STUDIES OF METHYLGLYOXAL SYNTHASE: THE DISTRIBUTION OF ENZYME AND CHEMICAL MECHANISM OF CATALYSIS

DISSERTATION

Presented to the Graduate Council of the
North Texas State University in Partial
Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

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May, 1978

MWS

Yuan, Pau-Miau, Studies of Methylglyoxal Synthase: The Distribution of Enzyme and Chemical Mechanism of Catalysis. Doctor of Philosophy (Chemistry), May, 1978, 85 pp., 8 tables, 13 illustrations, bibliography, 50 titles, 1 appendix.

Methylglyoxal synthase, which catalyzes the conversion of dihydroxyacetone phosphate to methylglyoxal and inorganic phosphate, has been found in several Enterobacteriaceae. The enzyme along with glyoxalase I and II and D-lactate oxidase, therefore, constitute a nonphosphorylated shunt of the normal glycolytic pathway.

A highly sensitive, radioisotope assay has been developed for methylglyoxal synthase. This and two additional methods were compared and shown to provide valid procedures for teh quantitative measurement of the enzyme in crude cell and tissue extracts. Based on all species tested thus far, it appears that the occurrence of the enzyme is restricted to certain Enterobacteriaceae and closely related species with the specific activity ranging from 0.02 to 0.5 units per mg of protein.

Methylglyoxal synthase from P. vulgaris was purified to homogeneity and utilized to assess the reaction mechanism. [^{14}C]dihydroxyacetone phosphate labeled in

either the C-1 and C-3 position was enzymatically synthesized, isolated and utilized as a substrate for the enzyme. After reaction with the enzyme, the methyl carbon of methylglyoxal was identified as CHI_3 by the iodoform reaction. The labeling pattern revealed that carbon 1 is dephosphorylated and reduced to the methyl group, while C-3 is oxidized to the aldehyde. Methylglyoxal was found to be noncompetitive with respect to dihydroxyacetone phosphate, while inorganic phosphate was competitive and transformed the dihydroxyacetone phosphate saturation kinetics from hyperbolic to sigmoidal. The enzyme was inactivated by freezing. Phosphate stabilized the enzyme toward both cold- and heat-induced denaturation. The phosphate moiety of the substrate appears to be required for binding, since the synthase is competitively inhibited by a variety of phosphorylated compounds, but not by their nonphosphorylated counterparts. 3-Bromo- and 3-iodoacetol phosphates irreversibly inactivate methylglyoxal synthase. Dihydroxyacetone phosphate and inorganic phosphate protect against the inactivation. Reduction of the enzyme-inactivator complex with $[^3H]$-$NaBH_4$, followed by amino acid analysis, suggested that the modification occurred through an acid-labile linkage, and the modified residue(s) was either glutamate or aspartate. No metal ion or cofactor was found to be involved in the enzyme-catalyzed reaction.

Based on these observations, a mechanism is proposed in which the enzyme first catalyzes the keto-enol tautomerization to the hydrogen-bonded enol which facilitates the internal oxidation-reduction and phosphoester cleavage with C-O bond breaking, followed by an ordered sequential release of products with methylglyoxal being the first product.

