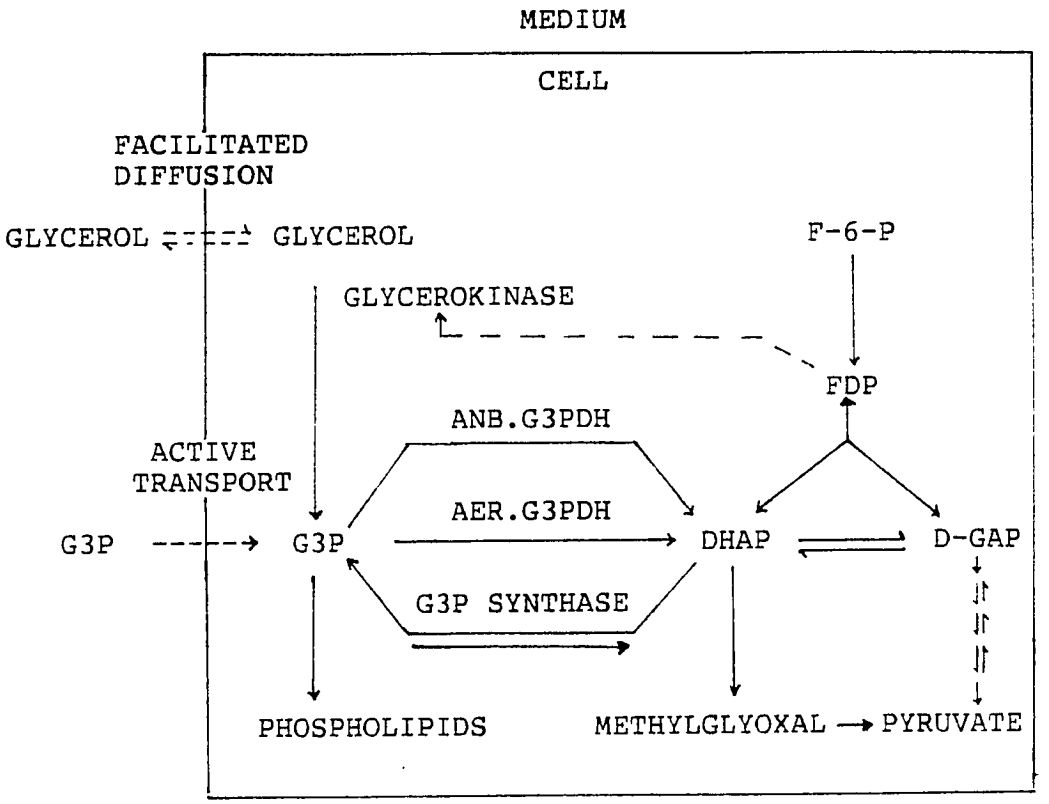

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FIG.3 PHOSPHOLIPIDS BIOSYNTHESIS

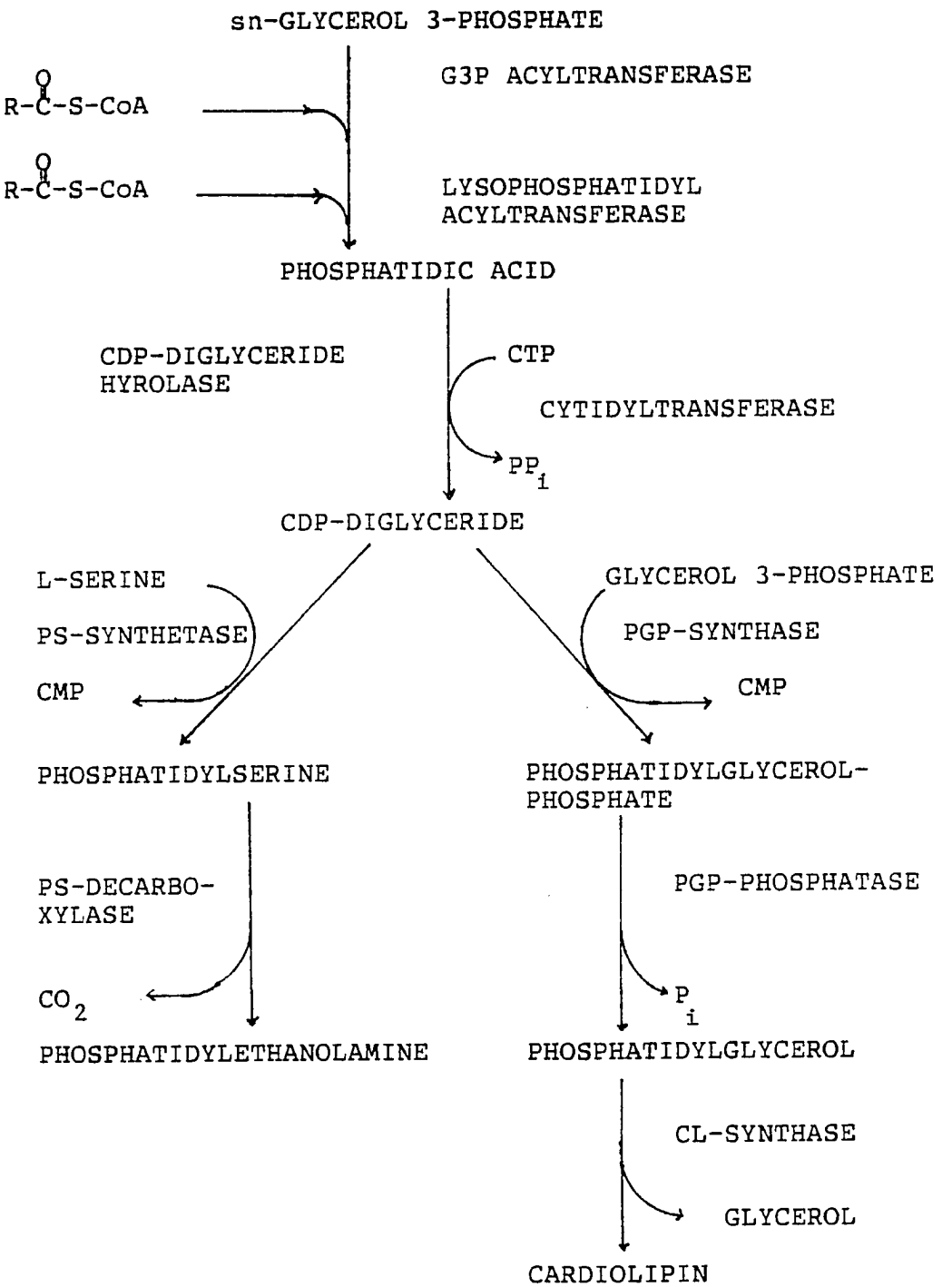

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Fig. 4 Chromatographic examination of the purity of crude L-[3-^{3}H]glyceraldehyde

Crude L-[3-^{3}H]glyceraldehyde (50 μCi, 44 μCi/μmol) was spotted on a pre-washed Whatman 3 MM chromatography paper. The spotting was done in two regions on the paper; a major region with 20 spots, located about 0.5 cm apart from each other and a minor region with a single spot located about 15 cm away from the center of the major region. The chromatogram was run in n-propanol : water : ethyl acetate (7:2:1) solvent system. The single spot region was scanned on a radioscanner. Peaks corresponding to L-glyceraldehyde and L-mannitol were identified by their R_f values, 0.53 and 0.29 respectively. (Unlabeled L-glyceraldehyde and L-mannitol were chromatographed at R_f values 0.52 and 0.29 in this solvent system).

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FIG.4 L-(3-^{3}H)GLYCERALDEHYDE, THE CRUDE PRODUCT

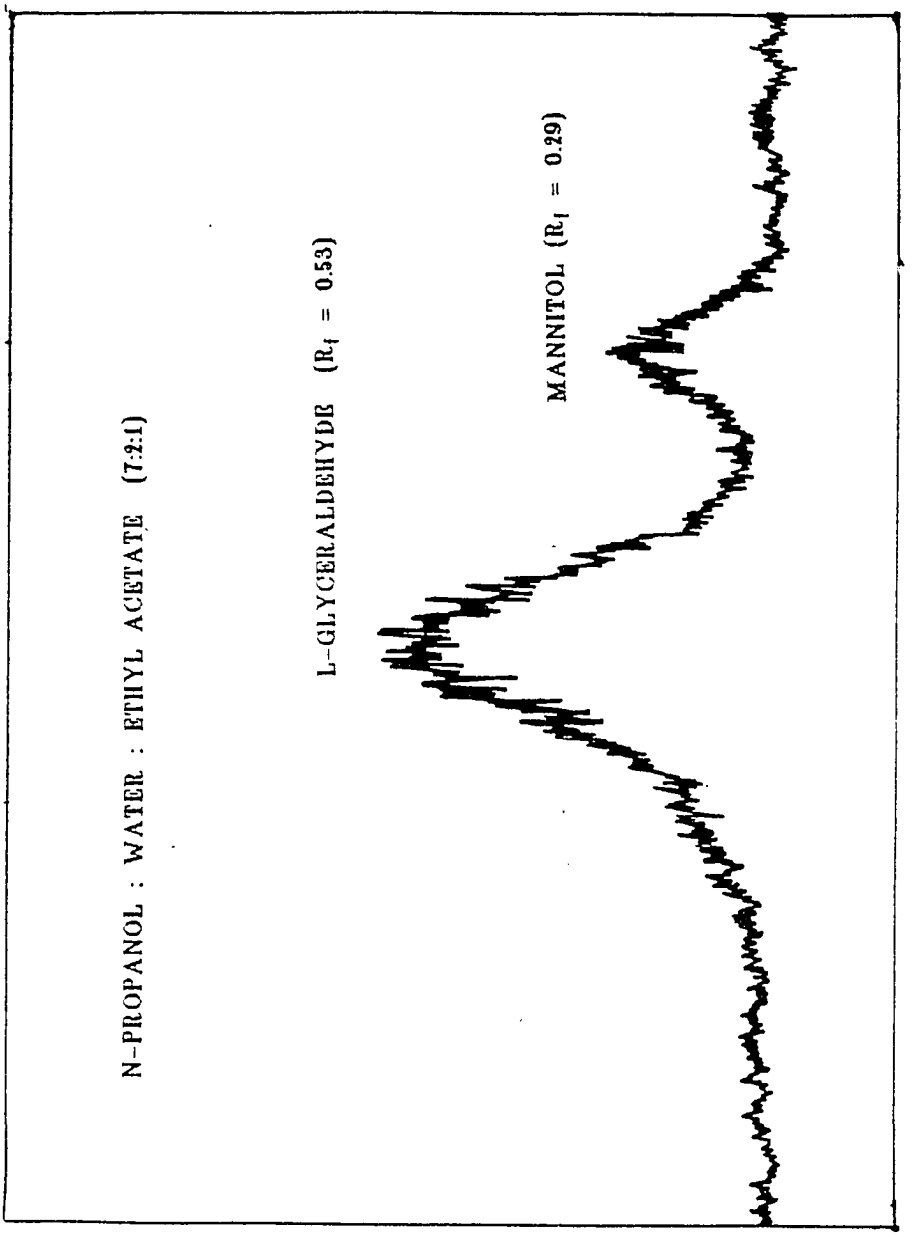

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Fig. 5 The purified L-[3-^{3}H]glyceraldehyde is free from [^{3}H]mannitol

In the experiment described under legend to Fig. 4, the area corresponding to L-glyceraldehyde in the major region of the chromatogram was cut out as a strip parallel to the solvent front. L-[3-^{3}H]Glyceraldehyde was eluted, from the strip, with distilled water, in a descending chromatography set up. The effluent was condensed in a rotary evaporator under partial vaccum and chromatographed in n-propanol : water : ethylacetate (7:2:1) solvent system. The chromatogram was scanned on a radio scanner.

1 --- authentic sample of L-glyceraldehyde.

2 --- authentic sample of mannitol.

*Slightly higher R_f values (0.57 and 0.32 for L-glyceraldehyde and mannitol respectively) were obtained here.

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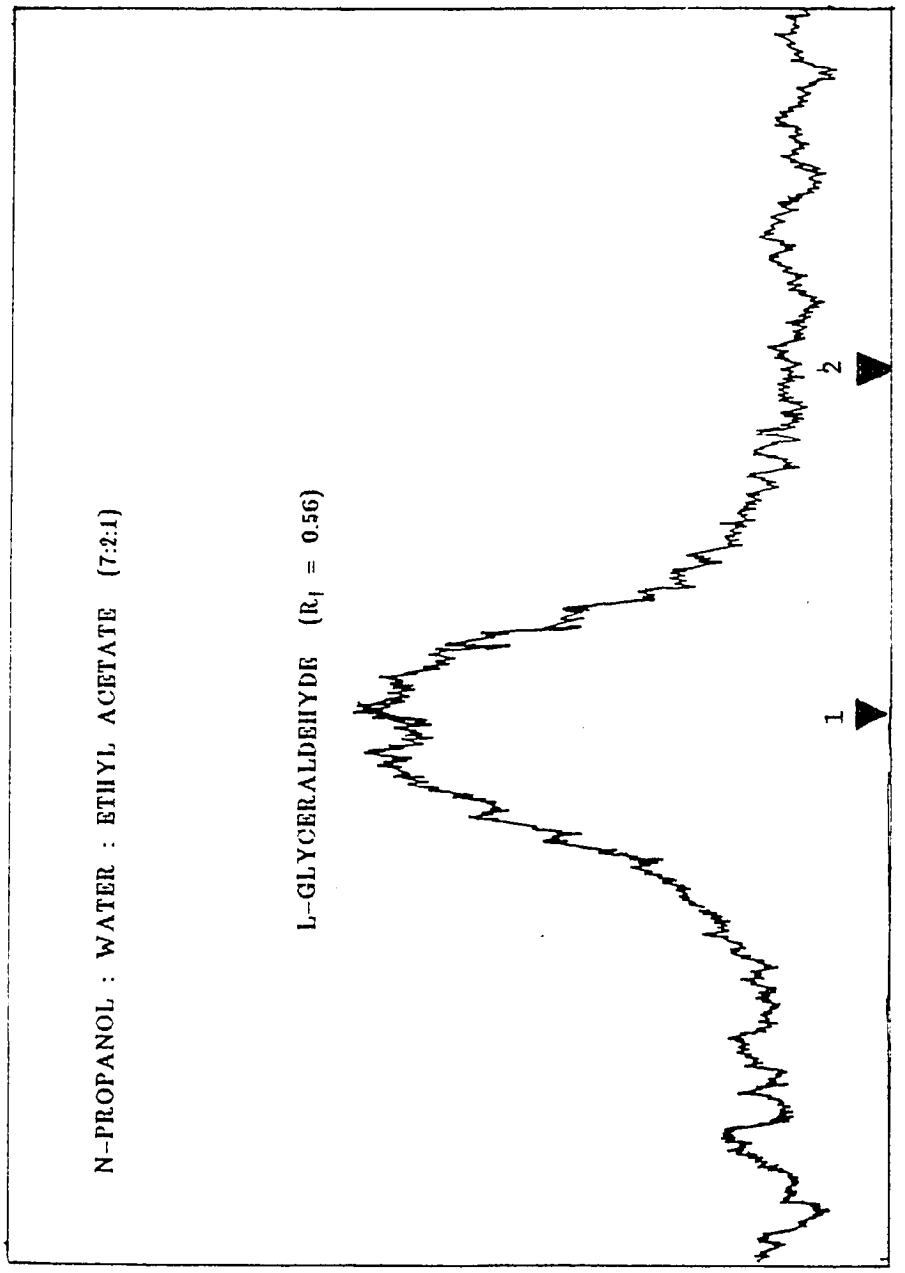

FIG.5 PURIFIED L-(3-^{3}H)GLYCERALDEHYDE

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Fig. 6 L-[3-^{3}H]glyceraldehyde preparation is free from [^{3}H]glycerol

(a) The crude product of L-[3-^{3}H]glyceraldehyde and an authentic sample of [2-^{3}H]glycerol were spotted together on a strip of Whatman 3 MM chromatography paper and developed in ethyl acetate : formic acid : water (7:2:1) solvent system. The radioscan of the chromatogram is shown here.

(b) The crude product of L-[3-^{3}H]glyceraldehyde was chromatographed in the solvent system described in Fig.6a. The chromatogram was scanned on a radio scanner.

1 ---- position of glycerol

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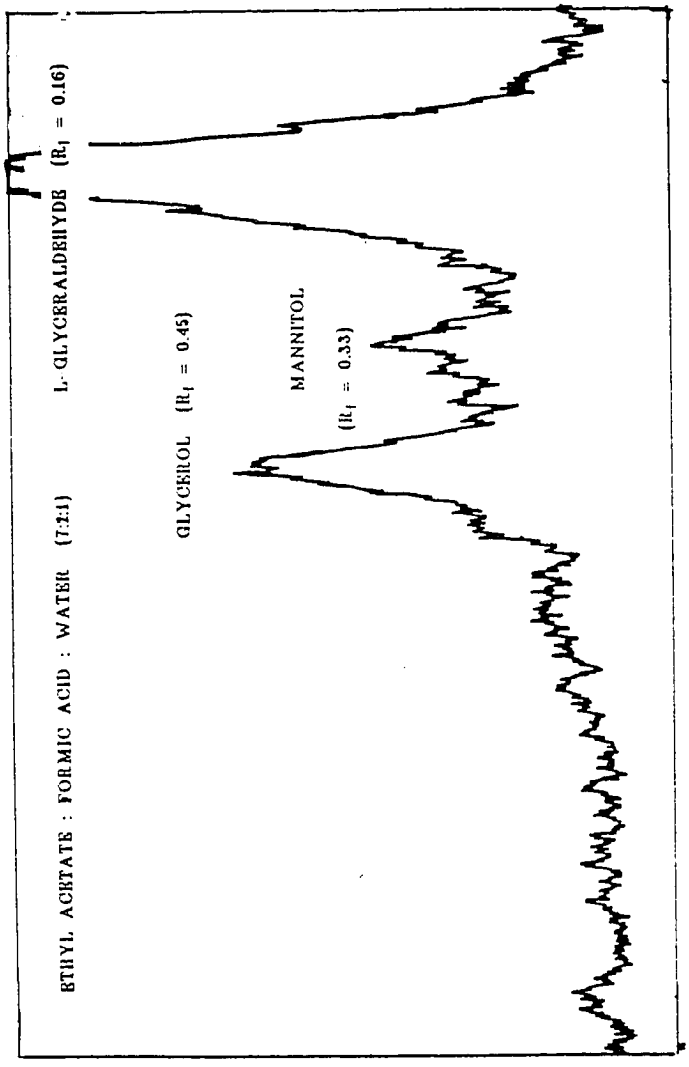

Fig.6a L-[3-^{3}H]GLYCERALDEHYDE, THE CRUDE PRODUCT AND AN AUTHENTIC SAMPLE OF [2-^{3}H]GLYCEROL

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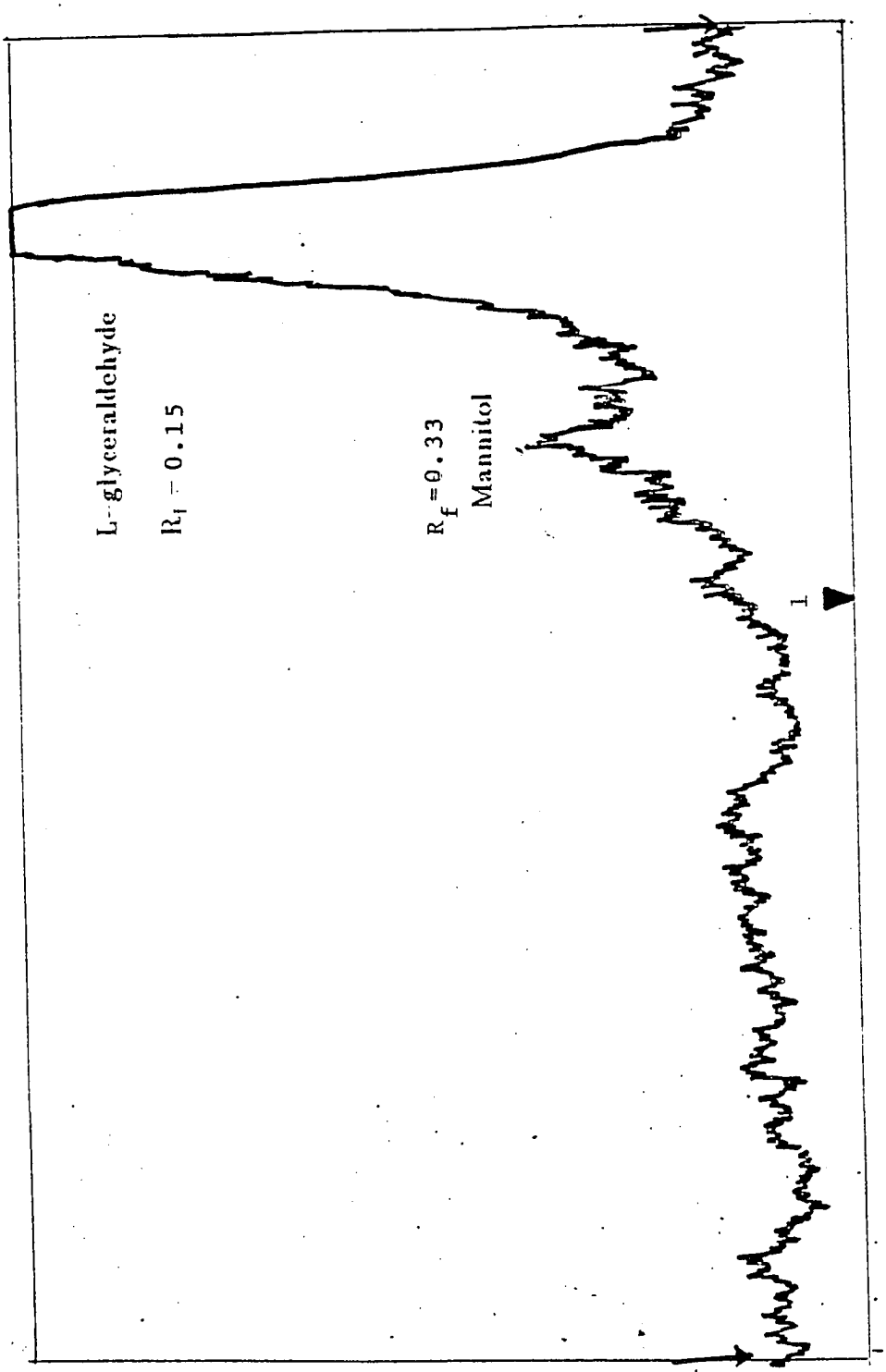

FIG. 6b L-[3-^{3}H]GLYCERALDEHYDE, THE CRUDE PRODUCT

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Fig.7 Phosphorylation of L-[3-^{3}H]glyceraldehyde, as visualized by paper chromatography

(a) About 0.1 μCi of crude L-[3-^{3}H]glyceraldehyde was chromatographed on Whatman 3 MM chromatography paper in n-butanol : glacial acetic acid : water (2:1:1) solvent system. The chromatogram was scanned on a radioscanner. Unlabeled DL-GAP, as identified by 2,4 - DNP spray, migrated with an R_f of 0.30 in this solvent system.

1 --- position of DL-GAP

(b) L-[3-^{3}H]Glyceraldehyde 3-phosphate was synthesized from crude L-[3-^{3}H]glyceraldehyde by the method described in sec 2.2.2. The product was chromatographed in n-butanol : acetic acid : water (2:1:1). Also chromatographed. Unlabeled DL-GAP was chromatographed on the same paper. The Chromatogram of the radioactive material was scanned on a radioscanner.

1 --- position of DL-GAP

2 --- position of L-glyceraldehyde

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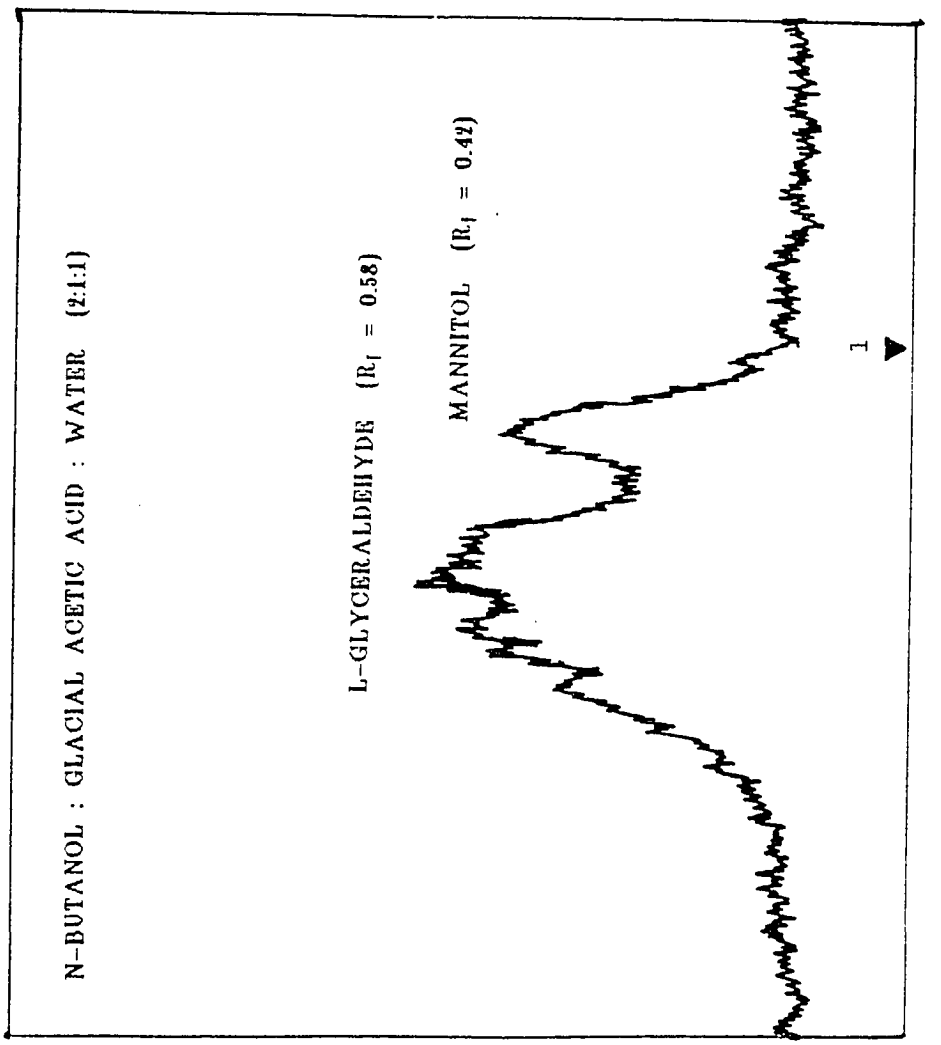

FIG.7a L-[3-^{3}H]GLYCERALDEHYDE, THE CRUDE PRODUCT

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FIG.7b L-(3-^{3}H)GLYCERALDEHYDE 3-PHOSPHATE

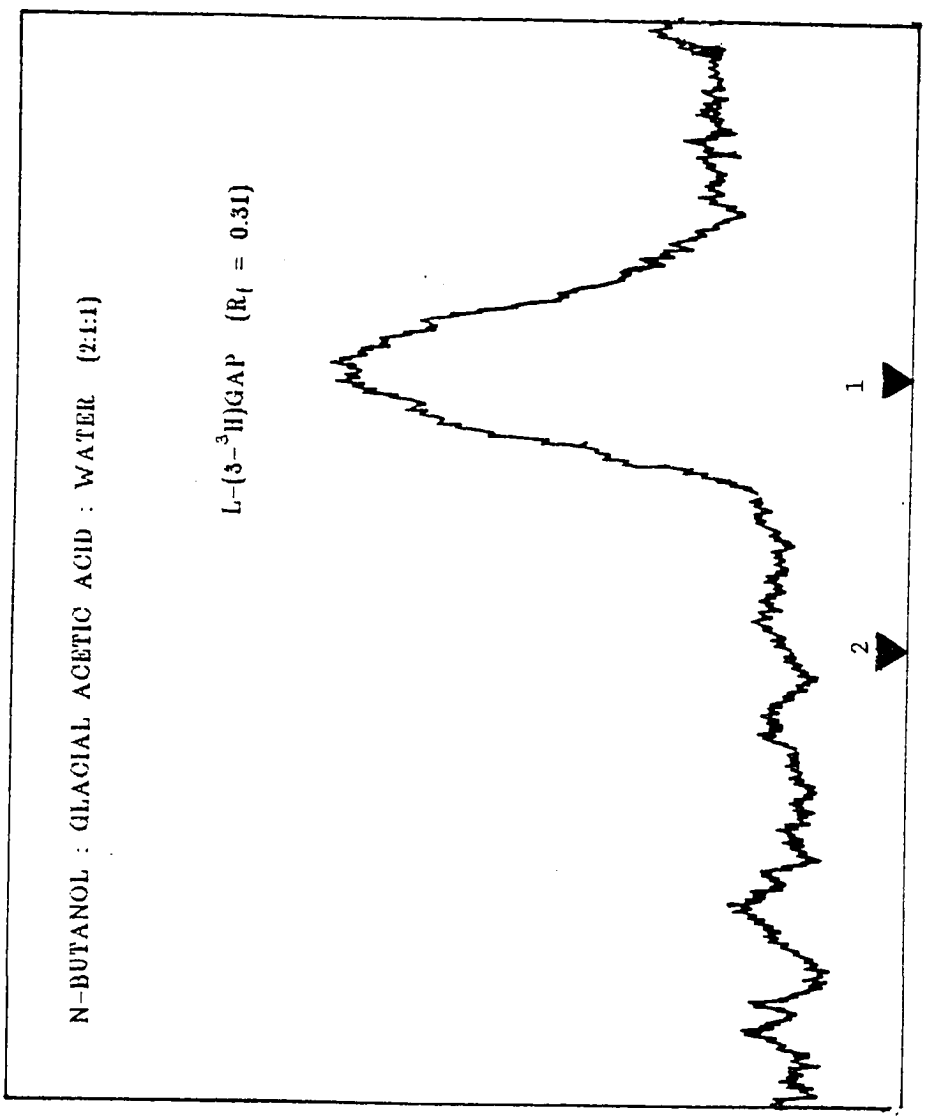

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Fig. 8 Additional chromatographic evidence for the chemical identity of L-[3-^{3}H]GAP

L-[3-^{3}H]Glyceraldehyde 3-phosphate preparation (see legend to Fig.7) was chromatographed in the upper phase of water : sec-butanol : t-butanol (48.4:43:8.6) solvent system. The chromatogram was scanned on a radioscanner. Unlabeled DL-GAP and mannitol were also chromatographed on the same paper, and the positions of the two compounds were identified (R_f , DL-GAP = 0.25 and R_f , mannitol = 0.43) by spraying the chromatograms with 2:4,DNP and $KMNO_4$ + Na_2CO_3 respectively.