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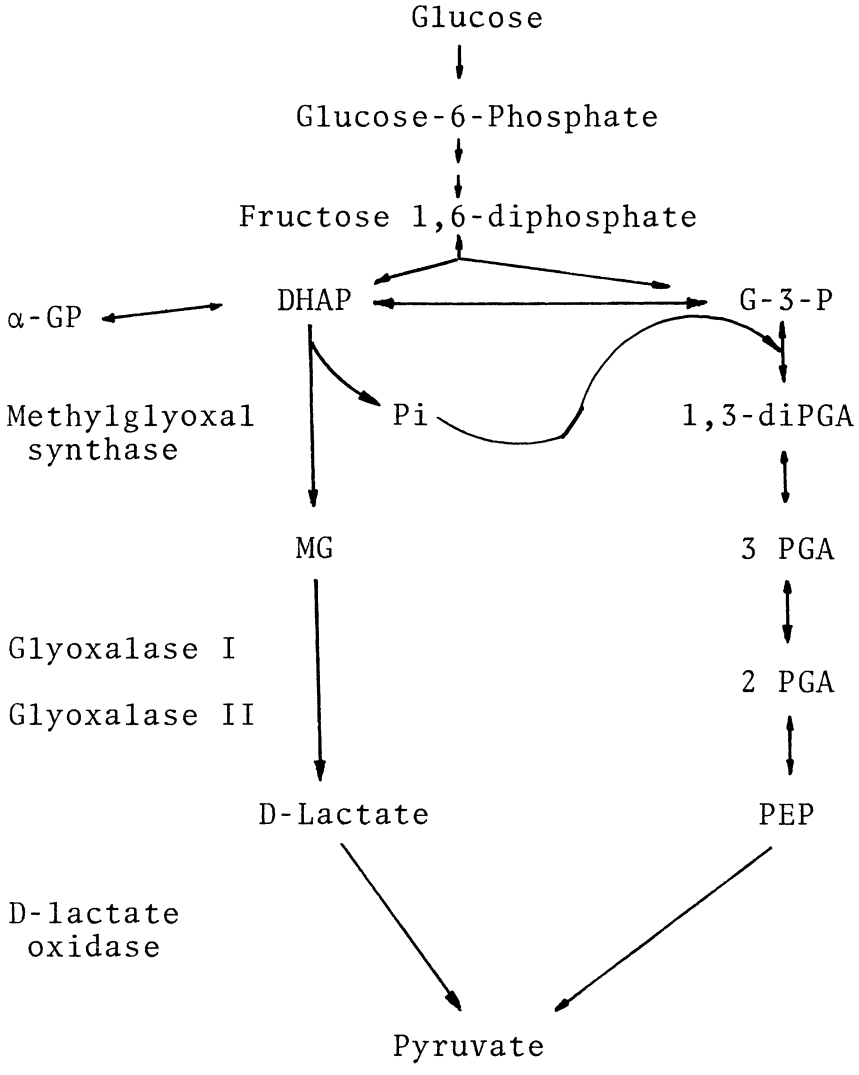
INTRODUCTION

Background

With the discovery of the glyoxalase system by Neuberg (1913) and Dakin et al. (1913), methylglyoxal was first considered to be a possible intermediate in normal glycolysis. Subsequently, Needham and Lehmann (1937) found that methylglyoxal was formed nonenzymatically from DL-glyceraldehyde when the triose was incubated at 37° in Ringer's Bicarbonate buffer. Elliot (1960) reported that methylglyoxal could be enzymatically formed from glycine and threonine via aminoacetone. Later, Bonsignore and his cowrkers (1968, 1970) provided evidence that the formation of methylglyoxal from glyceraldehyde in liver was due to catalysis by lysine. Riddle and Lorenz (1968) reported that the nonenzymatic formation of methylglyoxal from trioses was catalyzed by Tris and by certain polyvalent anions including phytate, phosphate, tetraborate, arsenate, arsenite, cacodylate, α,β-glycerophosphate, glucose-6-phosphate, fructose-6-phosphate, and fructose 1,6-diphosphate. Furthermore, they showed that the conversion took place under physiological conditions of pH and temperature. Therefore, by the late 1960's, it was generally concluded that methylglyoxal was a nonenzymic artifact that glycolysis rather than an authentic intermediate.

However, in 1970 Cooper and coworkers demonstrated an enzyme, methylglyoxal synthase (EC 4.2.99.11) in Escherichia coli which appeared to catalyze the direct conversion of dihydroxyacetone phosphate to methylglyoxal and inorganic phosphate. This finding provided convincing evidence that methylglyoxal might indeed be an intermediate in a pathway involving nonphosphorylated trioses. This metabolic bypass of glycolysis provided a potential route for the conversion of dihydroxyacetone phosphate to pyruvate (Figure 1). The nonphosphorylated bypass involved three additional enzymes (glyoxalase I, EC 4.4.1.5; glyoxalase II, EC 3.1.2.6; and D-lactate oxidase, EC 1.1.1.28), all of which had been clearly demonstrated in a variety of organisms. The only new unique enzyme to the pathway was the first enzyme, methylglyoxal synthase. Subsequently, methylglyoxal synthase was isolated from E. coli (Hopper and Cooper, 1971, 1972) and P. saccharophila (Cooper, 1974). Both enzymes were reported to exhibit molecular weights of approximately 67,000 daltons by gel filtration. On the basis of limited catalytic studies and the sigmoidal release of inorganic phosphate inhibition by dihydroxyacetone phosphate, the enzyme was proposed to possess three binding sites for dihydroxyacetone phosphate and to be regulated by inorganic phosphate. These observations led to the concept that this shunt might serve to replenish intracellular inorganic phosphate. However, in both cases involving the enzyme

Figure 1. Metabolic pathways involving methylglyoxal. Abbreviations are as follows: DHAP = dihydroxyacetone phosphate; MG = methylglyoxal; G-3-P = glyceraldehyde 3-phosphate; 1,3-di PGA = 1,3-diphosphoglycerate; 2-PGA = 2-phosphoglycerate; 3-PGA = 3-phosphoglycerate; PEP = phosphoenol pyruvate; αGP = α-glycerophosphate.