1 --- position of unlabeled DL-GAP

2 --- position of mannitol

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FIG.8 PURIFIED L-[3-^{3}H]GLYCERALDEHYDE 3-PHOSPHATE

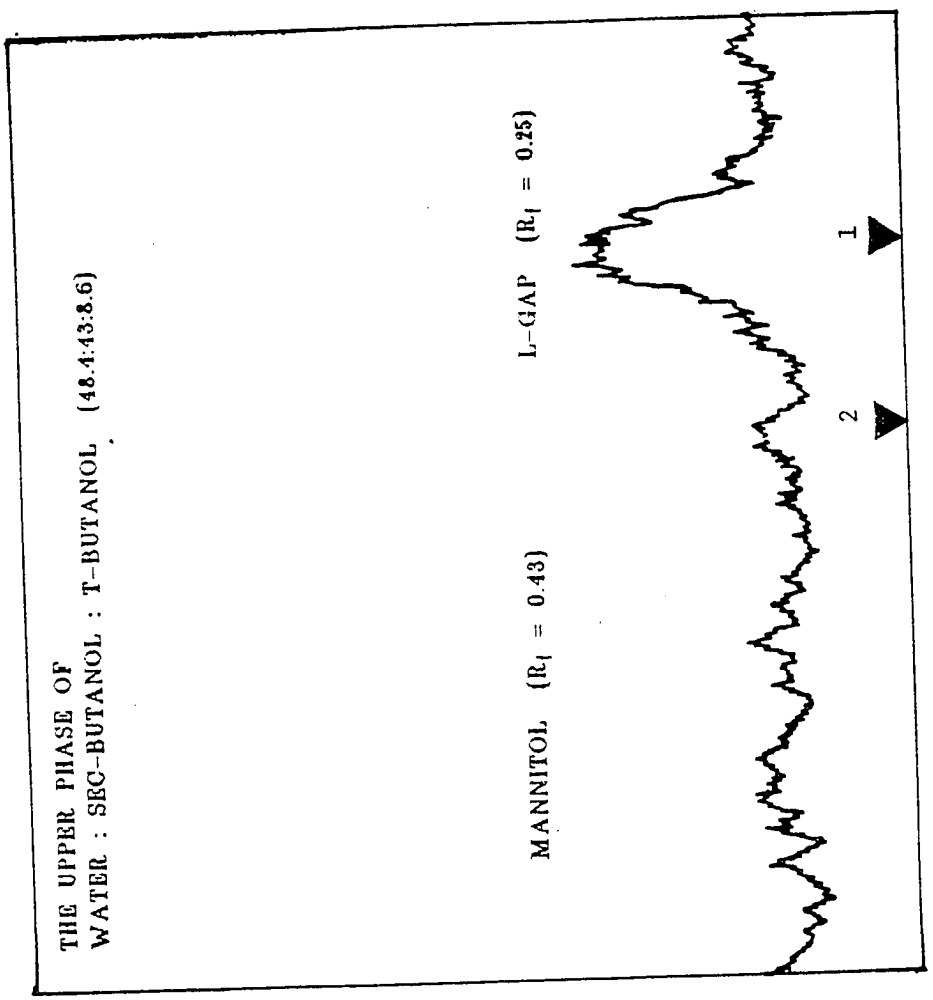

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Fig. 9 Phosphorylation of L-[3-^{3}H]glyceraldehyde takes place to near completion

The water effluent obtained during the purification of L-[3-^{3}H]GAP was condensed in a rotary evaporator and chromatographed in n-butanol : glacial acetic acid : water (2:1:1) solvent system.

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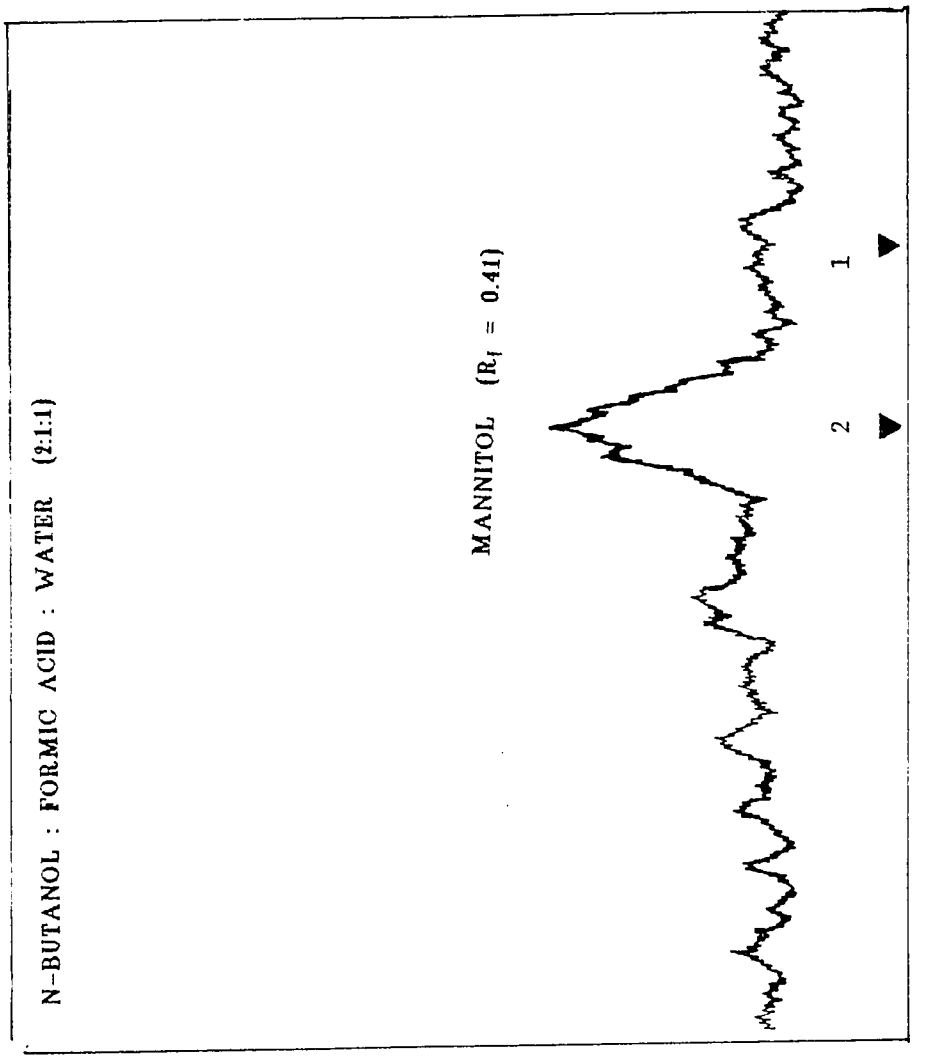

FIG.9 THE WATER EFFLUENT OBTAINED DURING THE PURIFICATION OF L-(3-^{3}H)GAP

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Fig. 10 Putative L-[3-^{3}H]GAP is free from the D isomer

Curve 1; Change of absorbance with time at 340 nm in a reaction mixture containing 23 nmol of DHAP, 200 nmol of NADH, 0.05 units of G3P-dehydrogenase and 10 units of triosephosphate isomerase in 1 ml of 40 mM triethanolamine buffer (pH 7.5).

Curve2; Same as curve 1, but instead of DHAP, 23 nmol of D-GAP was present, as the substrate.

Curve 3; Same as curve 1, but instead of DHAP, 23 nmol of L-[3-^{3}H]GAP (synthesized in sec.3.1.3) was present, as the substrate.

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FIG.10

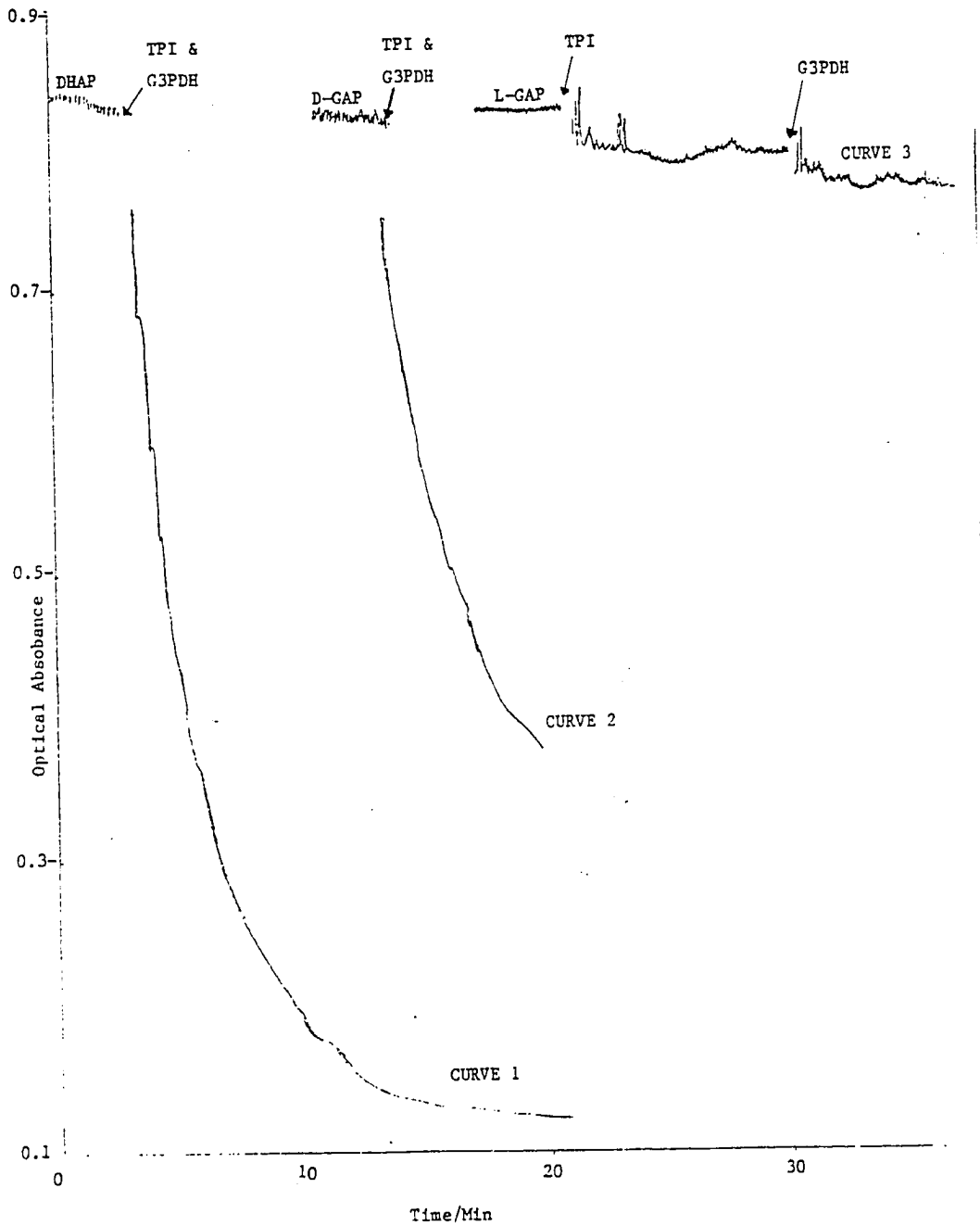

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Fig.11 Incorporation of L-[3-^{3}H]glyceraldehyde into E. coli - phospholipids

E. coli strains 8 and 9 were cultured in bicine + 0.5% potassium succinate medium. At 20 Klett, 5.5 ml of each culture were transfered into 25 ml flasks and treated either with 0.5 ml of [2-^{3}H]glycerol (8.5 µCi/ml, 8.5 µCi/umol) or 0.5 ml of L-[3-^{3}H]glyceraldehyde (8.6 µCi/ml, 8.6 µCi/umol). Incubated at 37°C in a water bath-shaker at 200 rpm. At the indicated times 0.5 ml samples were subjected to Bligh and Dyer extraction. An additional 2 ml sample was removed at 60 min for further analysis.

● --- ● ; strain 9, [2-^{3}H]glycerol treated

▲ --- ▲ ; strain 9, L-[3-^{3}H]glyceraldehyde treated

o --- o ; strain 8, [2-^{3}H]glycerol treated

Δ --- Δ ; strain 8, L-[3-^{3}H]glyceraldehyde treated

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FIG.11 INCORPORATION OF [2-^{3}H]GLYCEROL AND L-[3-^{3}H]GLYCERALDEHYDE INTO PHOSPHOLIPIDS

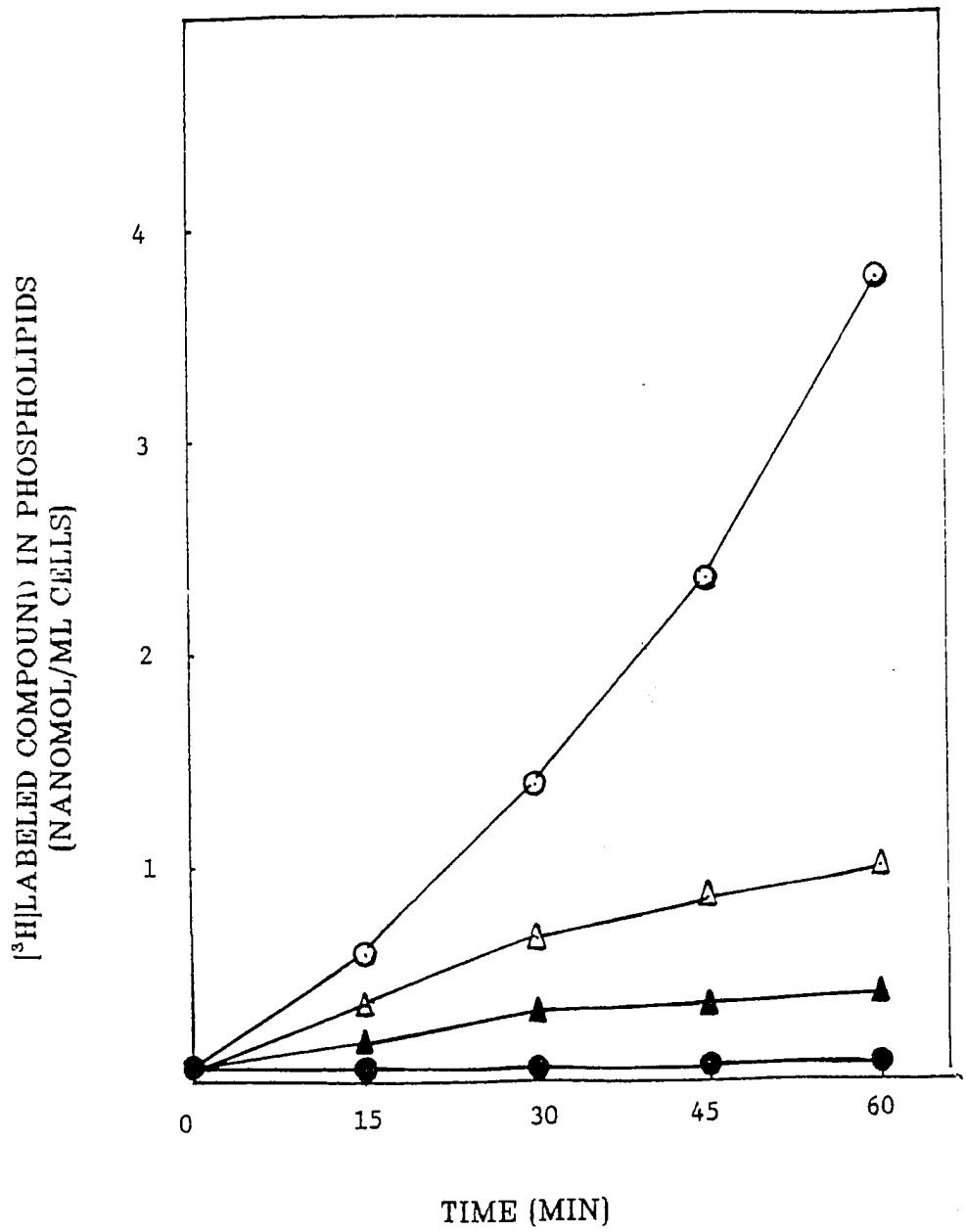

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Fig. 12a TLC of phospholipids from [2-^{3}H]glycerol treated cells

The phospholipid extracts obtained at 60 min from glycerol treated strain 8 was chromatographed on pre-heated (1 hr at 100 °C) sil G thin layer chromatography sheets, in chloroform : methanol : water (65:25:3) solvent system. The chromatograms were developed in an iodine tank. Regions stained by iodine were marked and the respective R_f values were calculated. Individual chromatograms were cut parallel to the solvent front, into approximately 1 cm wide strips. All the strips were counted in toluene scintillation fluid. The distance from the origin against the radioactivity was plotted. (Due to insufficient activity present in the extracts of [2-^{3}H]glycerol treated strain 9 cells, the peaks corresponding to individual phospholipids were not visible on the TLC).

Fig.12b TLC of phopspholipids from L-[^{3}H]glyceraldehyde treated cells

The experiment described in Fig.12a was carried out with the phospholipids extracted from L-[3-^{3}H]glyceraldehyde treated cells.

▨ ; strain9 □ ; strain 8

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FIG.12a TLC OF PHOSPHOLIPIDS LABELED WITH [2-^{3}H]GLYCEROL

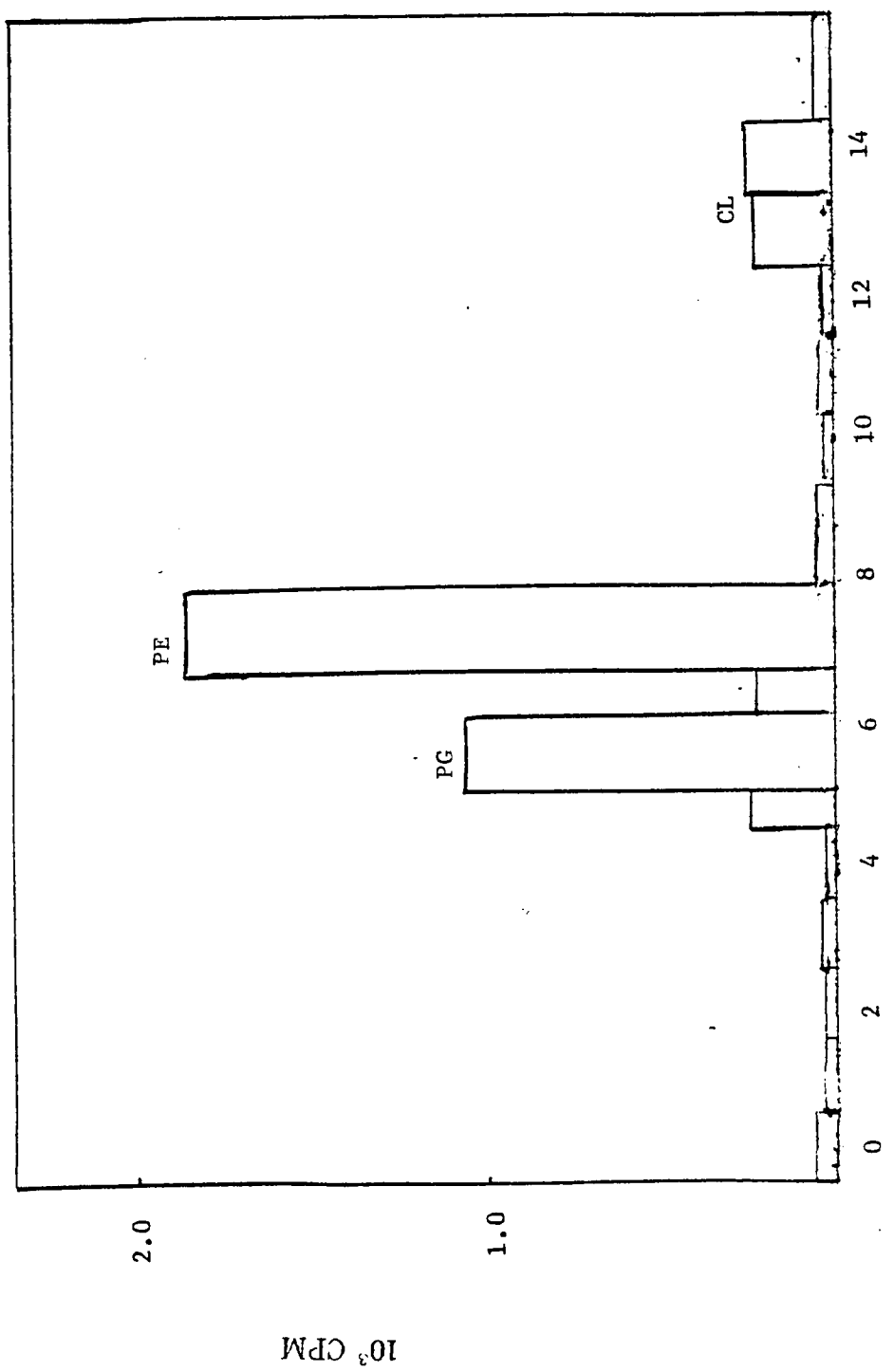

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FIG.12b TLC OF PHOSPHOLIPIDS LABELED WITH L-[3-^{3}H]GLYCERALDEHYDE

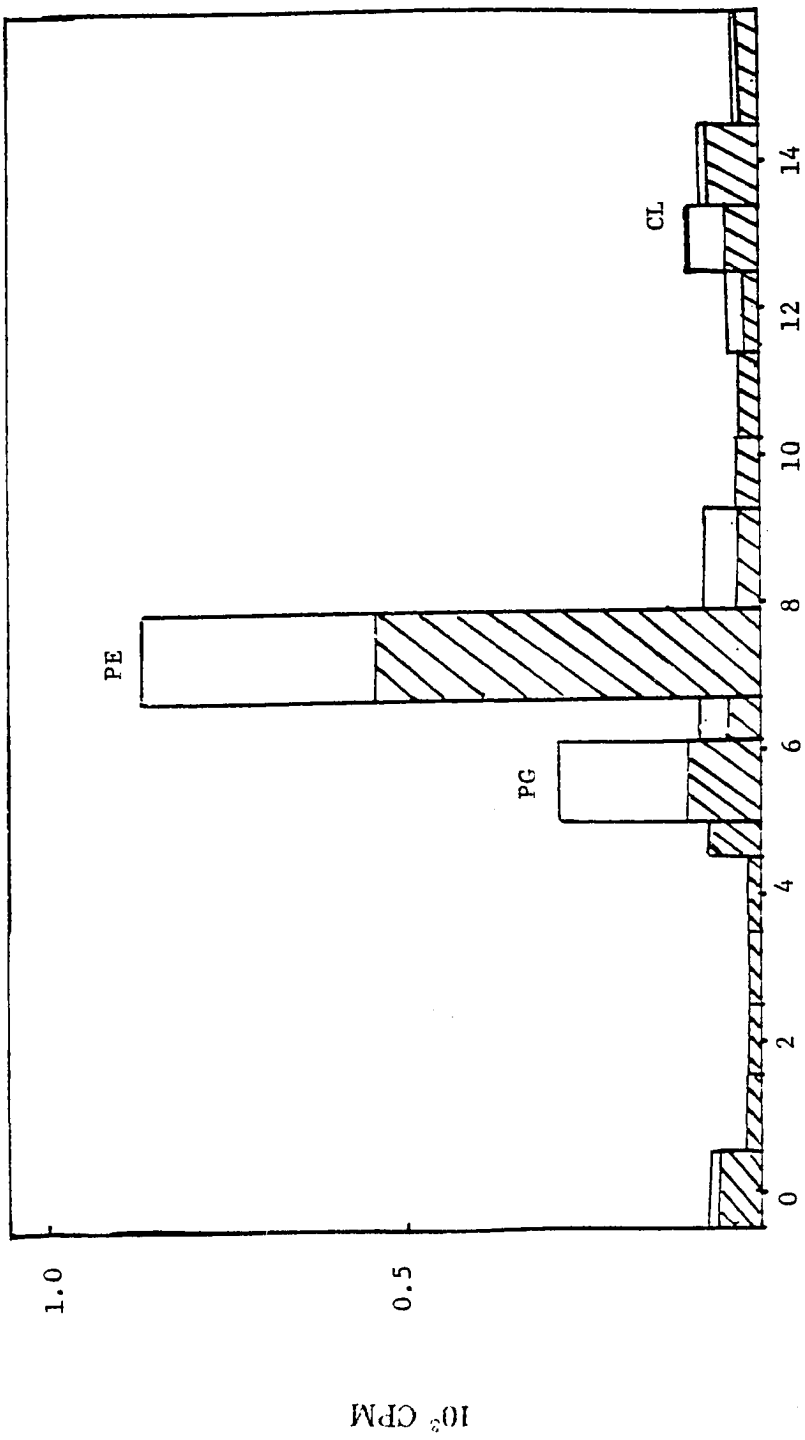

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Fig. 13 Incorporation of L-[3-^{3}H]GAP into E. coli phospholipids.

E. coli strains 8 and 9 were cultured in bicine + 0.5% pottassium succinate medium. At 20 Klett, 5.5 ml of each culture were placed into 25 ml flasks and treated either with 0.5 ml of L-[2-^{3}H]G3P (8.2 µCi/ml, 16.4 µCi/µmol) or 0.5 ml L-[3-^{3}H]GAP (8.4 µCi/ml, 16.8 µCi/µmol). Flasks were incubated at 37° C in a water bath-shaker at 200 rpm. At the indicated times, 0.5 ml samples were subjected to Bligh and Dyer extraction. An additional 2 ml sample was removed for further analysis.

● --- ● ; strain 9, L-[2-^{3}H]G3P treated

▲ --- ▲ ; strain 9, L-[3-^{3}H]GAP treated

o --- o ; strain 8, L-[2-^{3}H]G3P treated

Δ --- Δ ; strain 8, L-[3-^{3}H]GAP treated

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FIG.13 INCORPORATION OF L-[2-^{3}H]G3P AND L-[3-^{3}H]GAP INTO PHOSPHOLIPIDS

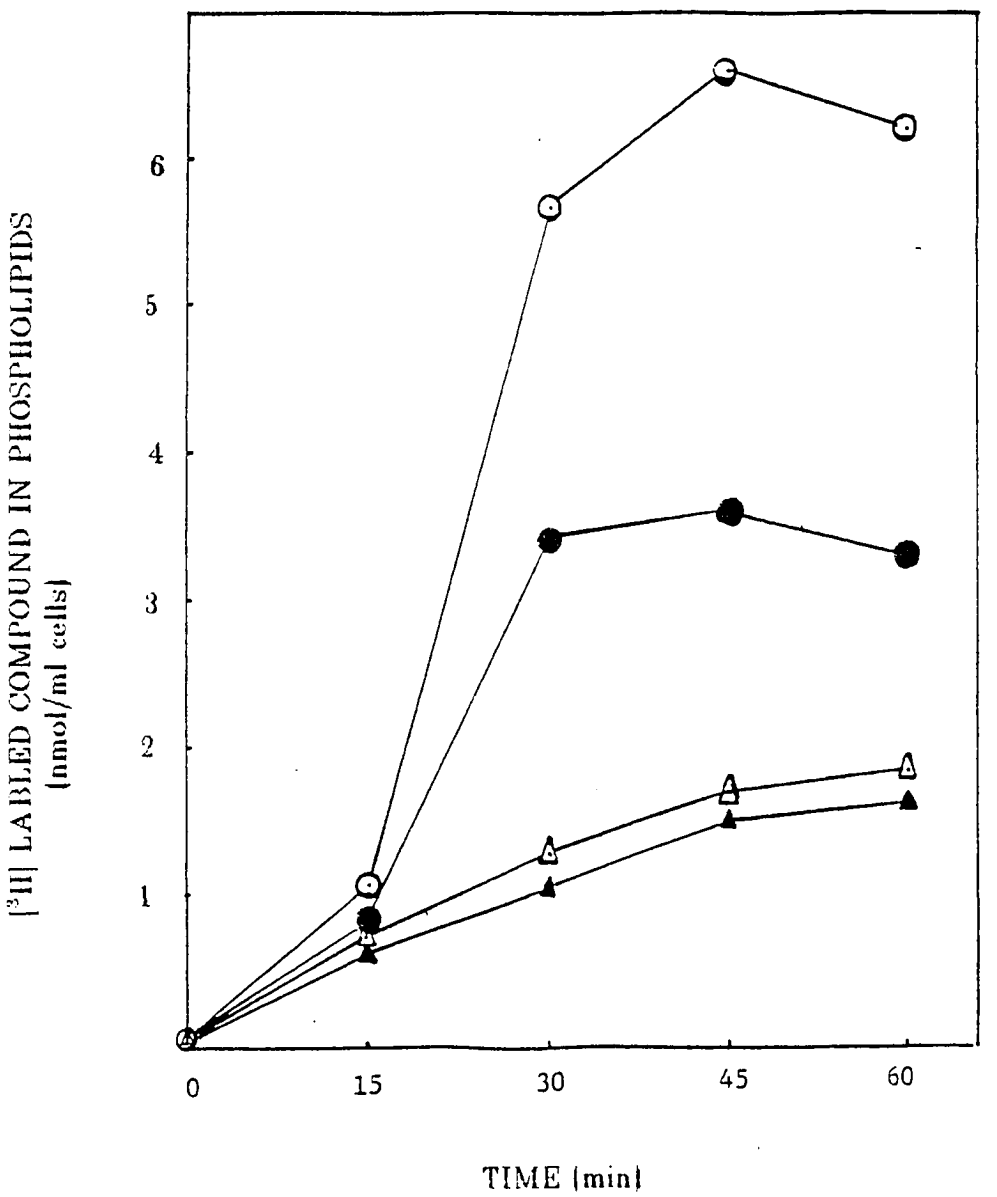

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Fig. 14a TLC of phospholipid extract of [2-^{3}H]G3P treated cells

An experiment simillar to the one described in Fig. 12a was carried out with the phospholipid extracted (legend to Fig. 13) from [2-^{3}H]G3P treated cells.

▨ ; strain 9

□ ; strain 8

Fig. 14b TLC of phospholipid extract of L-[3-^{3}H]GAP treated cells

An experiment simillar to the one described in Fig. 12a was carried out with the phospholipid extracted (60 min sample) from L-[3-^{3}H]GAP treated cells (legend to Fig. 13).

▨ ; strain 9

□ ; strain 8

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FIG.14a TLC OF L-[2-^{3}H]G3P LABELED PHOSPHOLIPIDS

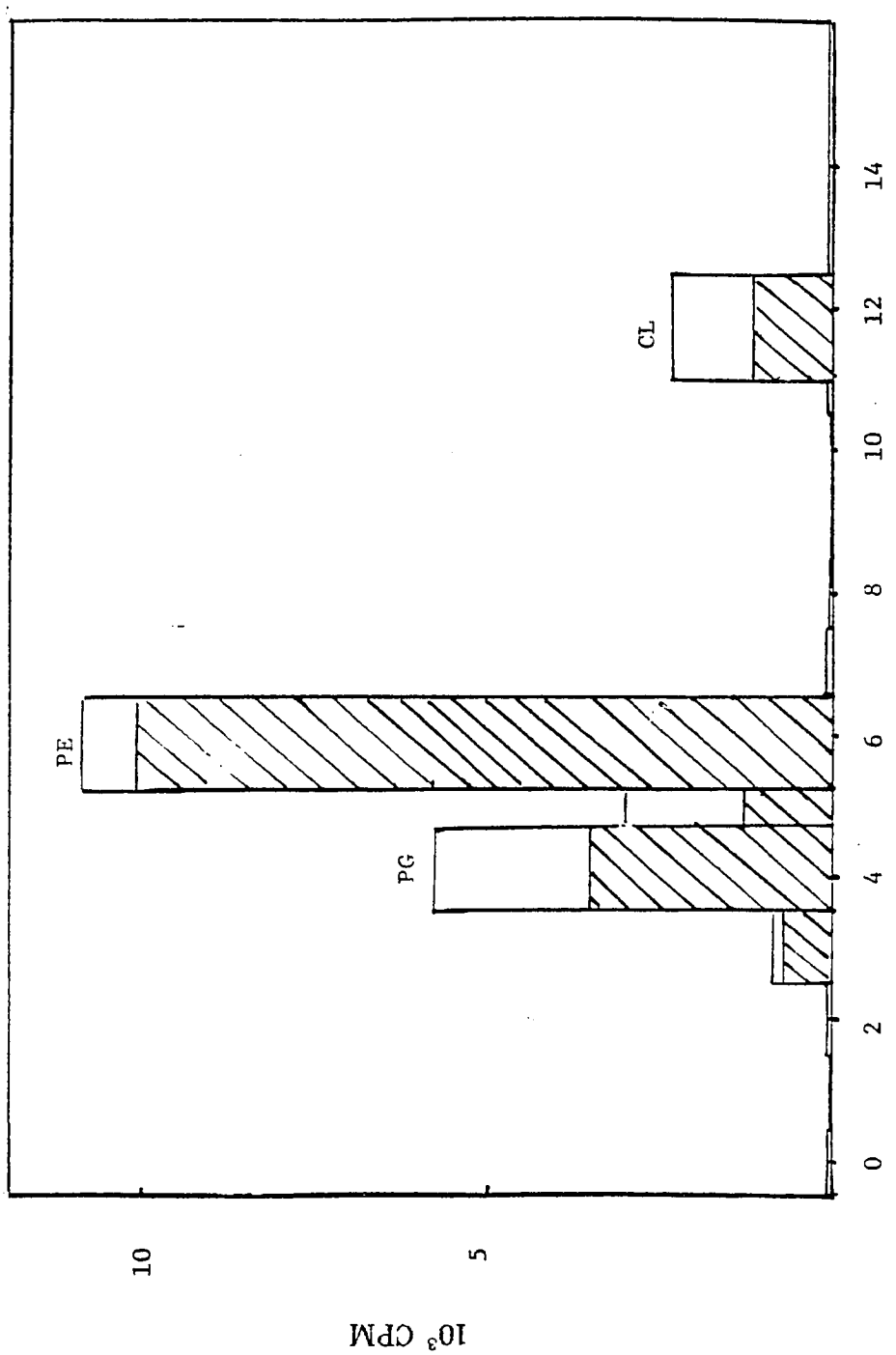

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FIG.14b TLC OF L-[3-^{3}H]GAP LABELED PHOSPHOLIPIDS

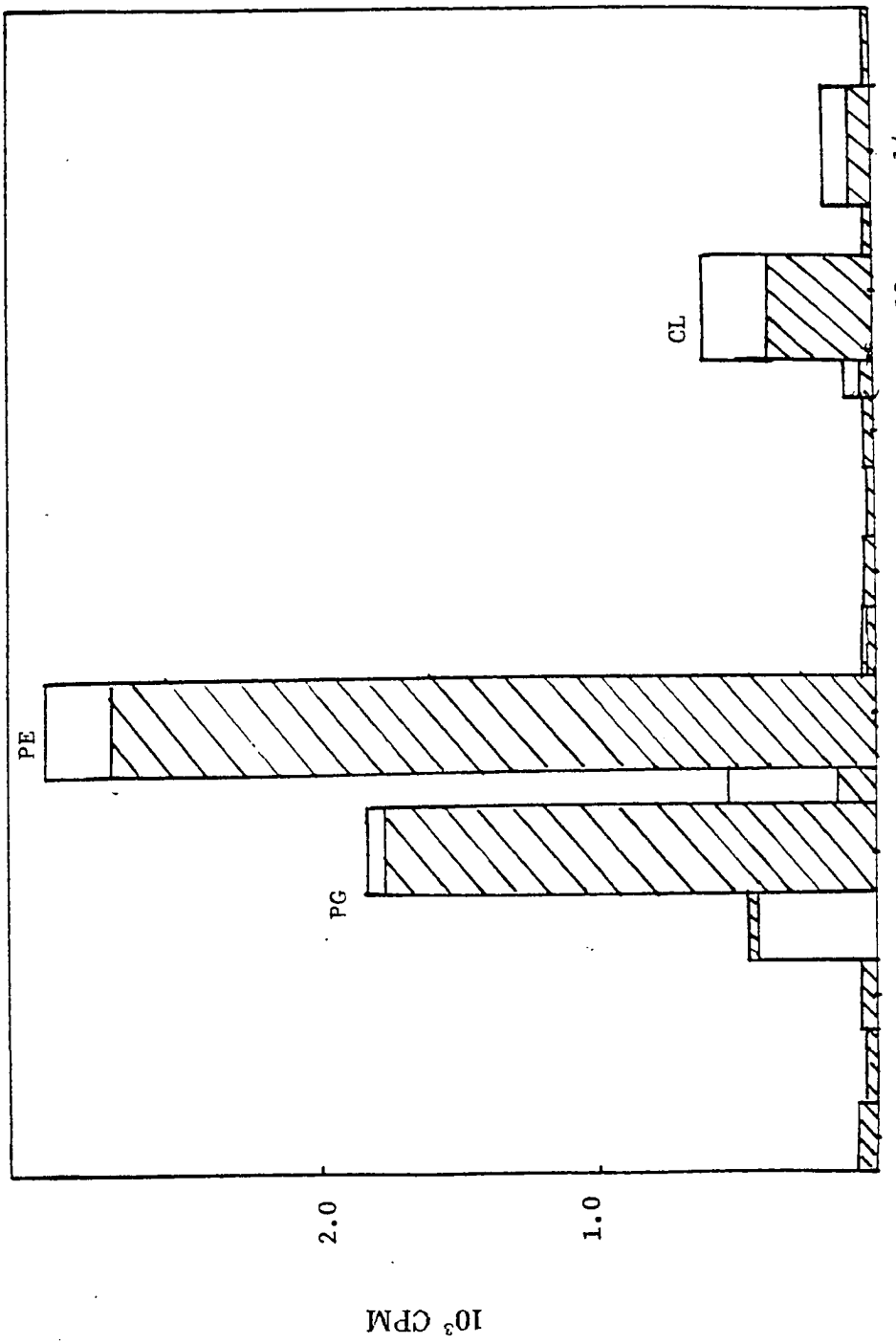

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Fig. 15 A solvent system that can separate a mixture of glycerol, G3P and GAP into individual components

Authentic samples of [2-^{3}H]glycerol, [2-^{3}H]G3P and [3-^{3}H]GAP were chromatographed in ethyl actate : formic acid : water (5:2:1).

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FIG.15 AUTHENTIC SAMPLES OF (2-^{3}H)GLYCEROL, L-(2-^{3}H)G3P AND L-(3-^{3}H)GAP

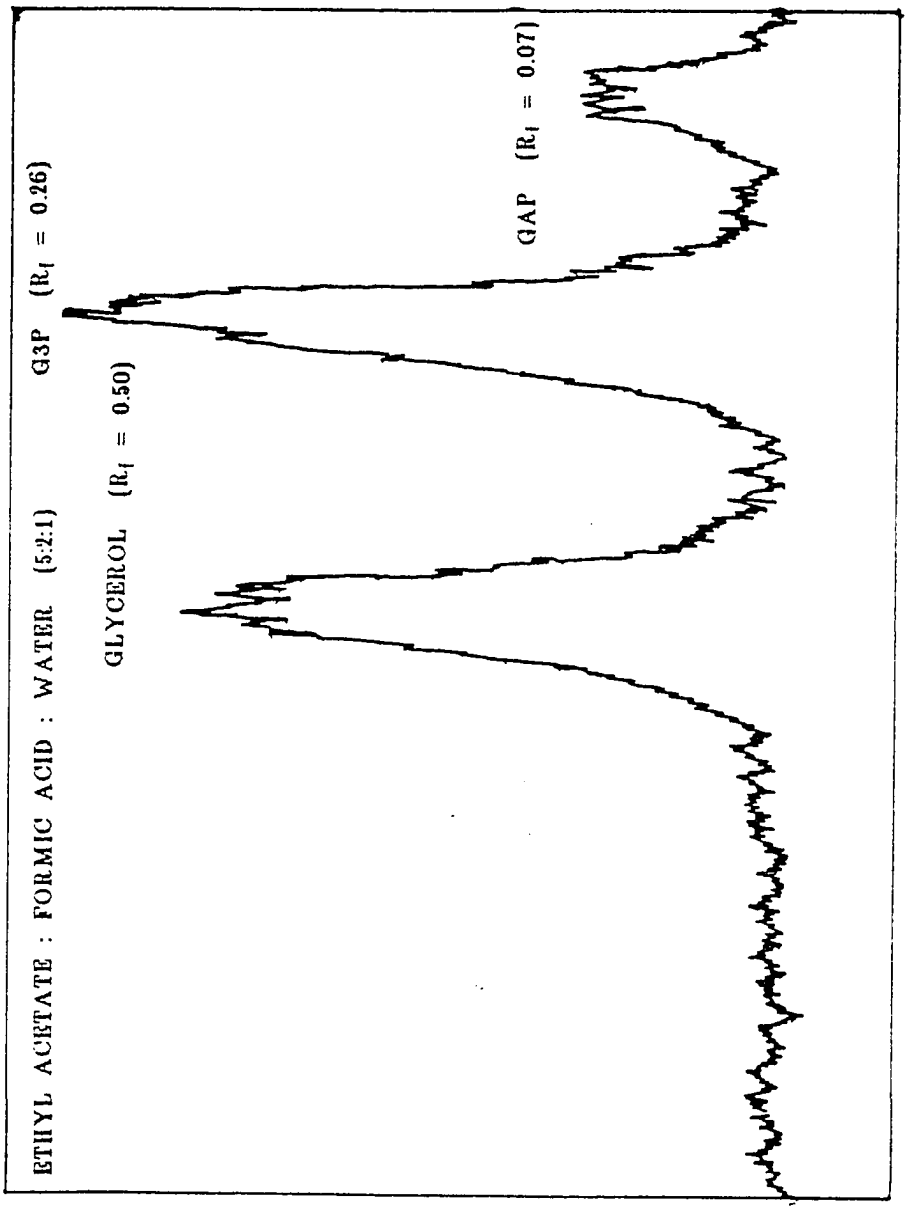

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Fig. 16 Conversion of L-GAP into G3P by E. coli cell extract

A reaction mixture, containing 11 nmol, L-[^{3}H]GAP (44 μCi/μmol), 60 nmol of NADPH, cell extract (45 μg protein) and 330 nmol bicine (pH 7.6) in a volume of 24 μl was incubated at 27°C for 3 hr. (The cell extract was prepared from E. coli strain 8 grown to 70 Klett in bicine medium supplemented with 0.5% K-succinate). The product was spotted on Whatman 3 MM chromatography paper in two regions; a major region consists of 10 spots spanning a length of about 5 cm and a minor region with a single spot. The chromatogram was run in ethyl acetate : formic acid : water (5:2:1) solvent system. The minor region of the chromatogram was scanned on a radioscanner.

1 --- position of glycerol

2 --- position of GAP

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FIG.16 CONVERSION OF L-GAP INTO L-G3P BY *E. COLI* CELL EXTRACT

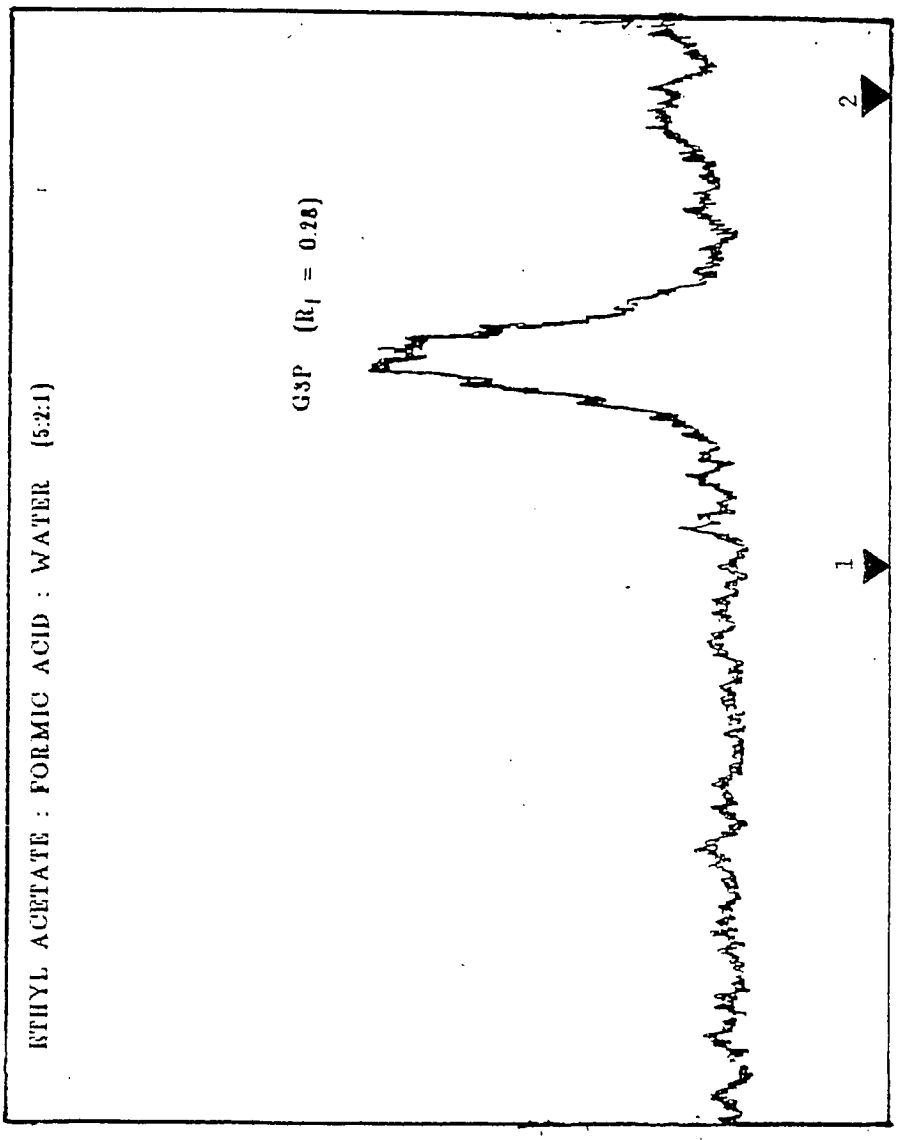

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Fig. 17 Further evidence for the identity of the product of Fig. 16 as G3P

The major region of the chromatogram, described under Fig. 16, was treated as follows. The area corresponding to the G3P peak on the major region of the chromatogram was located and cut out as a single peak. G3P was eluted from the paper with distilled water by descending chromatography set up. The effluent (about0.45 μCi in 7.5 ml) was condensed in a rotary evaporator, at about 35°C, to a final volume of about 1 ml. This was brought to complete dryness by blowing nitrogen over the tube. The product was dissolved in 10 μl of 0.1 M, CO_3^{-2} /HCO_3^- buffer, pH 10.1, and treated with 4 ug of alkaline phosphatase (0.0.05 units/μg) for 1 hr at 37°C. The product was chromatographed in ethyl acetate : formic acid : water (5:2:1) solvent system. The chromatogram was scanned on a radioscanner. The positions of authentic samples are also indicated.

1 --- position of GAP

2 --- position of G3P

3 --- position of glycerol

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FIG.17 THE PRODUCT SHOWN IN FIG.13 WAS HYDROLYZED WITH ALKALINE PHOSPHATASE.

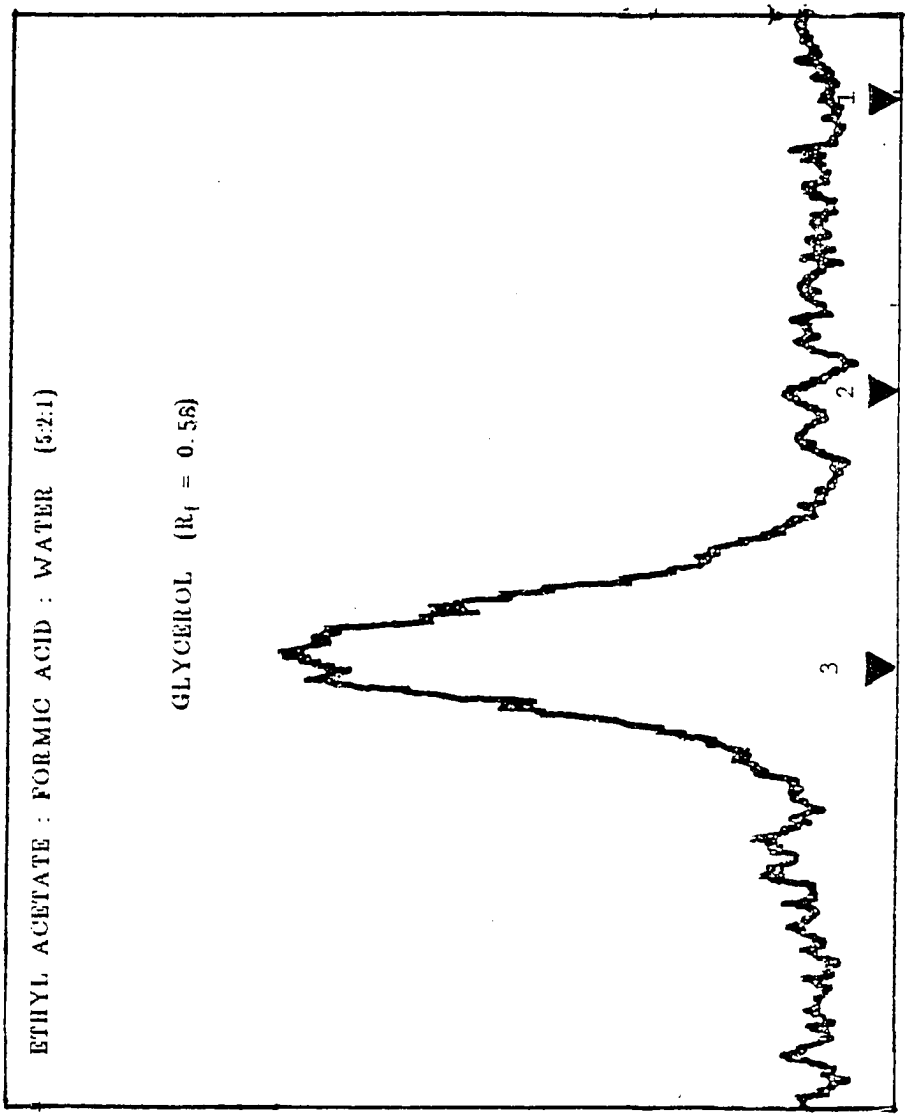

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Fig. 18 Identification of the reaction products of Fig. 16 using a second solvent system

(a) Authentic samples of L-[2-^{3}H]G3P and [2-^{3}H]glycerol were chromatographed in t-butanol : methyl ethyl ketone : water : ammonium hydroxide (4:3:2:1). The chromatogram was scanned on a radioscanner.

(b) The product obtained in Fig. 16 was also chromatographed in t-butanol : methyl ethyl ketone : water : ammonium hydroxide (4:3:2:1) solvent system. Positions of authentc samples are also indicated.