Glucose
Glucose-6-Phosphate
Fructose 1,6-diphosphate
α-GP
DHAP
G-3-P
Methylglyoxal synthase
Pi
1,3-diPGA
MG
3 PGA
Glyoxalase I
Glyoxalase II
2 PGA
D-Lactate
PEP
D-lactate oxidase
Pyruvate

from either E. coli or P. saccharophila, the degree of homogeneity of the enzyme was not assessed. Moreover, the extremely low recovery (2.6%) precluded most physical and chemical studies on the enzyme.

More recently, Tsai and Gracy (1976) reported the isolation and characterization of crystalline methylglyoxal synthase from Proteus vulgaris. A detailed study of the physical and catalytic properties of the enzyme has been conducted and is summarized in Table I. The work of Tsai and Gracy provided a better method for the isolation of methylglyoxal synthase in quantities suitable for structural-functional and mechanism studies.

Purpose of This Investigation

In spite of the suggested methylglyoxal bypass of glycolysis in some bacteria, little is known regarding the occurrence of the enzyme in both eukaryotic and prokaryotic organisms. Since dihydroxyacetone phosphate can be readily metabolized by several other enzymes such as triosephosphate isomerase, aldolase and α-glycerophosphate dehydrogenase, it is possible that these competing enzymes may interfere with the detection of methylglyoxal synthase. Furthermore, methylglyoxal synthase is allosterically inhibited by inorganic phosphate. Thus, a number of potential problems exist in the detection and accurate measurement of methylglyoxal synthase in tissue and cell extracts.

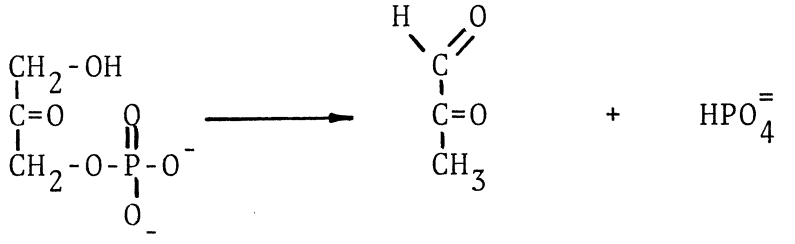
TABLE I

PROPERTIES OF METHYLGLYOXAL SYNTHASE OF PROTEUS VULGARIS

Properties	Method	Value
Molecular Weight	Gel Filtration	135,000
$S_{20,w}$	Sedimentation Velocity Ultracentrifugation	7.2S
Subunit Molecular Weight	SDS-Gel Electrophoresis	66,000
pH Optimum	Steady State Kinetics	7.7
Isoelectric Point	Isoelectric Focusing	4.9
K_m for DHAP	Steady State Kinetics	0.2 mM
K_i for Pyrophosphate	Steady State Kinetics	0.045 mM
K_i for Phosphoglycerate	Steady State Kinetics	0.0175 mM

Therefore, the first objective of this investigation was to develop and test several assay methods for accurately measuring the enzyme in extracts, and to survey a variety of organisms for the presence of the enzyme.

The second objective of this investigation was to explore the reaction mechanism. The overall reaction,



is unusual and appears rather complex, involving not only dephosphorylation but also an internal oxidation and reduction. The sequence of events and the overall course of the enzymatic conversion from dihydroxyacetone phosphate to methylglyoxal and inorganic phosphate appeared to pose intriguing questions. In the present studies I have attempted to answer these questions and to shed light on the reaction mechanism.

MATERIALS AND METHODS

Materials

Enzymes

Yeast glyoxalase I (4.4.1.5), specific activity 360 units/mg, phosphofructokinase (EC 2.7.1.11), specific activity 175 units/mg, phosphoglucose isomerase (EC 5.3.1.1), specific activity 550 units/mg, triosephosphate isomerase (EC 5.3.1.1), specific activity 6450 units/mg, aldolase (EC 4.1.2.13), specific activity 12 units/mg, and α-glycerophosphate dehydrogenase (EC 1.1.1.8), specific activity 175 units/mg were obtained from Sigma Chemical Company. Hexokinase (EC 2.7.1.1), specific activity 20 -30 units/mg, was from Nutritional Biochemical Corporation.