1 --- position of GAP

2 --- position of G3P

3 --- position of glycerol

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FIG.18a AUTHENTIC SAMPLES OF (2-^{3}H)GLYCEROL AND (2-^{3}H)GLYCEROL 3-PHOSPHATE

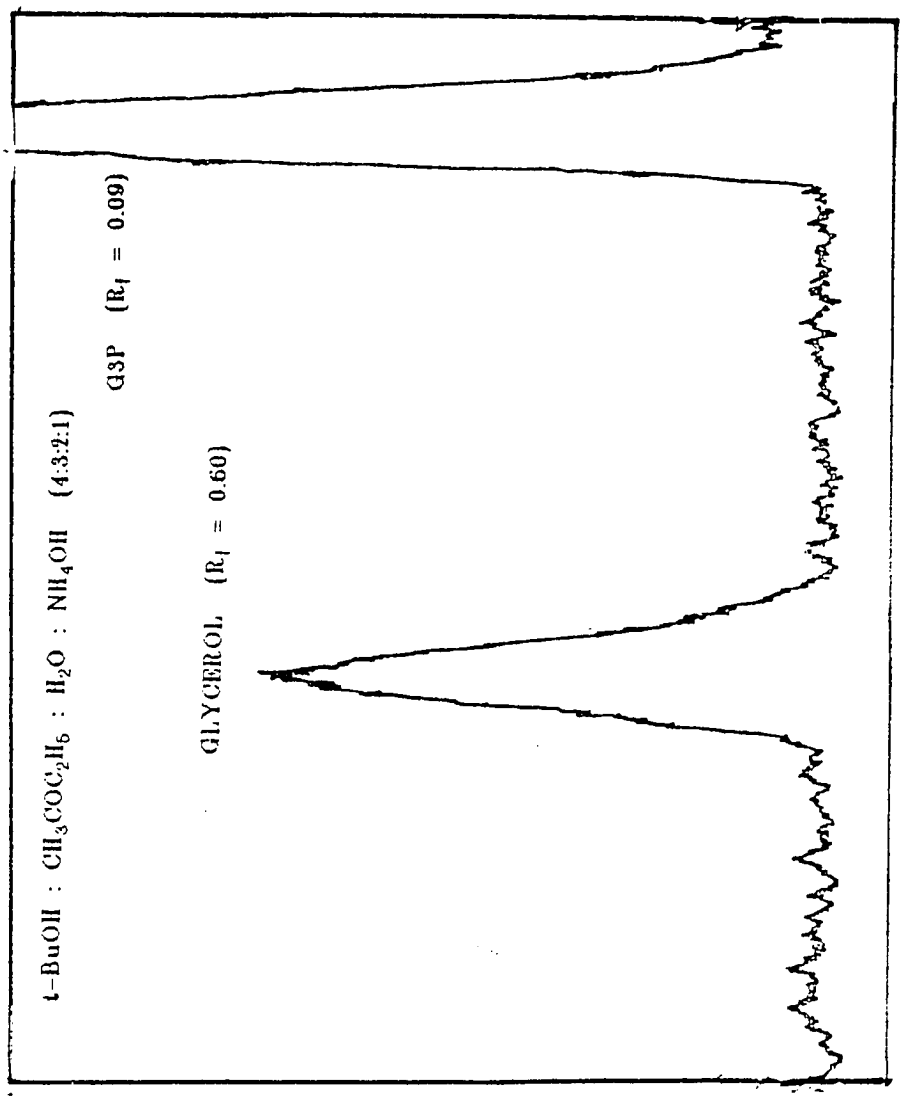

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FIG18b PRODUCT OF L-[3-^{3}H]GAP + NADPH + CELL EXTRACT

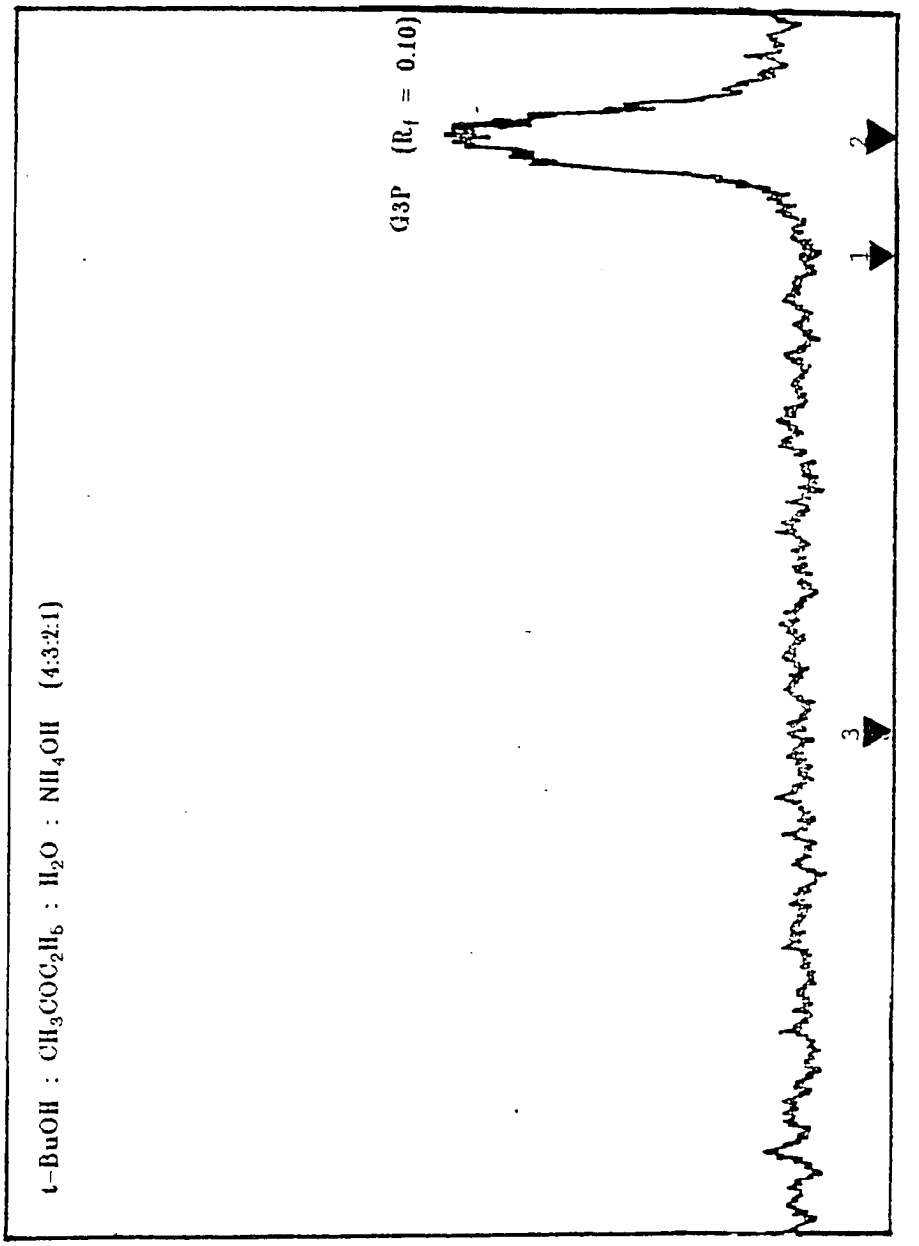

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Fig. 19 Identification of the product of Fig. 17 using a second solvent system

The product obtained in Fig. 17 was also chromatographed in t-butanol :methyl ethyl ketone : water : ammonium hydroxide (4:3:2:1). The chromatogram was scanned on a radioscanner. The positions of authentic samples are also indicated.

1 --- position of GAP

2 --- position of G3P

3 --- position of glycerol

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FIG.19 PRODUCT SHOWN IN FIG.15 WAS HYDROLYZED WITH ALKALINE PHOSPHATASE

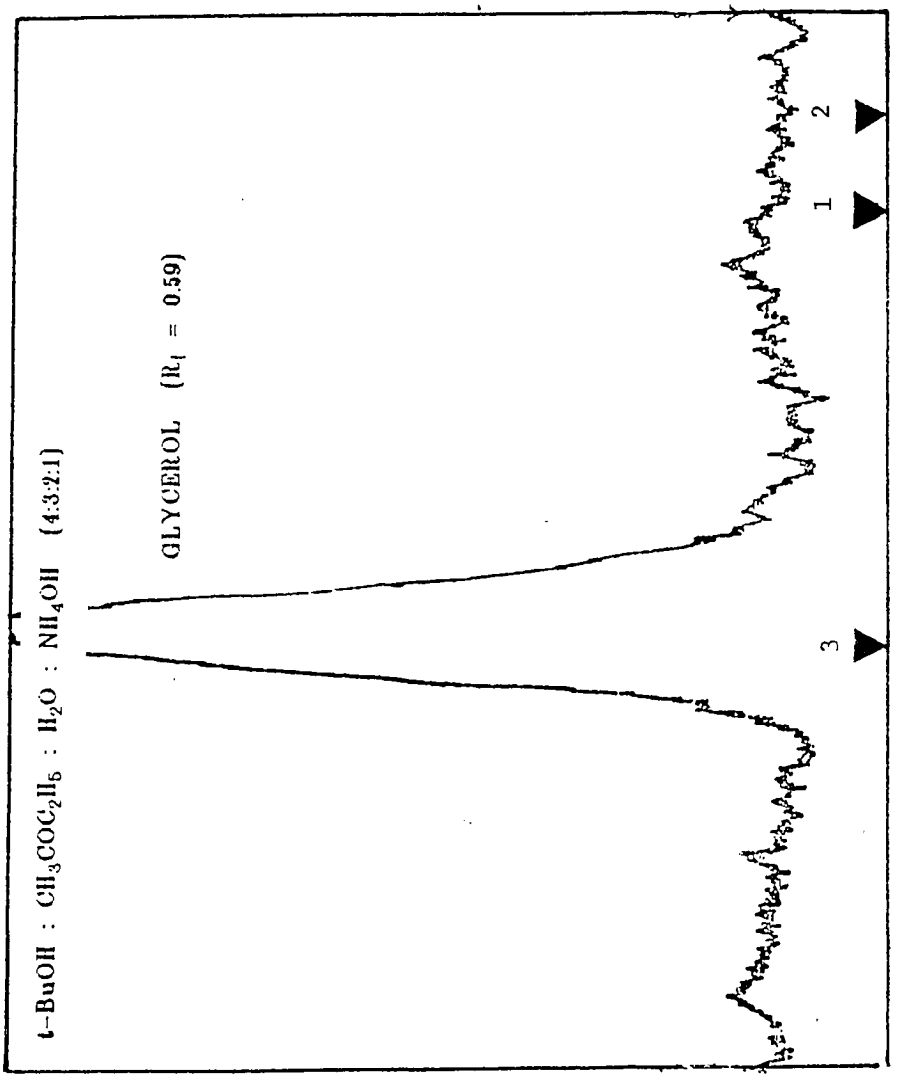

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Fig. 20 Demonstration of the NADPH requirement for the L-GAP reductase activity

A reaction mixture containing all the components, except NADPH, described in Fig. 16, was incubated for 3 hr at 27°C. The product was chromatographed in ethyl acetate : formic acid : water (5:2:1) solvent system. The chromatogram was scanned on a radio scanner.

1 --- position of GAP

2 --- position of G3P

3 --- position of glycerol

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FIG.20 PRODUCT OF L-[3-^{3}H]GAP + CELL EXTRACT

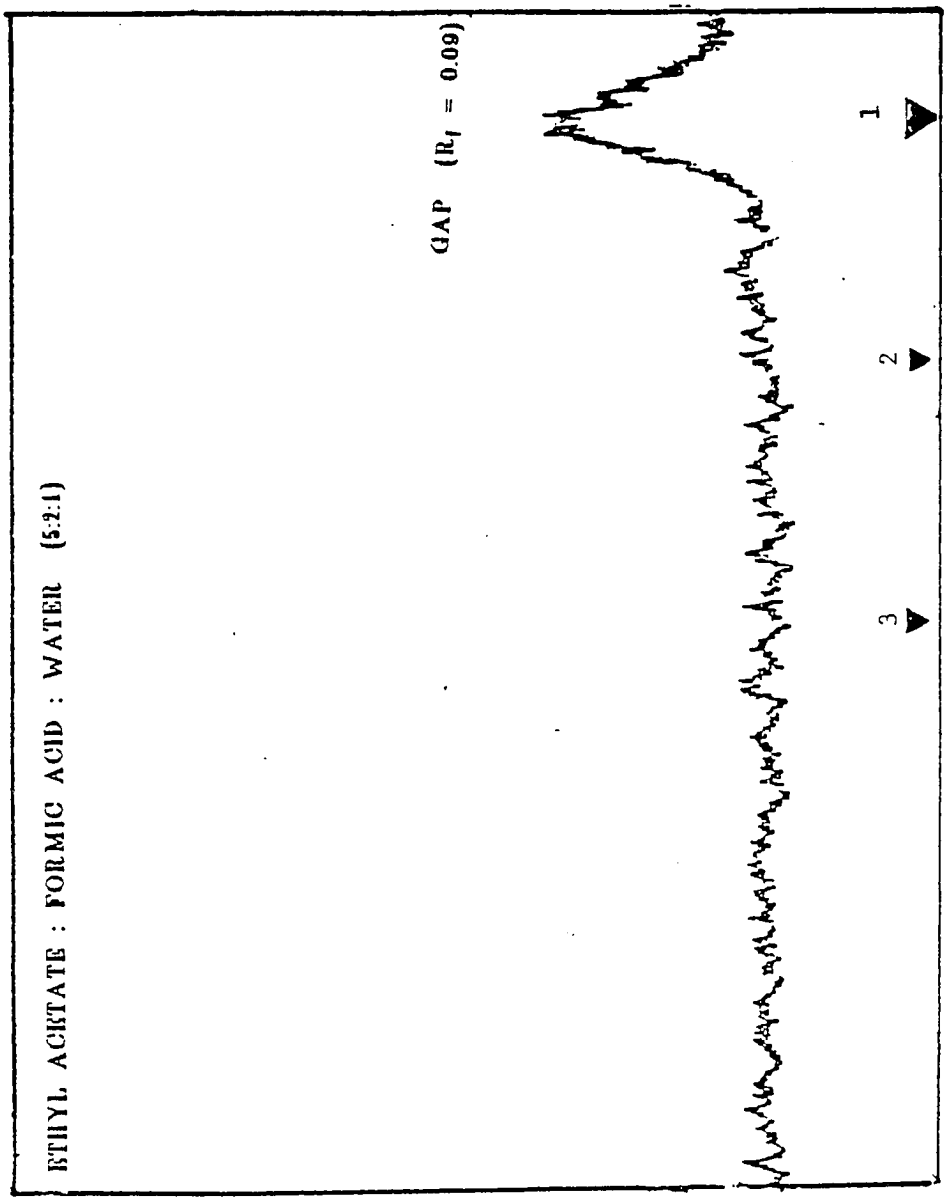

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Fig. 21 Calibration curve for the [^{3}H]G3P assay by solvent extraction method

Different amounts; 0, 5, 10, 15, 20 and 25 µl of [2-^{3}H]G3P (9.2 µCi/ml, 0.5 mM) were placed in small test tubes and added distilled water to bring the volume to 30 µl. Ten microliters of cell extract were added into each tube. Ten microliter samples from each tube were transfered into tubes containing 2,4 dinitrophenylhydrazine reagent and subjected to incubation followed by solvent extraction as described in sec.2.2.4.b. The quantity of G3P displayed by the assay data is plotted against the amount of G3P taken into the assay mixture. The quantities are expressed in CPM.

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FIG.21 CALIBRATION CURVE FOR THE ASSAY

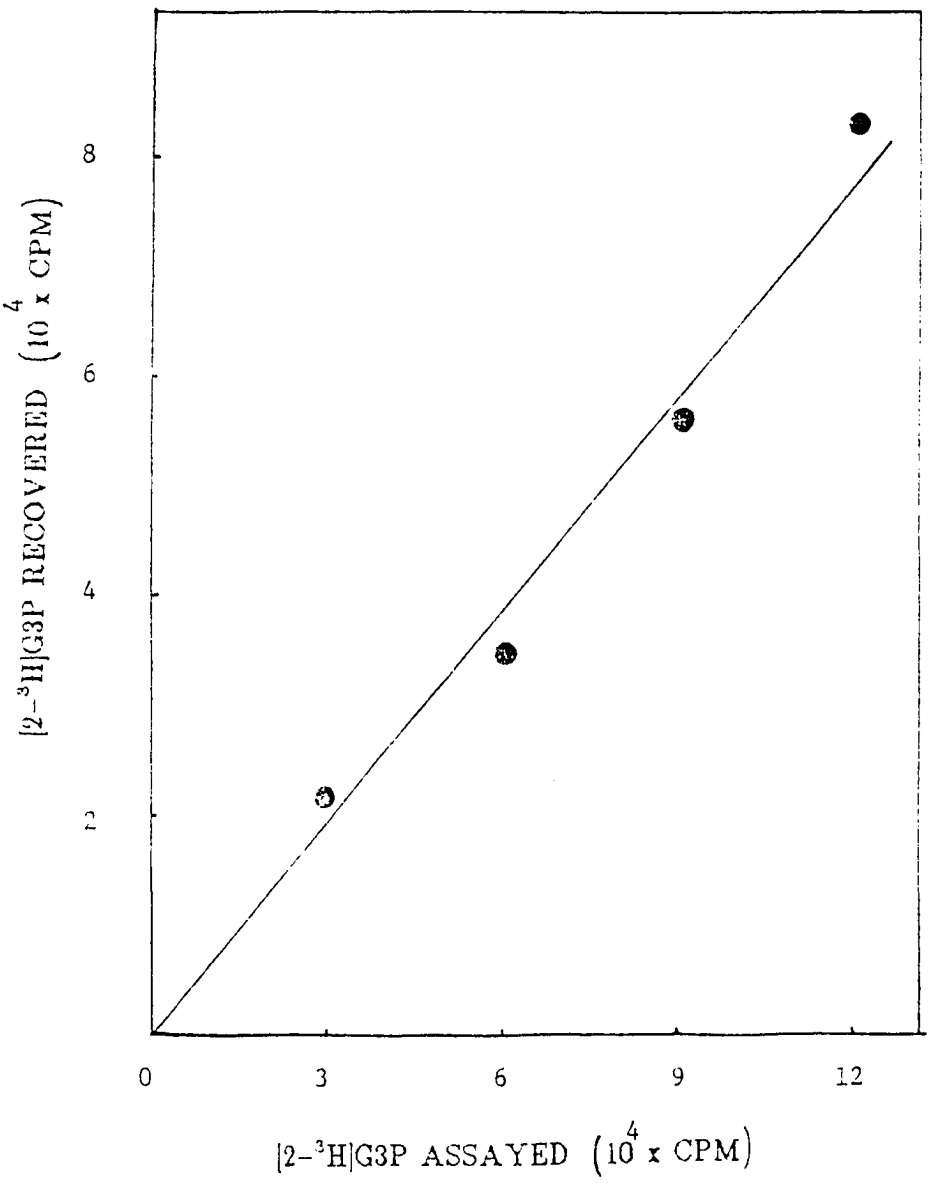

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Fig.22 Comparison of the solvent extraction method of assay with the chromatographic method

A time course study on conversion of L-[3-^{3}H]GAP into [3-^{3}H]G3P was carried out. The assay mixture contained 23.8 nmol, L-[3-^{3}H]GAP; 600 nmol, NADPH; 2.3 μmol, bicine (pH 7.6) and 22.5 μl of cell extract in a total volume of 215 μl. The reaction mixture was incubated at 27 ^{o} C. At indicated time intervals, samples were assayed by both chromatographic (sec 2.2.4.a) and solvent extraction (sec.2.2.4.b) methods.

G3P formed per 1 ml of the assay mixture is plotted against time.

O---- O Assayed by solvent extraction method

●---- ● Assayed by chromatographic method

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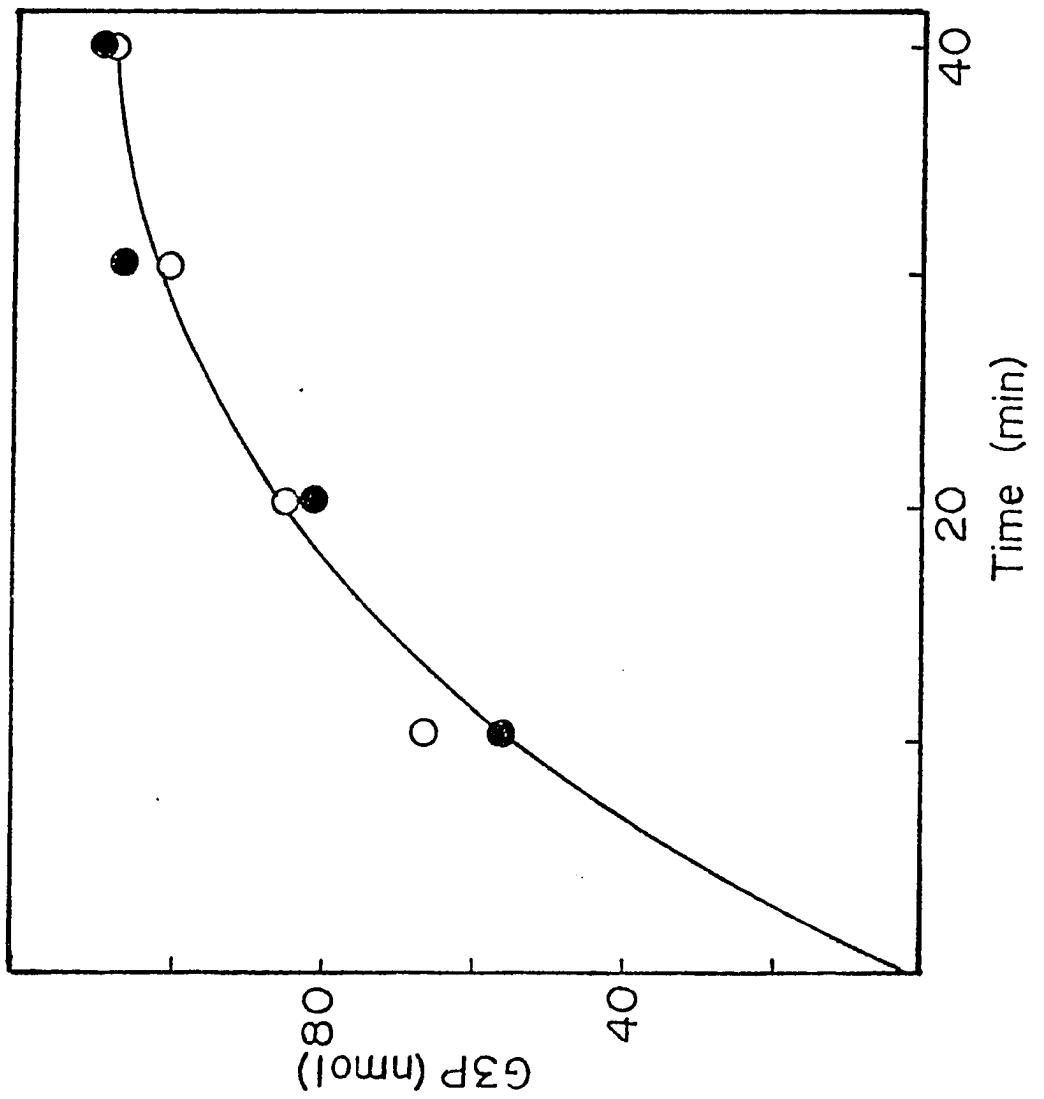

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Fig.23 Initial velocity vs enzyme concentration

Initial velocities of L-GAP ---> L-G3P reaction was determined at several different enzyme concentration. Tubes containing, 13.2 nmol, L-[3-^{3}H]GAP; 300 nmol, NADPH and 1.2 μmol bicine (pH 7.6) and indicated amounts protein (strain 8 cell extract) in a total volume of 107 μl were incubated at 27°C. Samples were assayed by the solvent extraction method.

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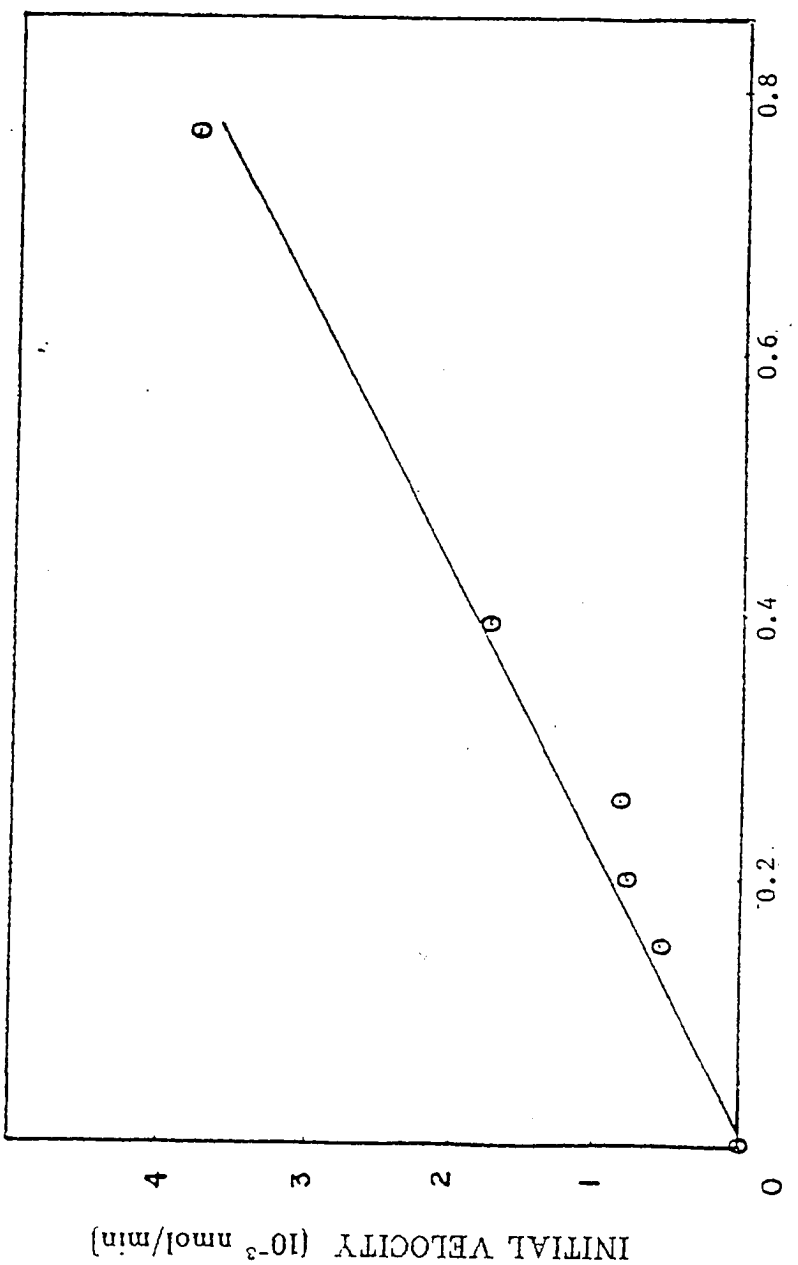

FIG.23 INITIAL VELOCITY vs ENZYME CONCENTRATION

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Fig. 24 Membrane fraction of the cell extract has no activity

E. coli strain 8 was grown in bicine + 0.5% potassium succinate. At 170 Klett, cells were harvested and the soluble and the membrane fractions of the cell extract were prepared. Both fractions were assayed for the L-GAP reductase activity by the solvent extraction method. The assay mixture contained 16 nmol, L-[3-^{3}H]GAP, 1.4 µmol, bicine (pH 7.6), 360 nmol, NADPH and 30 µl cell extract in a total volume of 132 µl.