Substrates

Dihydroxyacetone phosphate (dimethylketal cyclohexylammonium salt), DL-glyceraldehyde 3-phosphate (diethyl acetal monobarium salt), glutathione (reduced form), methylglyoxal [40% aqueous solution which was distilled and restandardized by the method of Kotsch and Bergmeyer (1965)] were from Calbiochem. D-[U-^{14}C]-glucose (1.49 mCi/mg) and D-[6-^{14}C]-glucose (329 μCi/mg) were from Amersham Searle, while D-[3,4-^{14}C]-glucose (80 μCi/mg) was from New England Nuclear.

Chromatographic and Electrophoretic Supplies

DEAE-Sephadex A-50 (exchange capacity 3.5 meq/g), DEAE cellulose (exchange capacity 0.8 meq/g) were obtained from Sigma Chemical Company. Dowex-1 (AG 1x8 formate 140-325 wet mesh), DEAE-Biogel, and reagents for polyacrylamide disc gel electrophoresis were from Bio-Rad. Ampholines and all reagents for isoelectric focusing were from LKB Instruments. Thin-layer cellulose sheets (No. 13255) were from Eastman Company.

Miscellaneous Reagents and Organisms

Trypticase Soy Broth and Sabaroud's media were from BioQuest. Tris-(hydroxymethyl)-aminomethane and EDTA were enzyme grade reagents obtained from Nutritional Biochemicals. Imidazole (Grade I), p-hydroxymercuribenzoate and iodoacetic acid were from Sigma Chemical Company. The iodoacetic acid was recrystallized from petroleum ether prior to use. [^{3}H]-$NaBH_4$ (706 mCi/mmole) was obtained from Amersham-Searle. Microrganisms were obtained from stock cultures of the Department of Biological Sciences, North Texas State University, and all living animals were from Carolina Biological Supply Company. Haloacetol phosphates were synthesized by F. C. Hartman as previously reported (1970a).

Methods

Synthesis of [^{14}C] Dihydroxyacetone Phosphate

D-[6-^{14}C] Glucose was used to synthesize C-1 labeled [^{14}C]dihydroxyacetone phosphate, while C-3 [^{14}C]dihydroxyacetone phosphate was synthesized from D-[3,4-^{14}C]glucose. Uniformly labeled [^{14}C]dihydroxyacetone phosphate was synthesized from D-[U-^{14}C]glucose. In each case, the following components were included in the reaction mixture: ATP, 10 mM; $MgCl_2$, 5 mM; KCl, 50 mM; $(NH_4)_2SO_4$, 5 mM; carrier D-glucose, 2 mM; D-[^{14}C]glucose, 10 μCi; 2-mercaptoethanol, 1 mM; and 10 units each of hexokinase, glucosephosphate isomerase, phosphofructokinase, aldolase and triosephosphate isomerase. The reaction was carried out in 20 ml of 50 mM triethanolamine (Cl), pH 8.3, at 30°C for 1.5 hours. The extent of reaction was monitored by removing a small aliquot at intervals and diluting into acetone to terminate the enzymatic reactions. The acetone was removed with a stream of dry N_2, and the aliquot was assayed for dihydroxyacetone phosphate by coupling with α-glycerophosphate dehydrogenase and measuring the oxidation of NADH at 340 nm. After the reaction was judged to be complete, the mixture was passed through a 1 x 15-cm column of AG 1x8 formate (140-325 wet mesh), which had been equilibrated in distilled water. The adsorbed sugar phosphates were fractionated according to Bartlett (1959) at a flow rate of 1.0 ml/min with a

2-liter linear gradient of 0 to 1.0 N formic acid. Fractions of 10 ml each were collected, and 50-µl aliquots were analyzed by liquid scintillation. Dihydroxyacetone phosphate appeared as the primary radioactive component and was eluted in fractions 110 to 125. These fractions were pooled, concentrated in vacuo to 5 to 10 ml, lyophilized to dryness, dissolved in 2 - 4 ml of distilled water. The yield and purity of [^{14}C]dihydroxyacetone phosphate were determined by enzymatic assay with α-glycerophosphate dehydrogenase and by chromatography on Eastman No. 13255 thin-layer cellulose sheets according to Bandurski and Axelrod (1951), using an acidic solvent system composed of methanol:formic acid:water (80:15:5) and a basic system of methanol:ammonium hydroxide:water (60:10:30).