G3P formed per 1 ml of assay mixture is plotted against time.

o -----o soluble fraction

x -----x membrane-bound fraction

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FIG.24 ACTIVITIES OF SOLUBLE AND MEMBRANE PORTIONS OF THE CELL EXTRACT

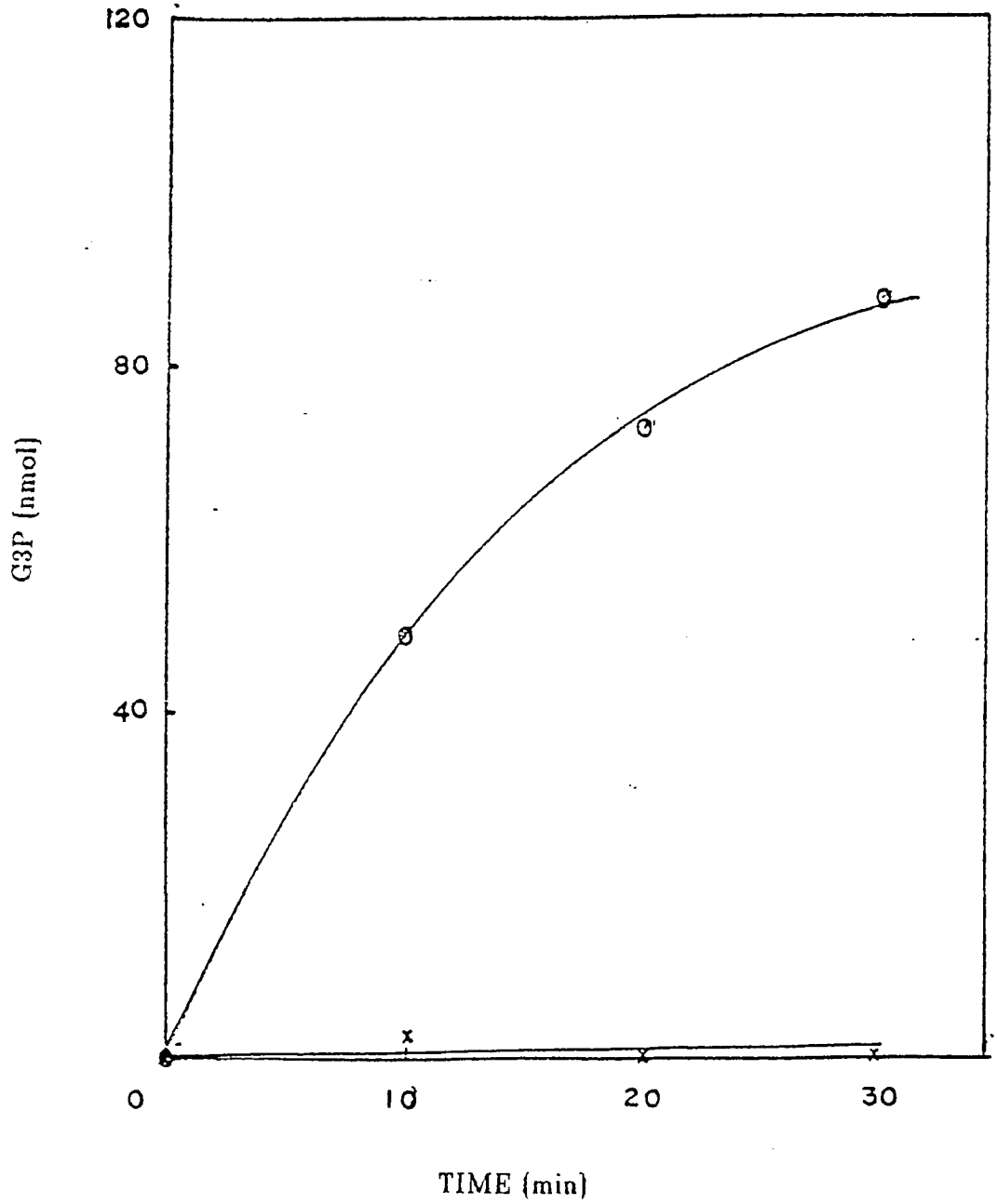

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Fig.25 L-GAP reductase activity in strain BB20

E. coli strains BB20 and strain 8 were grown in GL + K-succinate medium (for BB20 the growth medium was supplemented with 100 µM G3P). Cell extracts were prepared from cultures grown to about 140 klett units and the extracts were assayed for L-GAP reductase by the solvent extraction method. The assay mixture contained 9.5 nmol, L-[3-^{3}H]GAP; 0.72 µmol, bicine (pH 7.6); 180 nmol, NADPH; and 15 µl of cell extract in a total volume of 66 µl.
G3P formed per 1 ml of assay mixture is plotted against time.

O -- O ; Strain 8

Δ -- Δ ; BB20

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FIG.25 THE REDUCTASE ACTIVITY IN THE EXTRACT OF STRAIN BB20

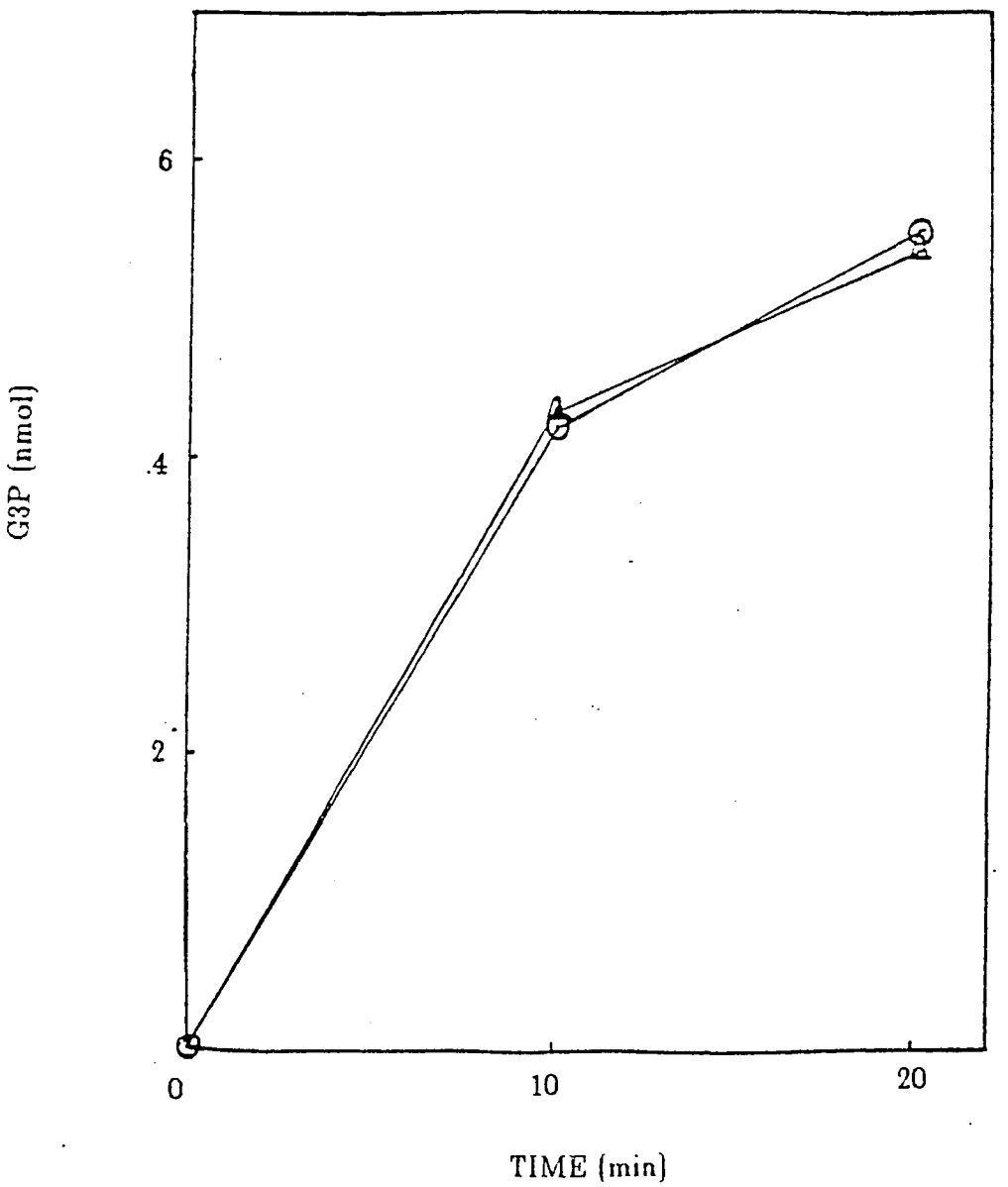

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Fig. 26 Effect of carbon source on the reductase activity

E. coli strain 8 was grown in 125 ml of 3 different media, CH, bicine medium + 0.5% glucose, and bicine medium + 0.5% potassium succinate to 70 Klett. Cell extracts were prepared in 5 ml bicine buffer (pH 7.6). Samples, of the three extracts, containing 130, 125, and 112 µg protein respectively, were assayed for L-GAP reductase activity by the solvent extraction method. The assay mixture contained 24 nmol, L-[3-^{3}H]GAP; 1.4 µmol, bicine (pH 7.6), 360 nmol, NADPH; and the cell extract in a total volume of 132 µl.

G3P formed per 1 ml of assay mixture is plotted against time.

● ----- ● casein hydrolysate

0 ----- 0 bicine + 0.5% glucose

Δ ----- Δ bicine + 0.5% potassium succinate

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FIG.26 EFFECT OF CARBON SOURCE ON THE REDUCTASE ACTIVITY

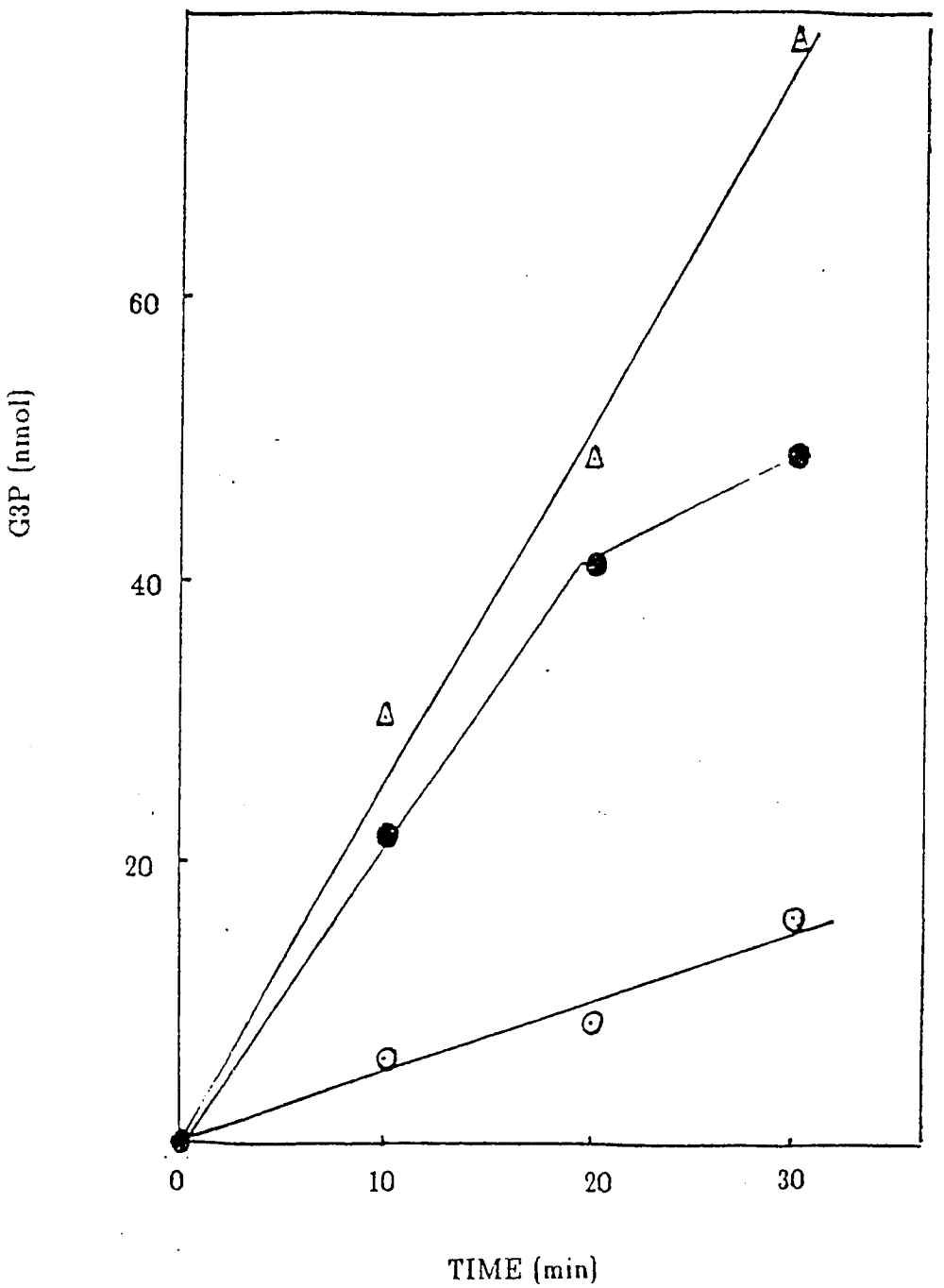

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Fig. 27 Effect of culture age on the reductase activity

E. coli strain 8 was grown to 70, 140 and 200 Klett in CH medium. Cell extracts were prepared in bicine buffer, pH 7.6. Samples of the three extracts containing 130, 204 and 270 µg protein were assayed for L-GAP reductase activity by the solvent extraction method. The assay mixture contained 24 nmol, L-[3-^{3}H]GAP; 1.4 µmol, bicine; 360 nmol, NADPH and the cell extract in a total volume of 132 µl. The results have been normalized for 1 mg of protein.

△ ----- △ 70 Klett

● ----- ● 140 Klett

○ ----- ○ 200 Klett

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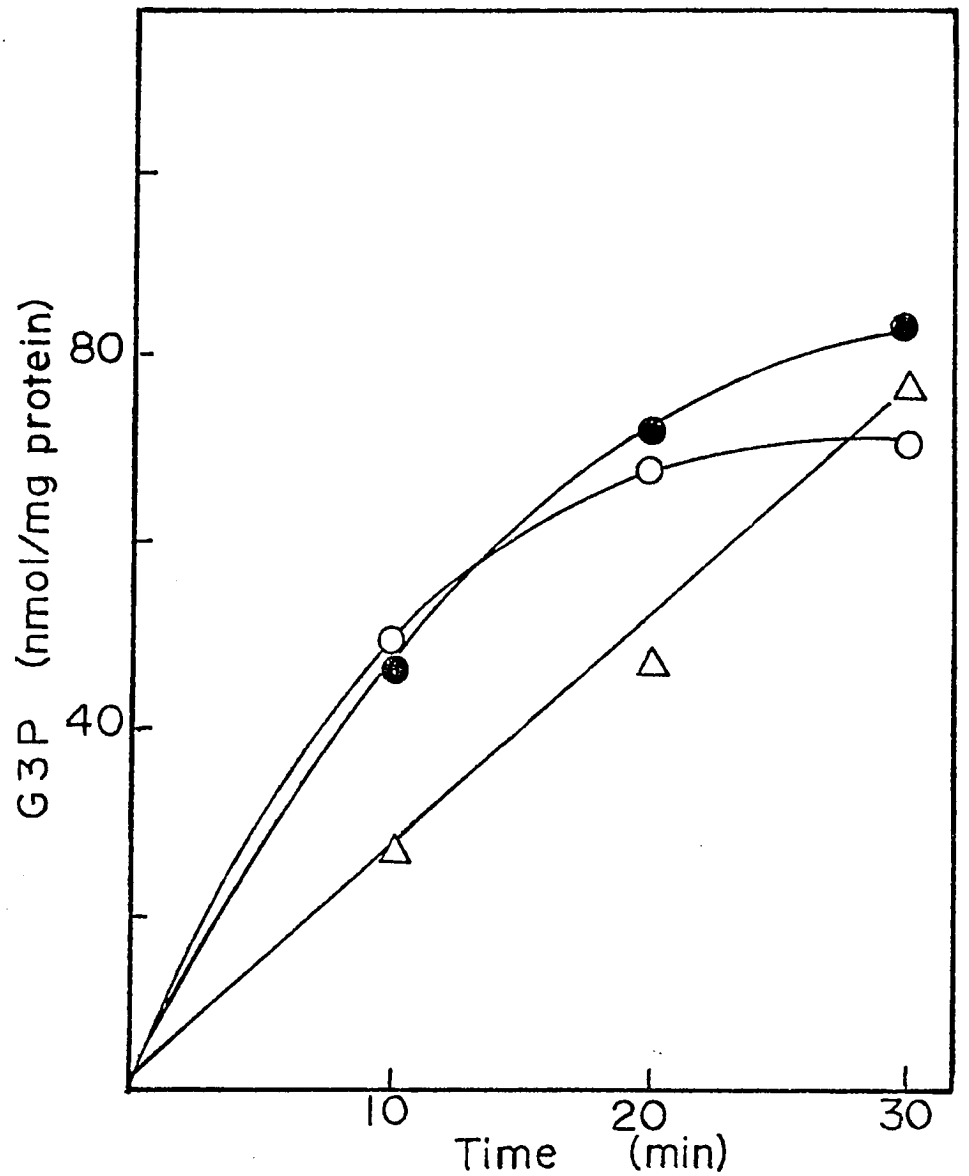

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Fig. 28 Effect of freezing of cells on the reductase activity

E. coli strain 8 was grown to 140 Klett in CH medium. Cells were harvested from 125 ml culture and stored, in a freezer at -20°C, for 4 days. Extracts were prepared from frozen cells in bicine buffer (pH 7.6). Extracts were also prepared from strain 8 cells harvested fresh at about 140 Klett from 125 ml CH medium. Samples of both extracts, were assayed, for L-GAP reductase activity, by the solvent extraction method. The assay mixture contained 24 nmol L-[3-^{3}H]GAP, 1.4 µmol, bicine (pH 7.6), 360 nmol, NADPH and 30 µl, cell extract (about 200 µg protein) in a total volume of 132 µl. The protein content of the two extracts was presumed to be equal (no protein determination was done on the extract from the frozen cells).

G3P formed per 1 ml of assay mixture is plotted against time.

O -----O fresh cells

X -----X frozen cells

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FIG.28 EFFECT OF FREEZING OF CELLS ON THE ACTIVITY

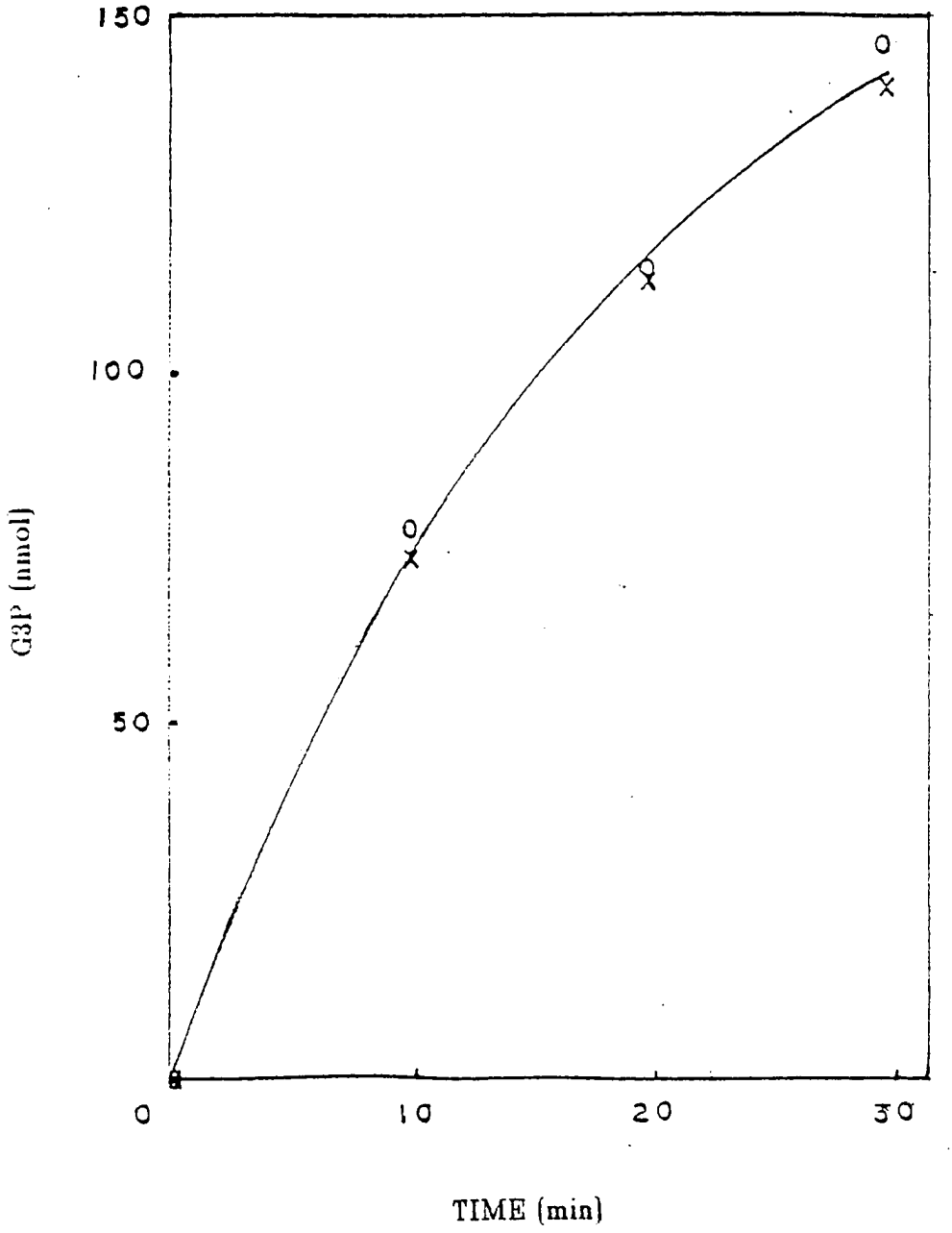

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Fig. 29 Effect of freezing of cell extract on the reductase activity

Cell extract was prepared from E. coli strain 8 grown to 70 Klett in bicine + 0.5% potassium succinate. A sample of the fresh extract (112 µg of protein) was assayed for L-GAP reductase activity by the solvent extraction method. The remainder was stored in a freezer at -20° C. The following day, the frozen extract was thawed and assayed for the reductase activity by the same method. The assay mixture contained 25 nmol, L-[3-^{3}H]GAP, 1.4 µmol, bicine (pH 7.6); 360 nmol, NADPH and the cell extract (112 µg protein) in a total volume of 132 µl.

G3P formed per 1 ml of assay mixture is plotted against time.

0 -----0 fresh extract

X -----X frozen extract

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FIG.29 EFFECT OF FREEZING OF CELL EXTRACT ON THE ACTIVITY.

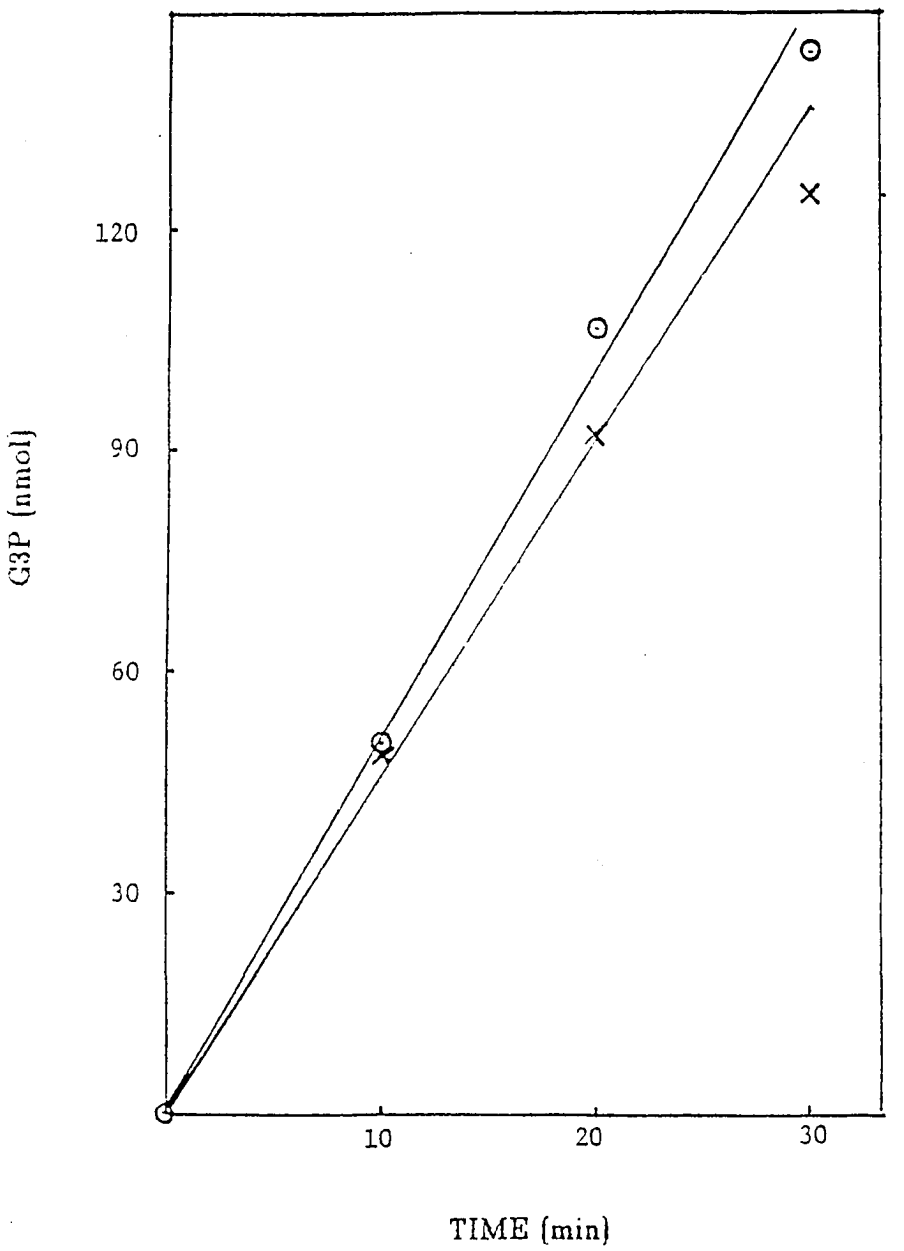

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Fig. 30 The reductase activity in extract of strain 7

E. coli strains 7 and 8 were grown in CH medium to 170 and 140 Klett units respectively. Cell extracts were prepared from equal volumes of the cultures in 5 ml bicine buffer (pH 7.6). Also prepared extracts from strain 7 cultured to 100 Klett units in bicine + 0.5% glucose medium . All three extracts were assayed for L-GAP reductase activity by the solvent extraction method. No protein determination was done on the extracts.

G3P formed per 1 ml of assay mixture is plotted against time.

△ -----△ strain 8 grown in CH

▲ -----▲ strain 7 grown in CH

▼ -----▼ strain 8,in bicine + 0.5% glucose

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FIG.30 L-GAP REDUCTASE ACTIVITY IN EXTRACTS OF STRAIN 7

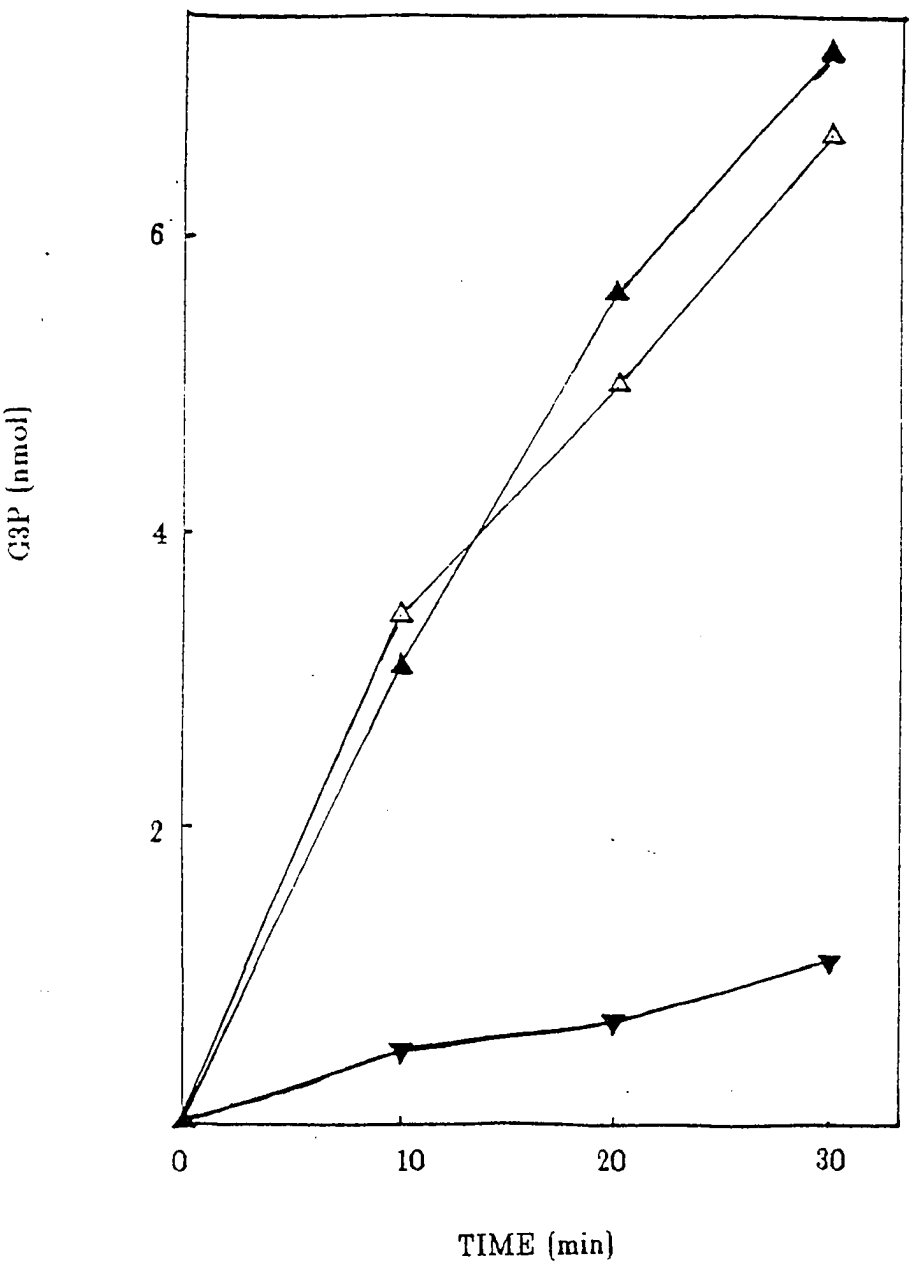

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Fig. 31 DEAE-Sephadex A-50 column chromatography; 1 g cell experiment

The 26-37 % ammonium sulfate fraction, obtained from 1 g cells, was applied on a 17.5 x 0.7 cm DEAE-Sephadex A-50 column. The column was first eluted with 2 ml of buffer and then eluted with a convex gradient of 0-220 mM KCl. The fractions were assayed for L-GAP reductase activity by the solvent extraction method. The total protein was determined by the Lowry's method.

o --o ; L-GAP reductase activity

△ --△ ; protein concentration

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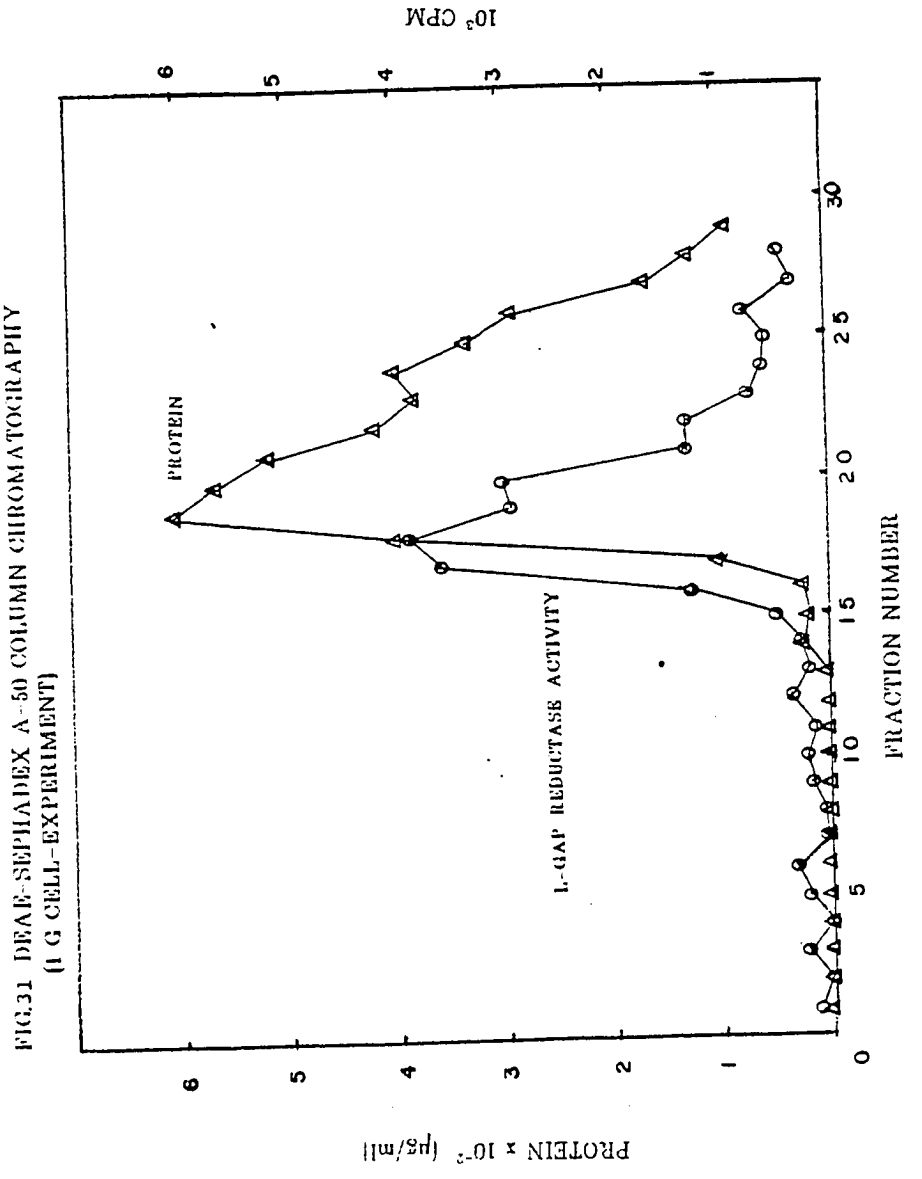

FIG.31 DEAE-SEPHADEX A-50 COLUMN CHROMATOGRAPHY
(1 G CELL-EXPERIMENT)

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Fig. 32a Sephadex A-50 column chromatography

(60 g cell experiment)

The details of the column chromatography is described in the text. The indicated column fractions were assayed for L-GAP reductase (spectrophotometric method), triosephosphate isomerase, G3P synthase and protein content. Activity per 10 µl of fraction is indicated.

0 ----- 0 L-GAP reductase activity

▲ -----▲ triosephosphate isomerase activity

△ -----△ G3P synthase activity

0 ----- 0 protein concentration

Fig. 32b KCl concentration in column fractions

The ionic strength of Sephadex A-50 column fractions was determined by conductivity measurments of individual fractions. Fractions 93 through 108 were not available for the experiment, because they were already combined to be used as the purified enzyme in future experiments.

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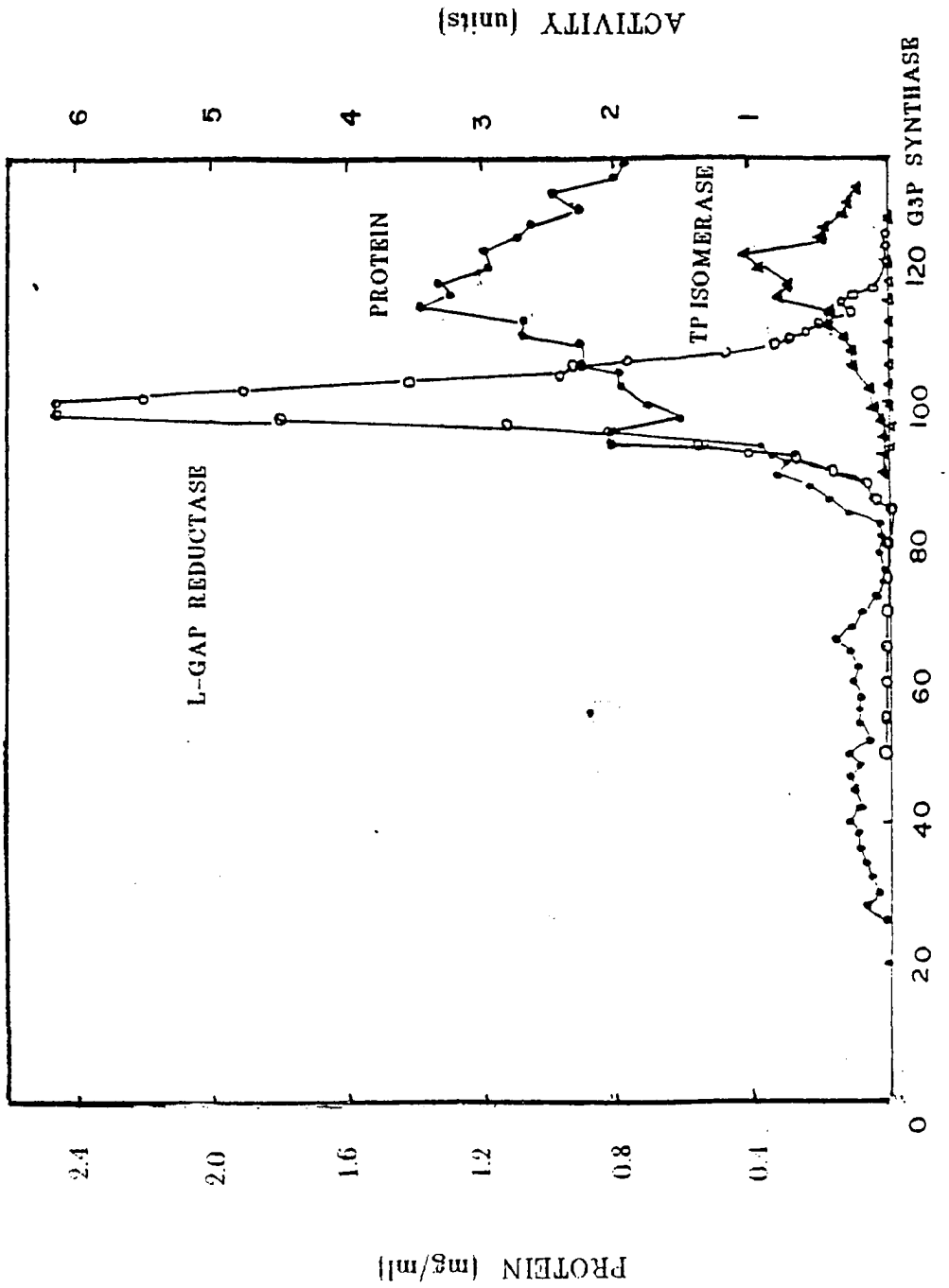

FIG.32a DEAE SEPHADEX A-50 COLUMN CHROMATOGRAPHY

(60 G CELL-EXPERIMENT)

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FIG. 32b

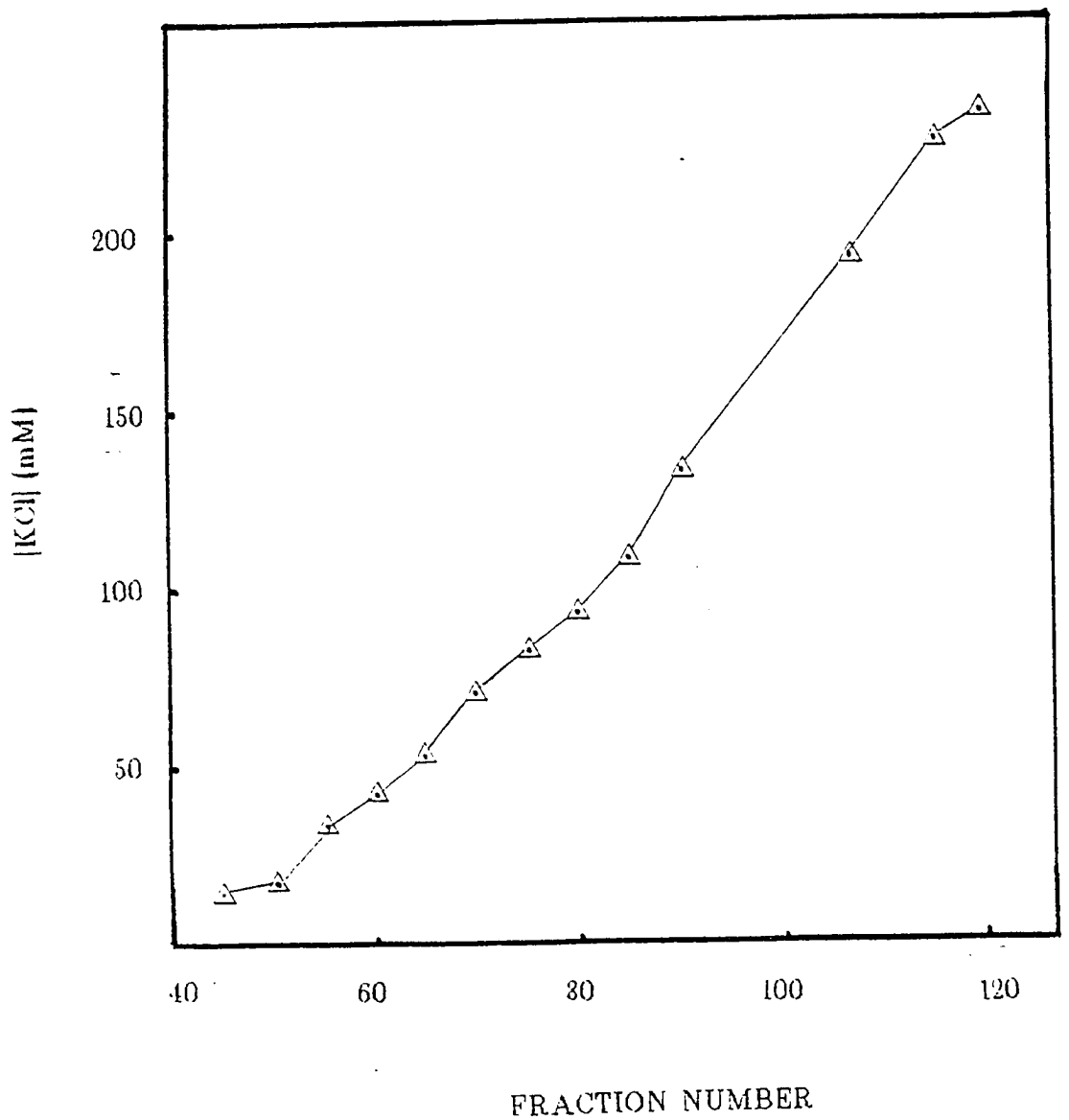

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Fig. 33 pH vs activity

The purified enzyme was assayed, by the spectrophotometric method, at several different pH values, in 50 mM cacodylate buffer and in 50 mM triethanolamine.HCl buffer. Triethanolamine.HCl was not employed below pH 5.8 due to its poor buffering capacity at low pHs. After each assay the pH of the reaction mixture was measured using a microelectrode pH meter.

The assay mixture contained 50 µmol triethanolamine HCl or cacodylate (indicated pHs), 160 nmol NADPH, 1.6 µmol, DL-GAP and 15 µl (6 µg protein) of purified enzyme in a total volume of 1 ml.

o ----- o in cacodylate buffer

△-----△ in triethanolamine.HCl buffer

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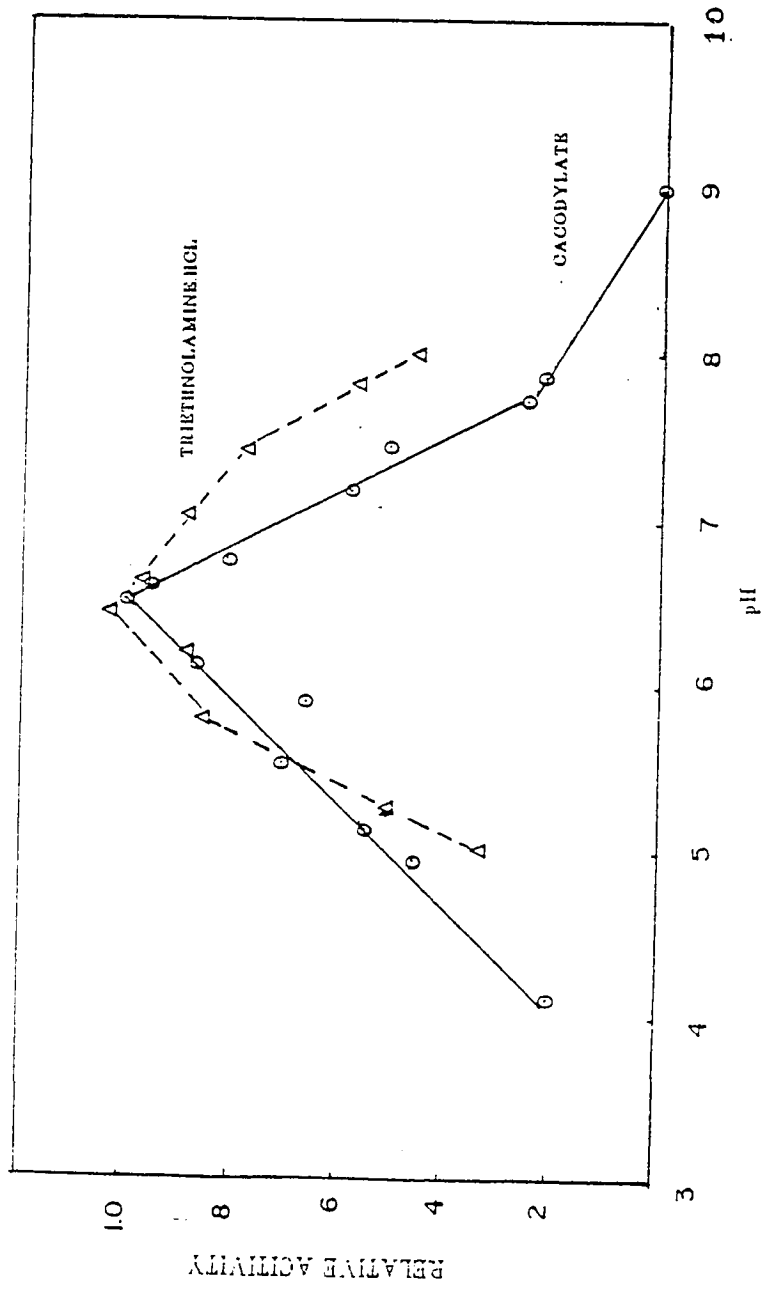

FIG.33 pH vs ACTIVITY

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Fig. 34 Enzyme concentration vs initial rate

Rate of conversion of L-GAP into G3P was measured at different concentrations, of the purified enzyme, spectrophotometrically. The assay mixture contained 50 µmol, triethanolamine.HCl; 150 nmol, NADPH; and varying amounts of the purified enzyme in a total volume of 1 ml at pH 6.8. DL-GAP was added last, to initiate the reaction. The rest of the assay method was same as that described in sec.2.2.4.c.

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FIG.34 ENZYME CONCENTRATION vs ACTIVITY

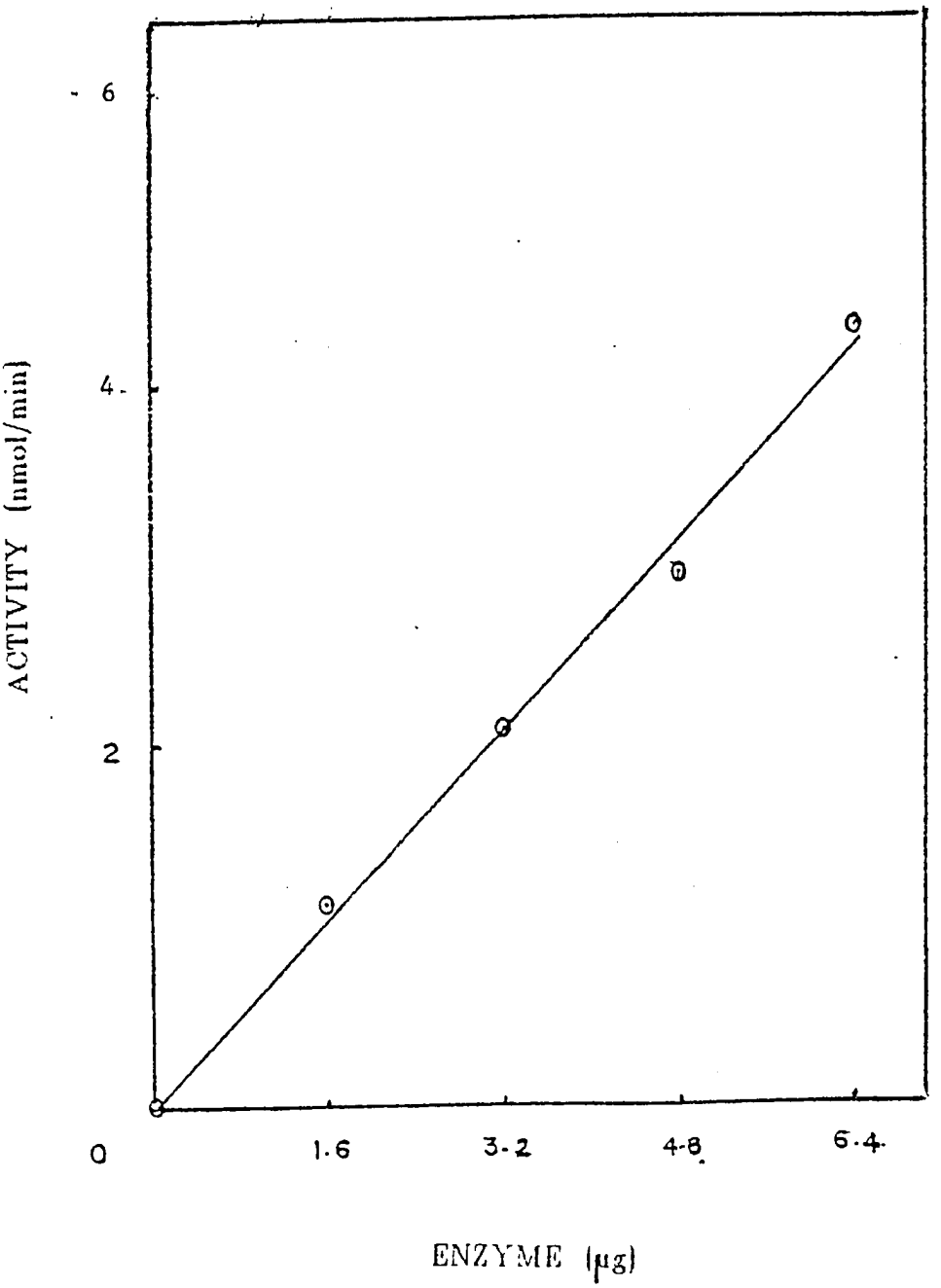

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Fig. 35a $K_{M\ (L\text{-}GAP)}$ (apparent) for the reduction of L-GAP

Initial rate of L-GAP ----> G3P was measured spectrophotometricallty at several different concentrations of L-GAP. The assay mixture contained 50 umol, triethanolamine.HCl(pH 6.8); 150 nmol NADPH; 10 µl of the purified enzyme (4 µg protein) and varying amounts of DL-GAP in a total volume of 1 ml at pH 6.8. DL-GAP was added last to initiate the reaction. The rest of the method is same as that described in sec.2.2.4.c.

Fig. 35b $K_{M(NADPH)}$ (apparent) for the reduction of L-GAP

Initial rate of L-GAP ----> G3P was measured at 44, 66, 110 and 198 µM concentrations of NADPH, spectrophotometrically. The reaction mixture for the assay contained 50 µmol, triethanolamine.HCl; 158 nmol, DL-GAP; 10 µl (4 µg of protein), purified enzyme; and varying amounts of NADPH in a total volume of 1 ml, at pH 6.8. DL-GAP was added last, to initiate the reaction. The rest of the assay method was same as that described in sec.2.2.4.c.

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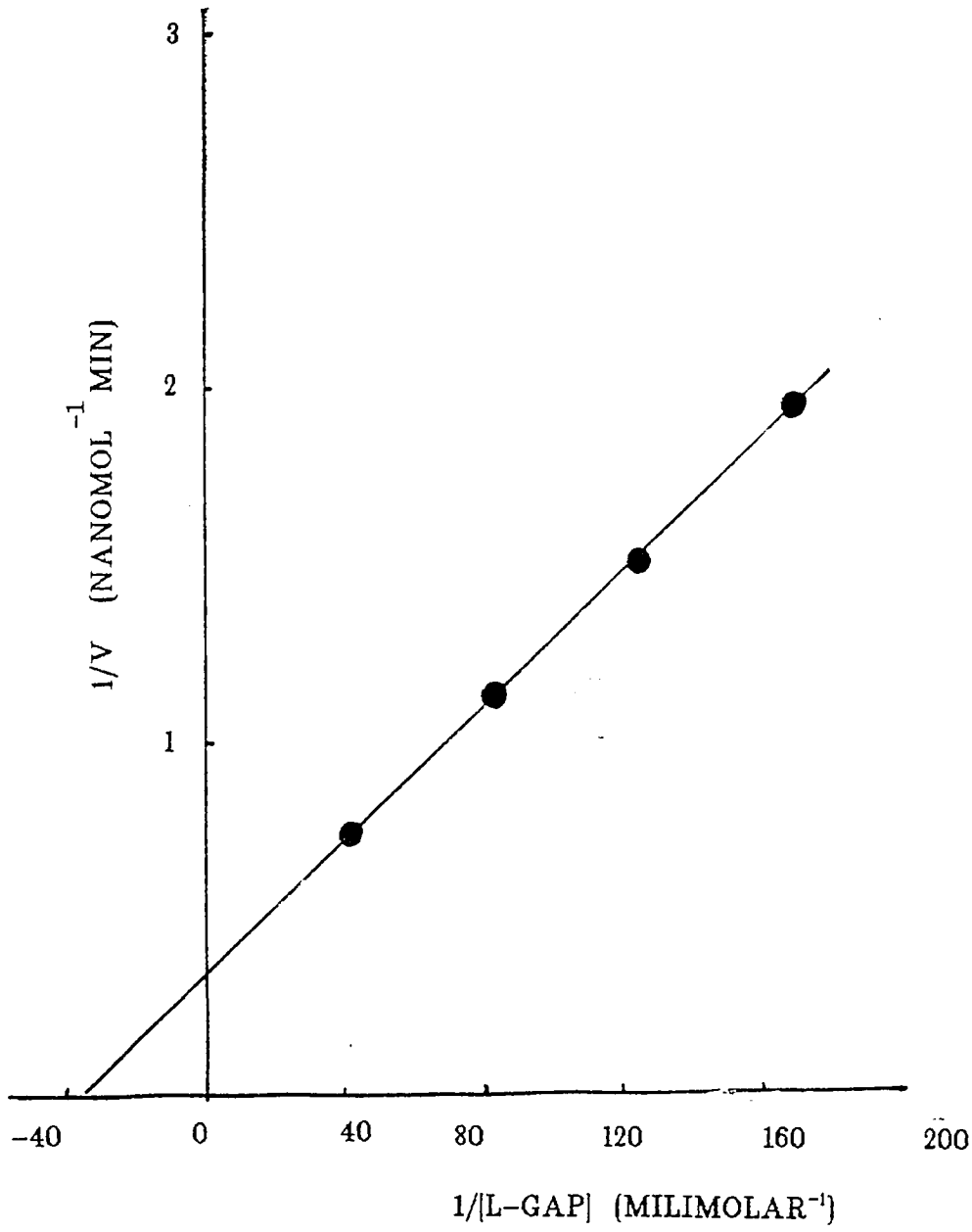
FIG.35a



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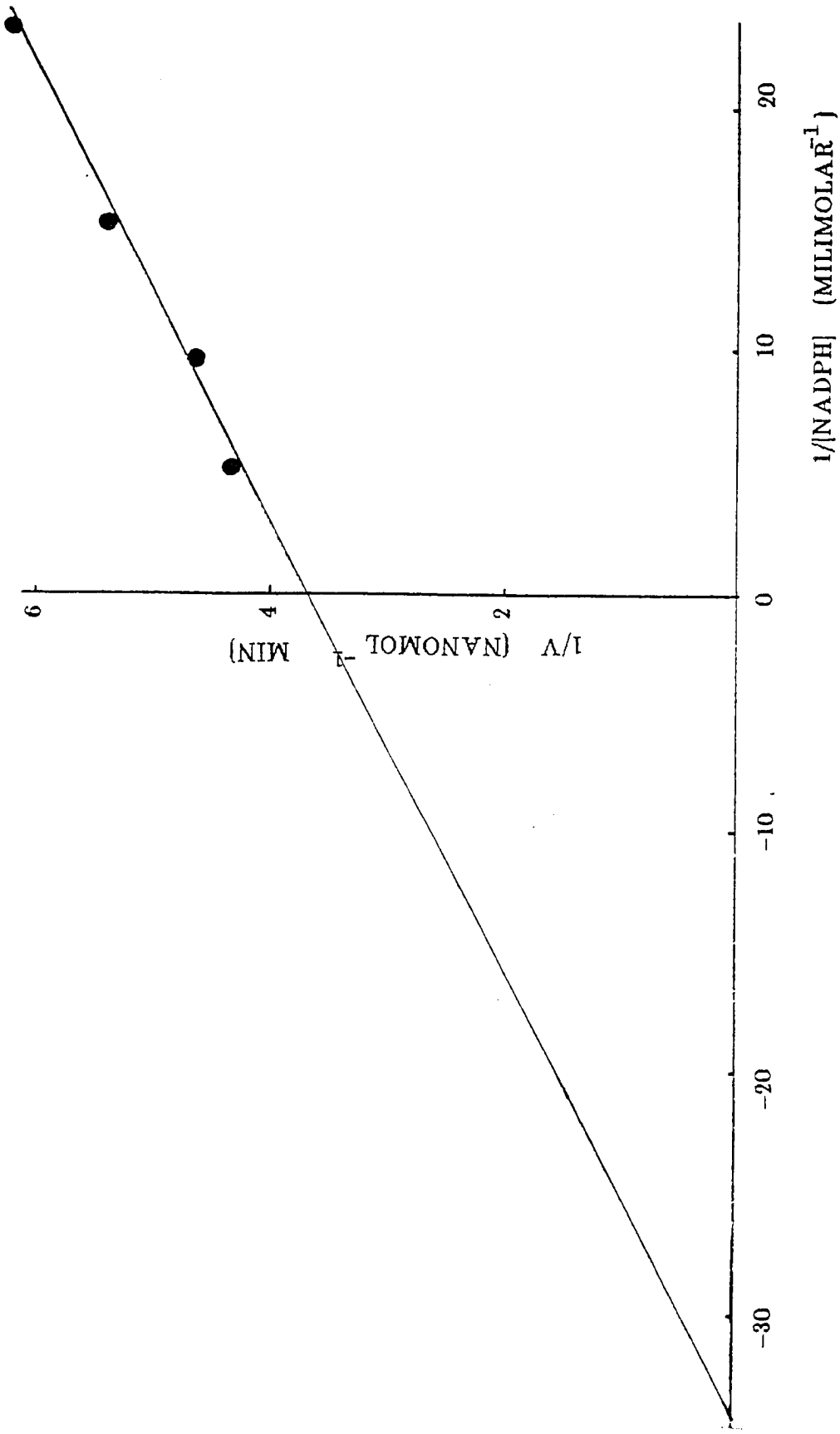

FIG.35b

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Fig. 36a $K_{M\ (GAPA)}$ (apparent) for the reduction of GAP analog

The initial reaction rate of L-GAP -----> G3P was measured at several different concentrations of glyceraldehyde 3-phosphate analog (GAPA),DL-3-hydroxy-4-oxobutyl-1-phosphonate. The reaction mixture was similar to the one described in Fig. 35a, but contained GAP analog in the place of DL-GAP.

Fig. 36b $K_{M\ (NADPH)}$ (apparent) for the reduction of GAP analog

Initial rate of the L-GAP -----> G3P reaction was measured at several different NADPH concentration. The reaction mixture was same as that described in Fig. 35b, but contained 1.26 umol, GAP-analog in the place of DL-GAP.

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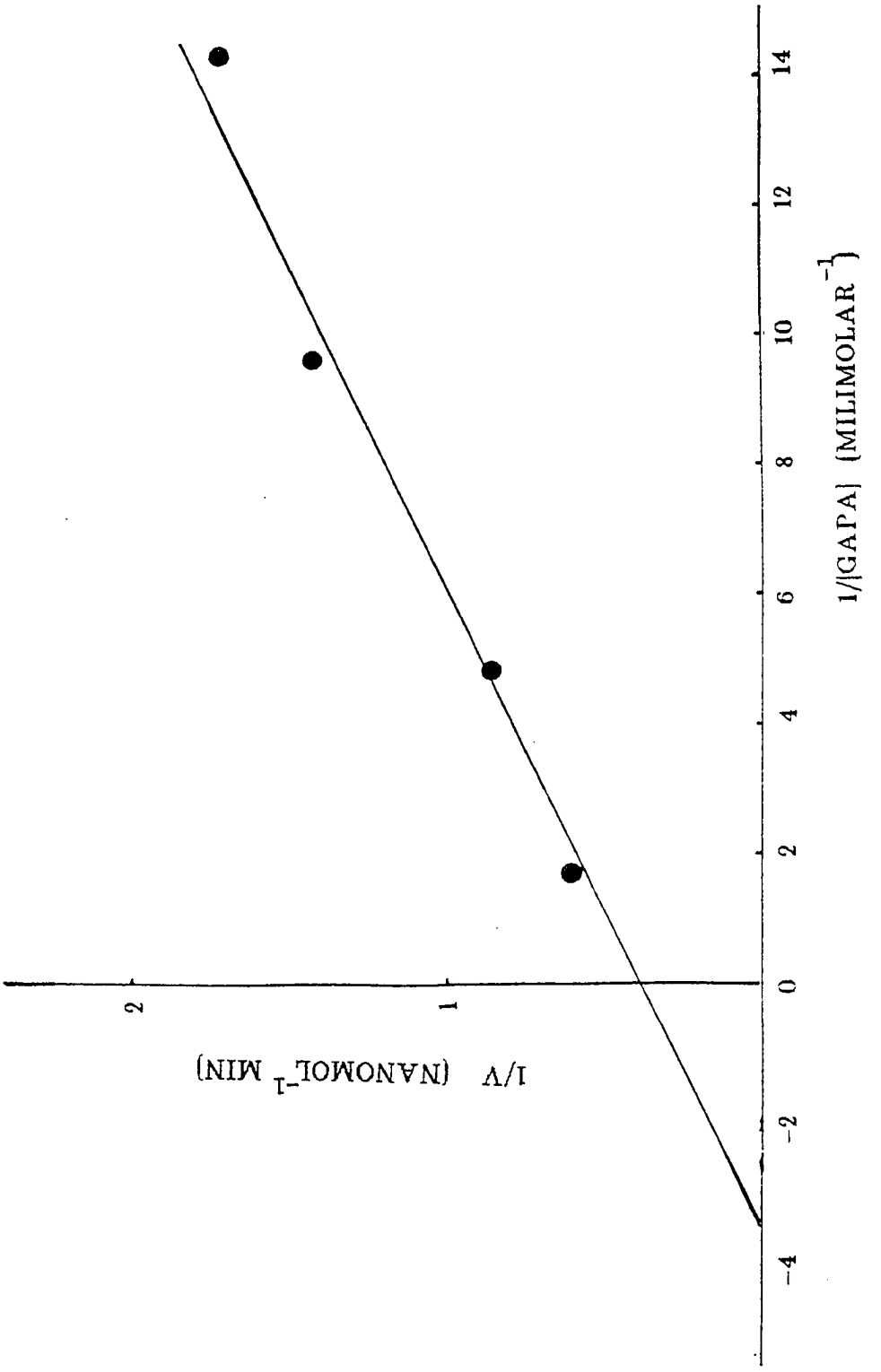

FIG. 36a

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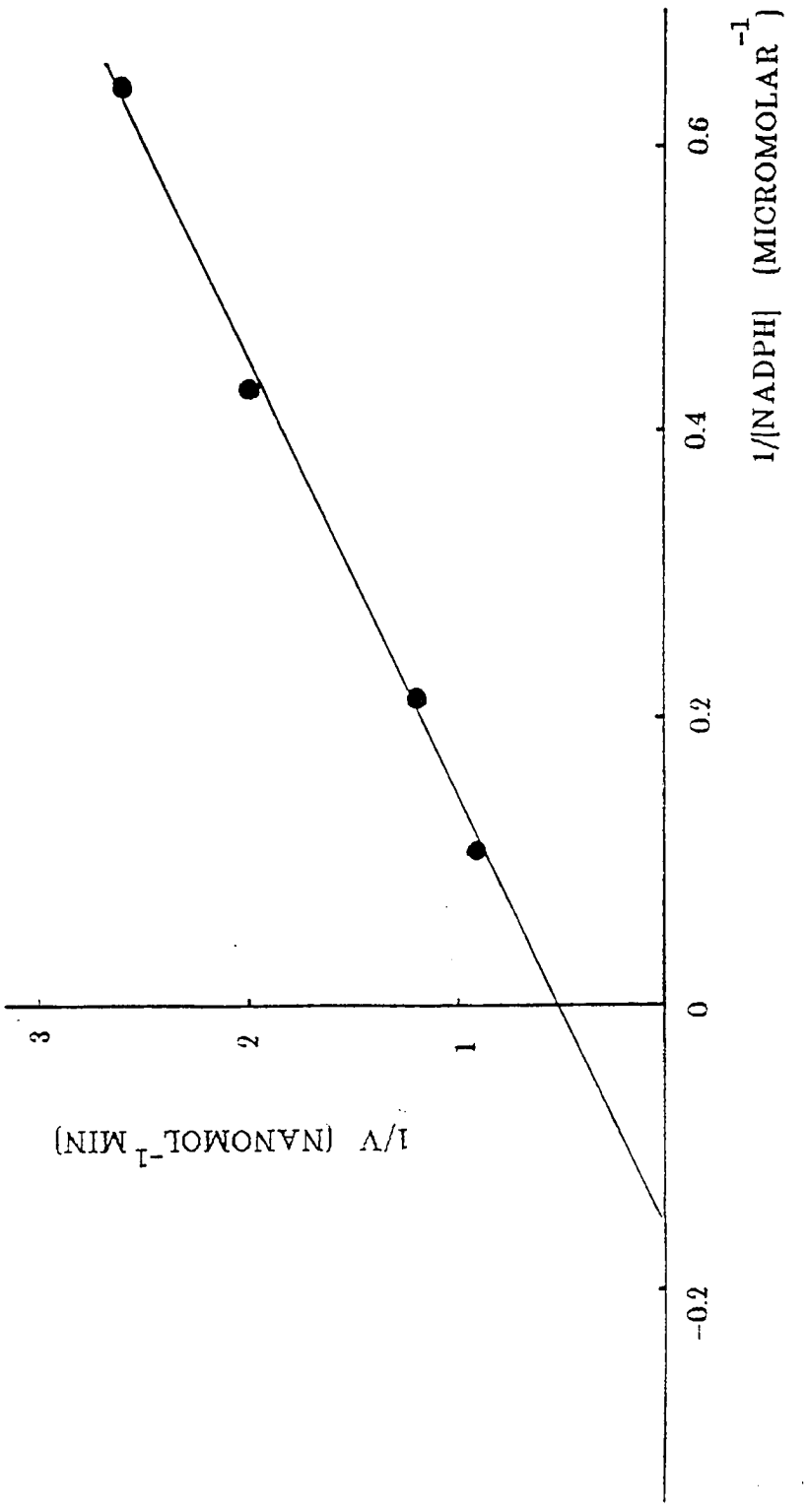

FIG.36b

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Fig. 37a $K_{M\ (MeG)}$ (apparent) for the reduction of methylglyoxal

The initial rate of L-GAP ------> G3P was measured at several different concentrations of methylglyoxal. The reaction mixture was same as that in Fig. 35a but methylglyoxal was used in the place of DL-GAP

Fig. 37b $K_{M\ (NADPH)}$ (apparent) for the reduction of methylglyoxal

The initial rate of L-GAP ----> G3P reaction was measured at several different concentrations of NADPH, in the presence of methylglyoxal as the substrate. The reaction mixture was that described in Fig. 35b, but contained 46 µmol, methylglyoxal in the place of DL-GAP.

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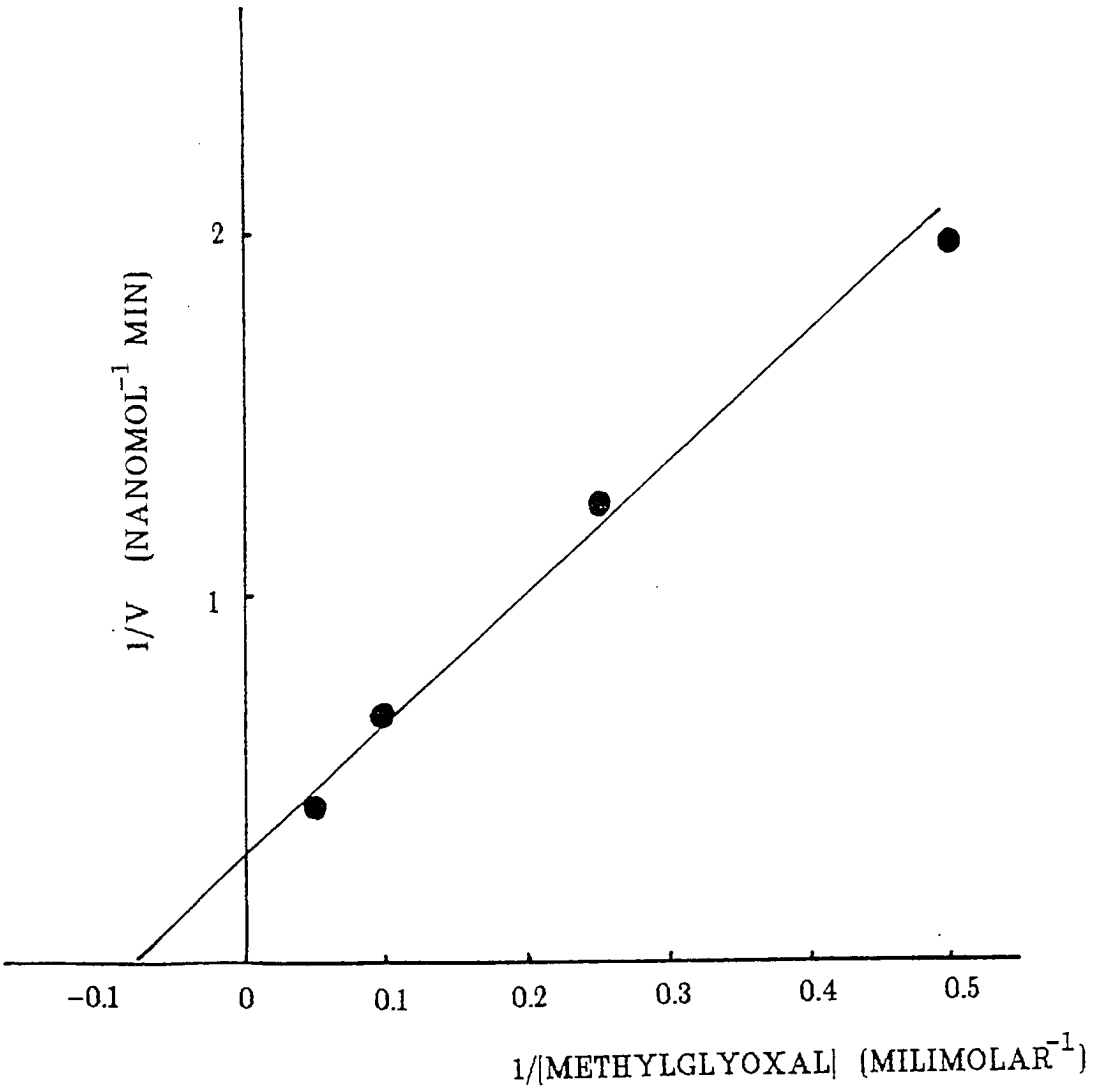
FIG.37a



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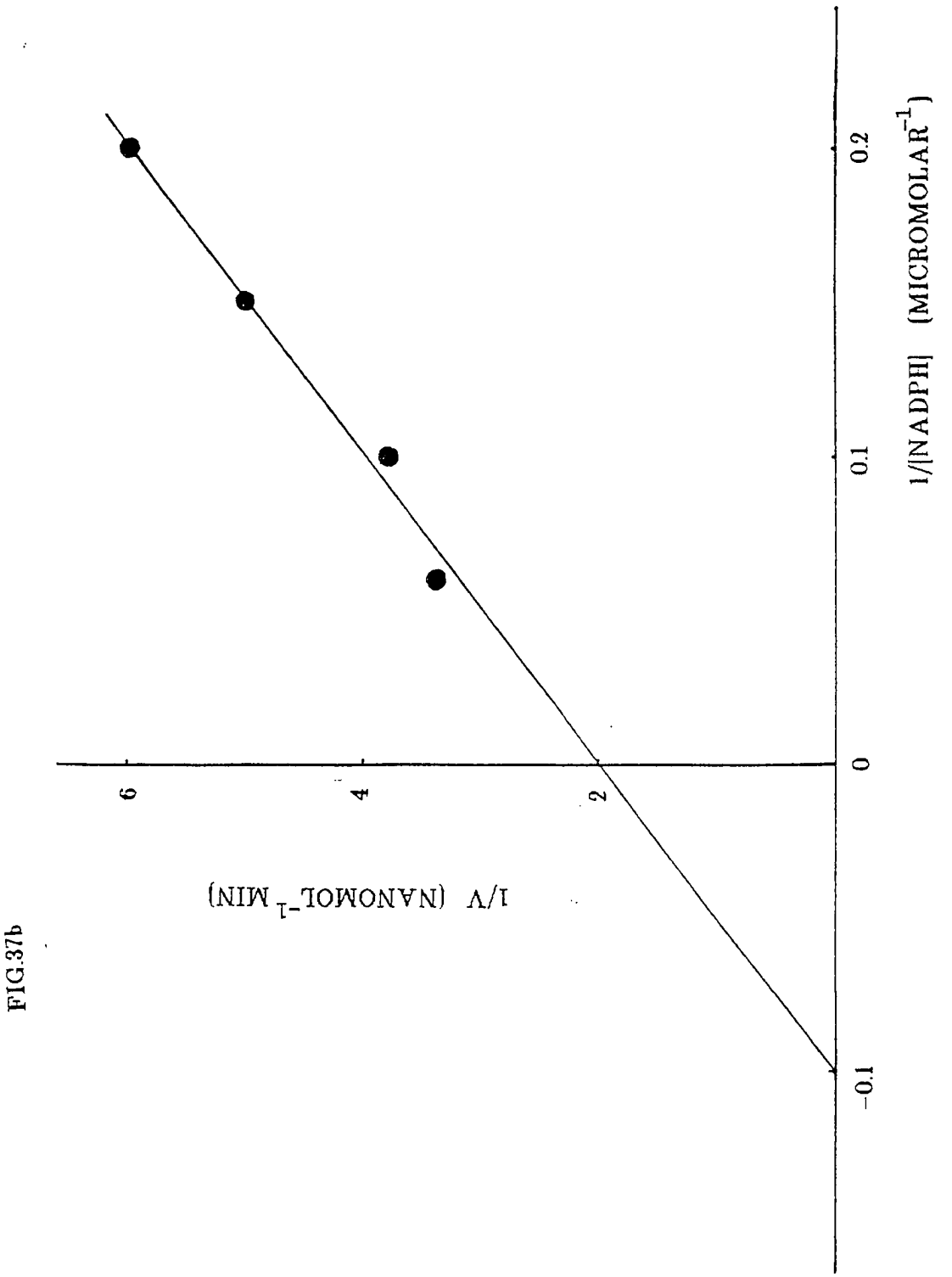

FIG.37b

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Fig. 38a $K_{M\ (L\text{-glyceraldehyde})}$ (apparent) for the reduction of L-glyceraldehyde

The initial rate of L-GAP -----> G3P was measured at several different concentrations of L-glyceraldehyde. The reaction mixture was same as that in Fig. 35a but contained L-glyceraldehyde as the substrate.

Fig.38b $K_{M\ (NADPH)}$ (apparent) for the reduction of L-glyceraldehyde

The initial rate of the L-GAP ----> G3P reaction was measured at several different concentrations of NADPH, in the presence of L-glyceraldehyde as the substrate. The reaction mixture is that described in Fig. 35b, but contained 23.6 µmol, L-glyceraldehyde in the place of DL-GAP.

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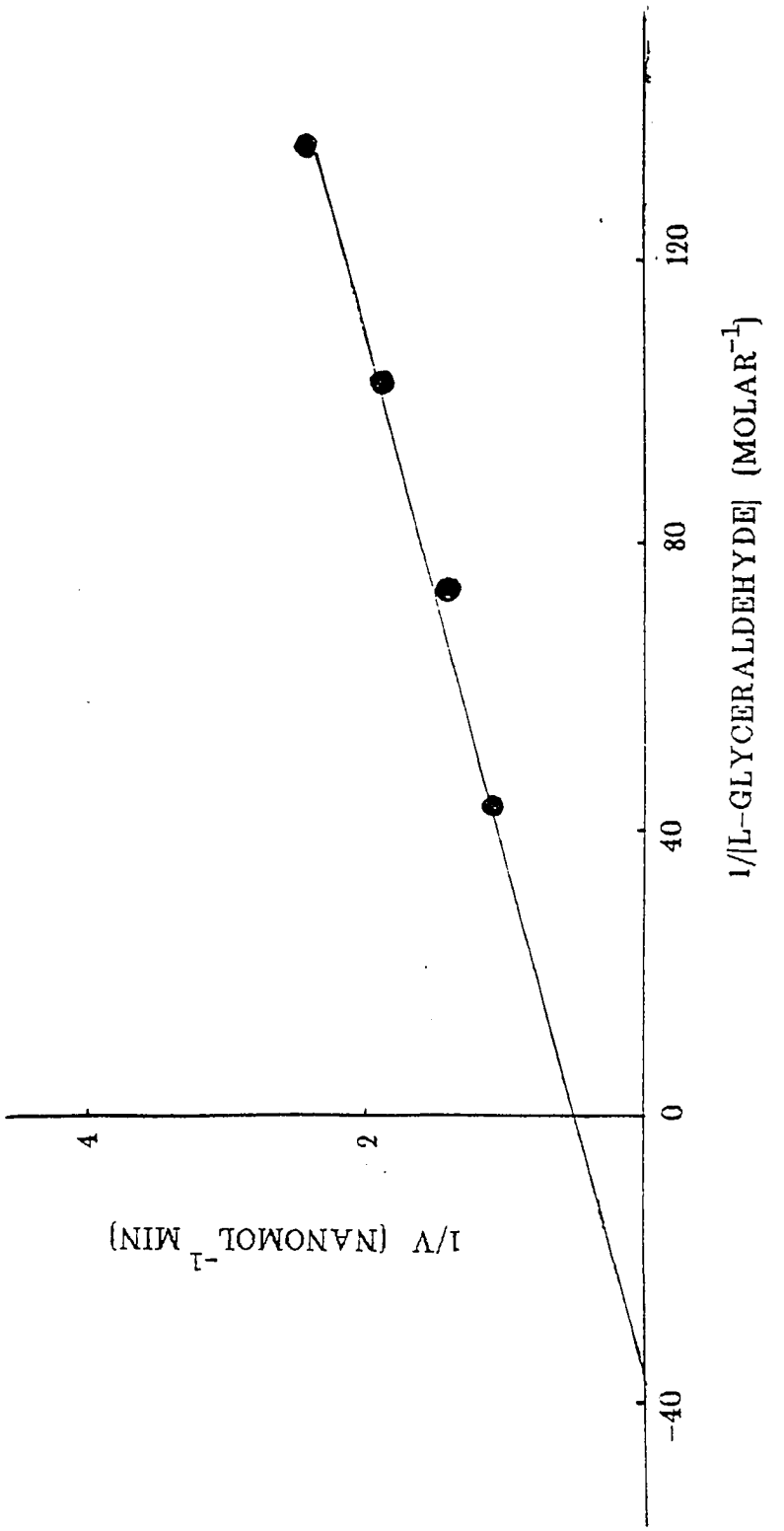

FIG.38a

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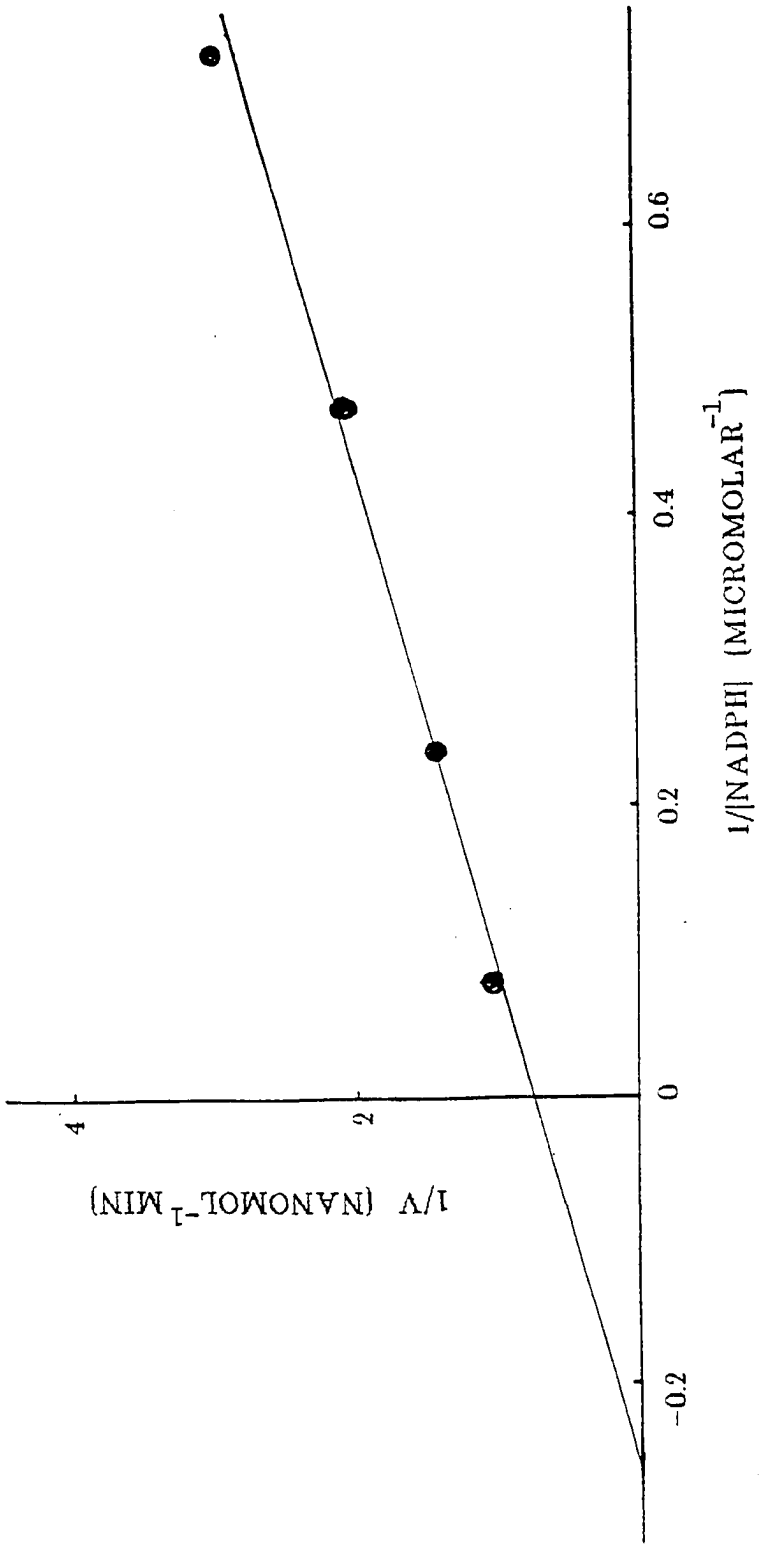

FIG.38b

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Fig. 39a $K_{M\ (D\text{-glyceral})}$ (apparent) for the reduction of D-glyceraldehyde

A study simillar to the one described in Fig. 35a was carried out with D-glyceraldehyde as the substrate.

Fig. 39b $K_{M\ (NADPH)}$ (apparent) for the reduction of D-glyceraldehyde

A study simillar to the one described in Fig. 35b was carried out with D-glyceraldehyde as the substrate. The reaction mixture is similar to the one in Fig.35b, but contained 24 µmol, D-glyceraldehyde in the place of DL-GAP.

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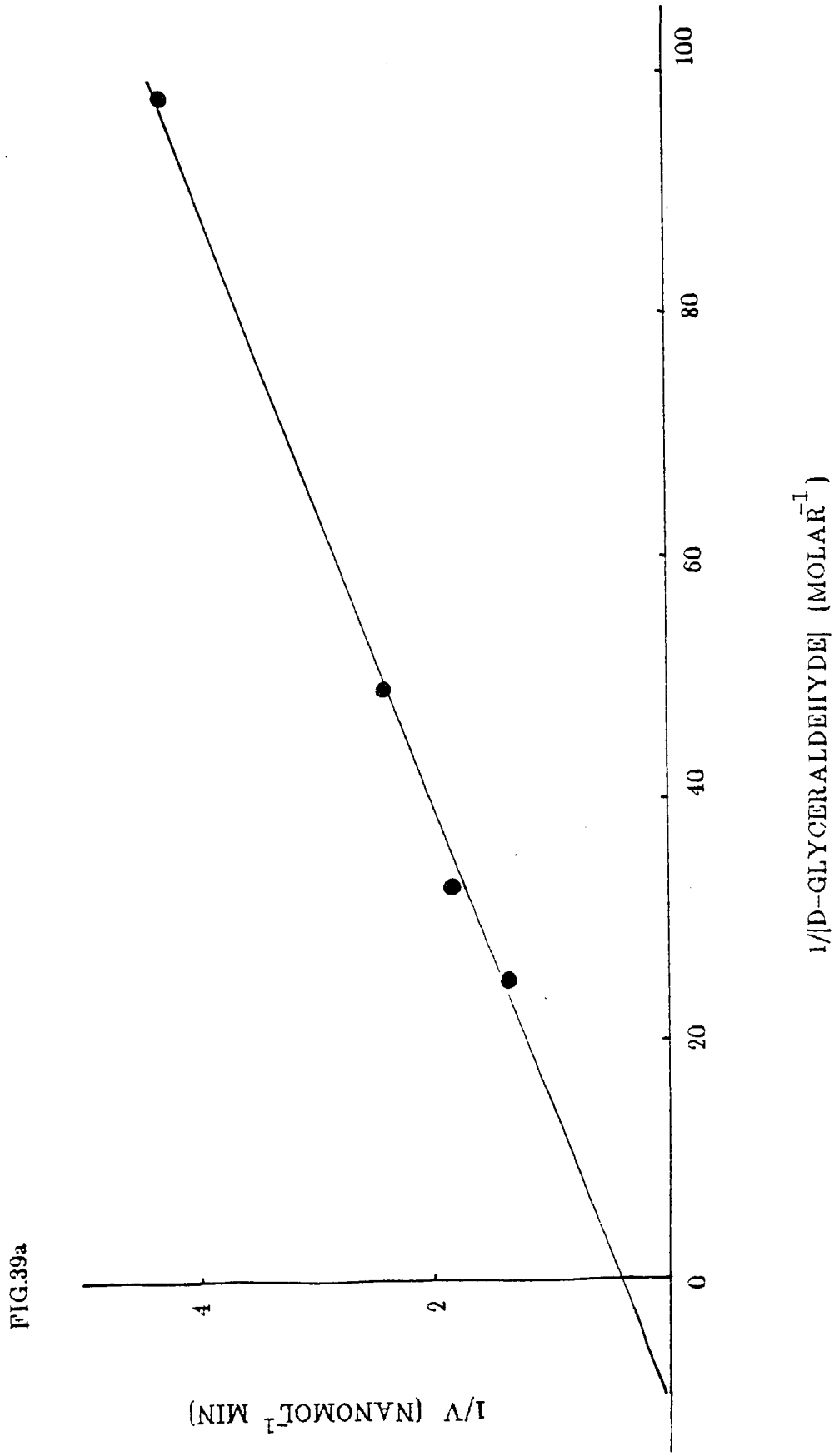

FIG.39a

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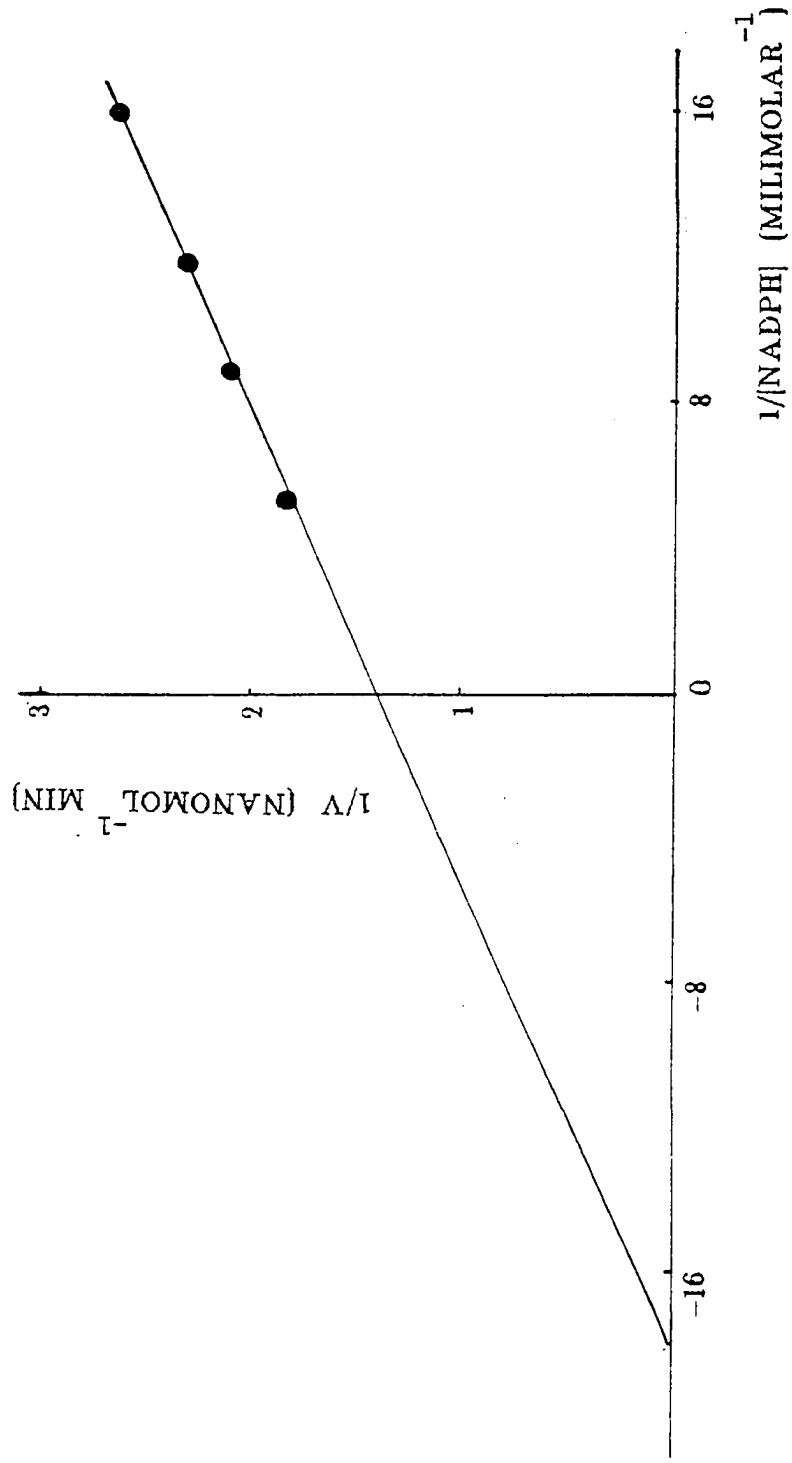

FIG.39b

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Fig. 40 Resistance of E. coli KL-21 to 1 mM methylglyoxal

E. coli strains 7 and KL-21 were grown in phosphate minimal medium supplemented with 0.2% glucose. At 20 Klett, cultures were treated with 1 mM methylglyoxal. Untreated cultures of the two strains were used as controls. Growth of the cultures, at 37 °C, was followed turbidimetrically.

● ---● ; strain 7, control

o ---o ; strain 7, 1 mM methylglyoxal

▲ ---▲ ; KL-21, control

△ ---△ ; KL-21, 1 mM methylglyoxal

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FIG.40 RESISTANCE OF STRAIN KL-21 TO 1 MM METHYLGLYOXAL

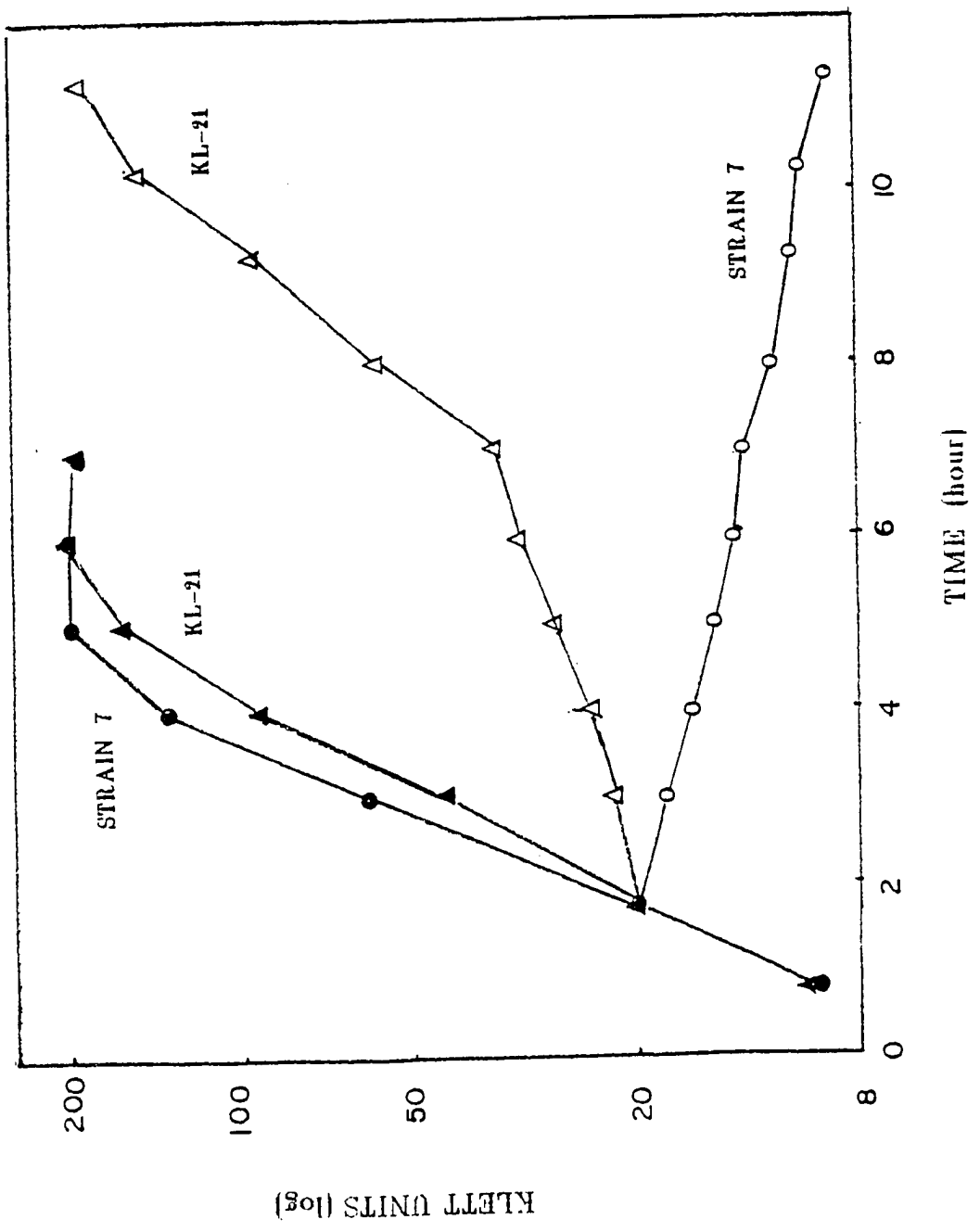

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Fig. 41 Resistance of E. coli KL-21 to 2.5 mM DL-GAP

E. coli strains 7 and KL-21 were grown in bicine medium suplemented with 0.5% glucuronate. At 21 Klett, cultures were treated with 2.5 mM DL-GAP. Untreated cultures of the two strains were used as controls. Growth of the cultures, at 37 °C , was followed turbidimetrically.

● --- ● ; strain 7, control

0 --- 0 ; strain 7, 2.5 mM DL-GAP

▲ --- ▲ ; KL-21, control

△ --- △ ; KL-21, 2.5 mM DL-GAP

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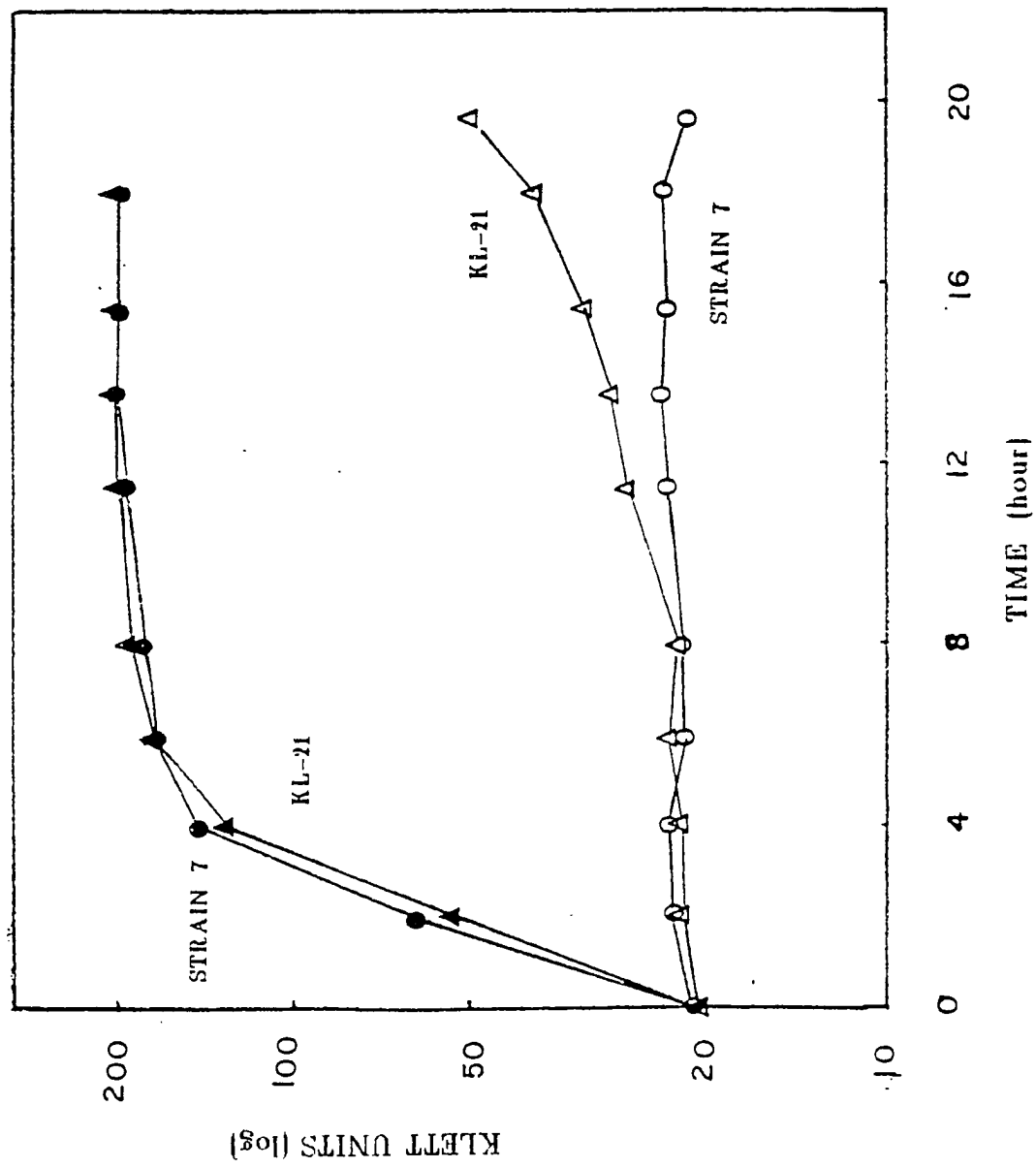

FIG.4f RESISTANCE OF STRAIN KL-21 TO 2.5 MM DL-GAP

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Fig. 42 Resistance of methylglyoxal treated E.coli KL-21 to methylglyoxal and DL-GAP

E. coli Kl-21 (methylglyoxal resistant) was cultured in bicine + 0.5% Na-glucuronate. At 18 Klett, methylglyoxal was added to make the medium 1 mM and continued incubation for another six hours. When the turbidity reached 50 Klett cells were harvested by centrifugation and resuspended in fresh bicine + 0.5% Na-glucuronate so that the final turbidity was 17 Klett. Ten ml portions of the cell suspension was treated either with 1 mM methylglyoxal, 2.5 mM Dl-GAP or none. Untreated culture of E. coli KL-21, which has not seen any methylglyoxal were also harvested at 50 Klett and treated exactly the same way as a control. All the cultures were incubated at 37°C and followed the growth by monitoring the turbidity change.

symbol	harvested from	treatment
◇	-------- untreated	control
△	-------- untreated	1 mM MeG
○	-------- untreated	2.5 mM GAP
◆	------ 1 mM MeG treated	control
▲	------ 1 mM MeG treated	1 mM MeG
●	------ 1 mM MeG treated	2.5 mM GAP

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FIG.42

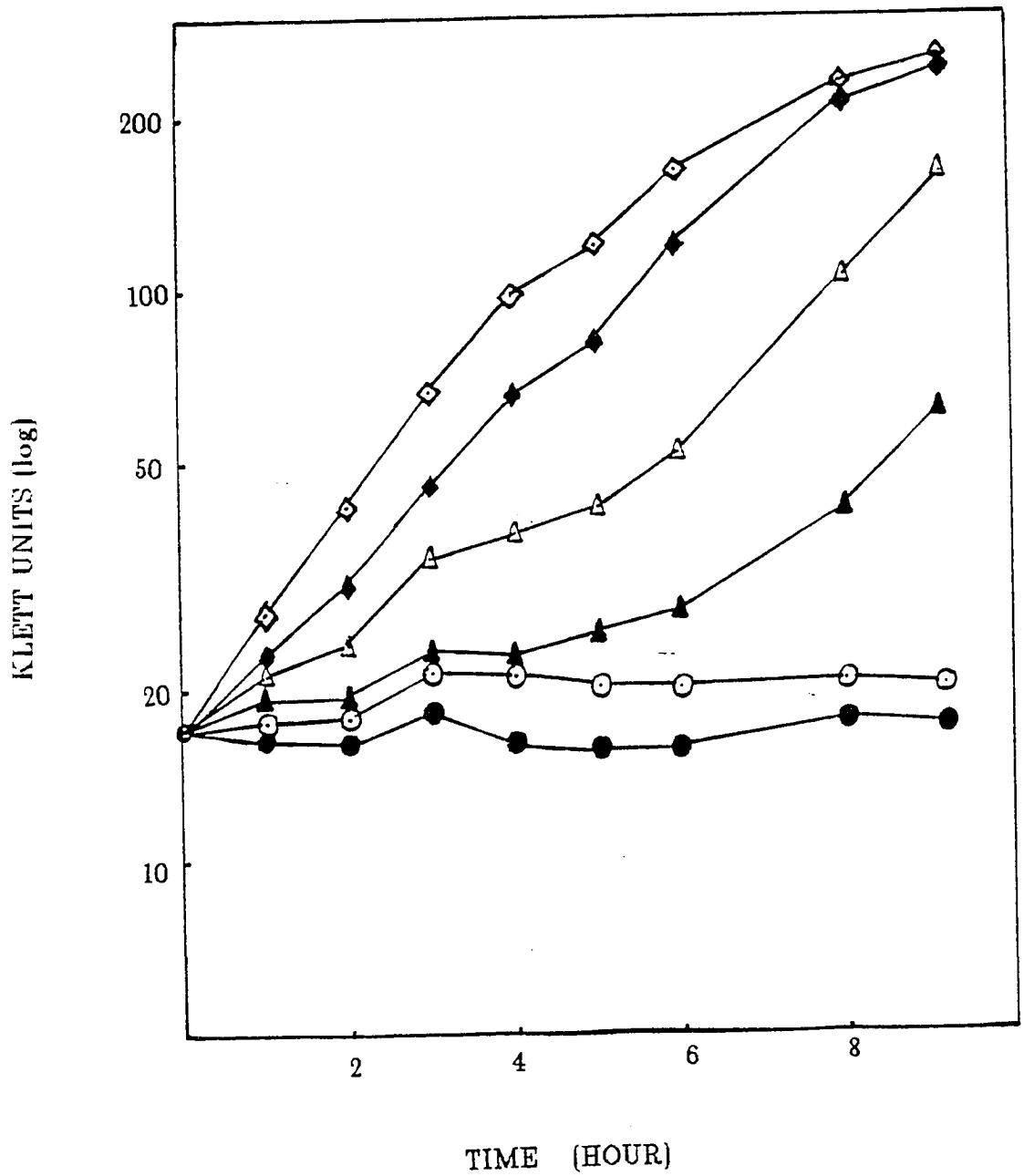

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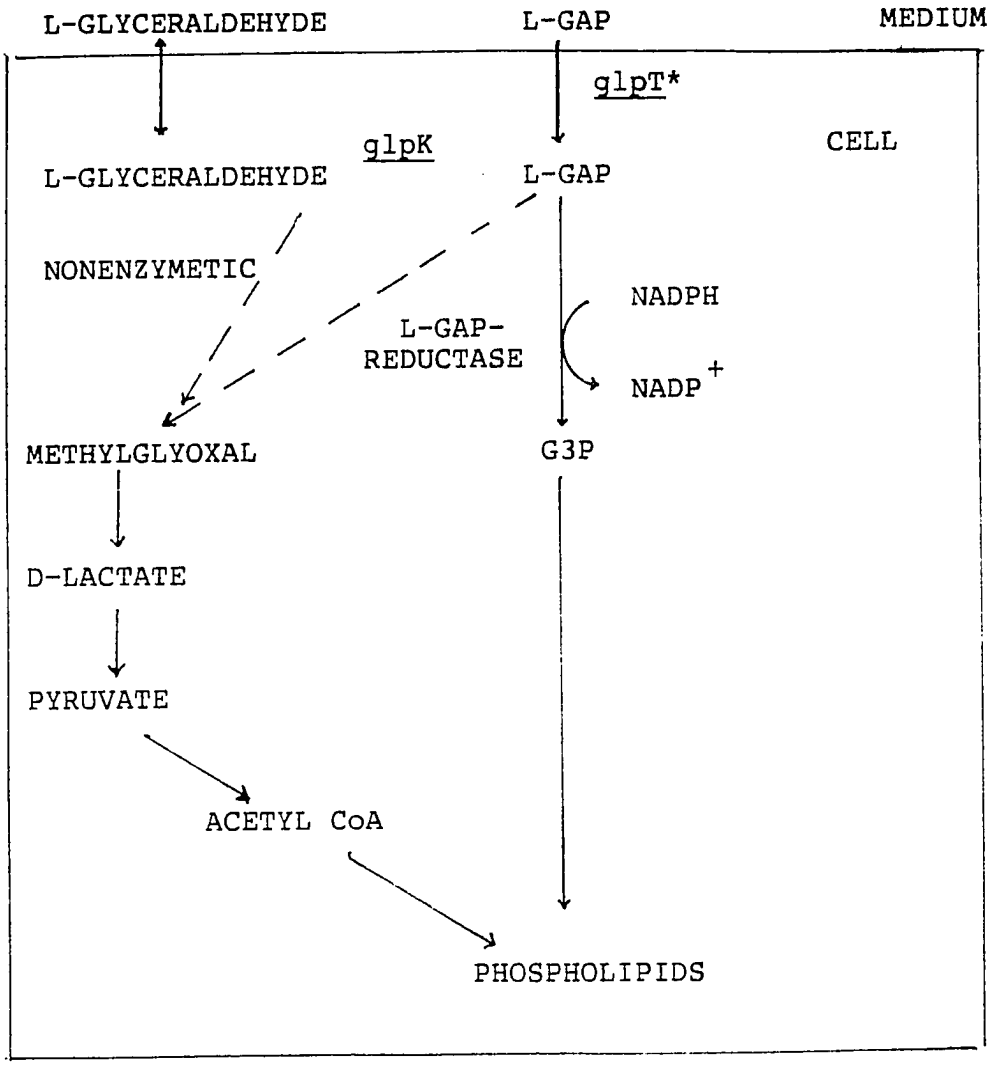
FIG.43 PATHWAYS FOR THE METABOLISM OF L-GAP IN E. COLI



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Abreviation

ADP : Adenosine 5'-diphosphate

ATP : Adenosine 5'-triphosphate

NAD^+ : β Nicotinamide adenine dinucleotide

NADH : β Nicotinamide adenine dinucleotide (reduced)

$NADP^+$: β Nicotinamide adenine dinucleotide phosphate

NADPH: β Nicotinamide adenine dinucleotide phosphate reduced form

G3P : snGlycerol 3-phosphate

GAP : Glyceraldehyde 3-phosphate

DHAP: Dihydroxyacetone phosphate

L-gly: L-Glycerldehyde

MeG : Methylglyoxal

PPO : 2,5-diphenyl oxazole

POPOP: Phenyl-oxazolyl phenyl-oxazolyl-phenyl

3T3: This is an established cell line of mouse embryo fibroblast. The cell line has been established by transfering 3×10^5 cells, every third day, into fresh medium.

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