Enzyme Assays

Methylglyoxal synthase was routinely assayed by the glyoxalase I-coupled spectrophotometric method described by Tsai et al. (1976). The assay mixture contained in a volume of 1.0 ml: 45 mM imidazole buffer (ph 7.0), 1.0 mM dihydroxyacetone phosphate, 1.67 mM reduced glutathione, glyoxalase I (1.0 unit), and methylglyoxal synthase. The rate of formation of S-lactoyl glutathione was determined from the increase in absorbance at 240 nm. In this system, 1 µmole per ml of S-lactoyl-glutathione has an absorbance at 240 nm of 3.4 (Racker, 1957).

A colorimetric assay for methylglyoxal was also carried out according to the method of Cooper et al. (1970). The reaction mixture at 30° contained in 0.5 ml: 45 mM imidazole (Cl), pH 7.0; dihydroxyacetone phosphate (1 mM); and methylglyoxal synthase. The methylglyoxal formed was measured colorimetrically by first adding 0.33 ml of 2,4-dinitrophenylhydrazine (0.1%, 2,4-dinitrophenylhydrazine in 2 N HCl) to the reaction mixture plus 0.5 ml of water. After incubated at 30° for 15 minutes, 1.67 ml of 10% (w/v) NaOH was added, and after 15 minutes, the absorbance at 555 nm was measured. Control solutions without the synthase were required as references. A molar extinction coefficient of 4.48×10^4 was used to convert readings into micromoles of methylglyoxal formed per minute (Wells, 1966).

For the direct isotope assay for methylglyoxal synthase, [^{14}C]dihydroxyacetone phosphate was diluted with carrier substrate to achieve a specific radioactivity of approximately 10^4 to 10^5 cpm/μmole. The substrate (5 μmoles) was added to 5 ml of 10 mM imidazole, pH 7.0. The solution was preincubated at 30° for 5 minutes, and an aliquot (1.0 ml) removed prior to the addition of the enzyme. The enzyme was added to the remaining 4.0 ml, rapidly mixed on a Vortex stirrer, and immediately returned to the water bath. Aliquots (1.0 ml) were withdrawn at specific intervals and immediately mixed with 0.5 g of AG 1X8 (140 - 325 mesh formate form). The mixture was

stirred for 30 seconds and quantitatively transferred to a 0.5 x 2 cm Pasteur pipette which had been plugged with a small wad of glass wool. The product was allowed to filter through the ion exchanger, and then 1.0 ml of nonradioactive 10 mM methylglyoxal was added to wash the ion exchanger free of any nonspecifically adsorbed [^{14}C]methylglyoxal. The filtrate was transferred to a vial containing 10 ml of scintillation fluid, and the [^{14}C]methylglyoxal measured. The enzyme activity was calculated from the specific radioactivity of the substrate.

For each of the three above assay methods, one unit of methylglyoxal synthase activity is defined as the amount catalyzing the production of one micromole of methylglyoxal per minute at 30°.

Protein Determination

The protein content of crude extracts was determined by either the Biuret method (Gornall et al., 1949) or the Bradford method (Bradford, 1976). During chromatographic fractionation the absorbance at 280 nm was measured. An absorbance index at 280 nm ($E^{1\%}_{1cm}$) of 9.0 was determined for P. vulgaris methylglyoxal synthase by comparison with bovine serum albumin using the fluorescamine method of Udenfriend (1973).

Growth of the Organisms

P. vulgaris (ATCC 13315) was grown on a rotary shaker in 1-liter Erlenmeyer flasks containing 250 ml of Trypticase Soy Broth at 32°, pH 7.3. After 24 hours the inoculum was transferred to a fermentor containing 10 liters of Trypticase Soy Broth medium, and the organism grown for another 36 hours at 32°C, pH 7.3. The cells were then harvested with a Sharples centrifuge (23,000 rpm), washed twice with deionized water, and the cell pellet stored in a freezer at -15°C. All bacteria utilized in the screening procedure were grown to late log phase at 32°C. Yeast and fungi were grown in Sabaroud's media for 36 to 72 hours and also harvested in late log phase.

Preparation of Crude Extracts

Frozen bacterial cells were thawed in 2 volumes of buffer (50 mM imidazole, pH 7.5) and disrupted by sonication. Yeast and fungi were disrupted in a tissue grinder with Teflon pestle. Tissues and animals were homogenized in a Waring blendor. The extracts were centrifuged at 30,900 x g for 1 hour at 4°, and the supernatant solutions collected.

Isolation of Methylglyoxal Synthase

Methylglyoxal synthase was purified from Trypticase Soy Broth-grown Proteus vulgaris cells as described

previously by Tsai and Gracy (1976). In order to botain homogeneous protein, two to three isoelectric focusing steps were routinely carried out.

Electrophoresis and Electrofocusing

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was performed as described by Fairbanks et al. (1971). Isoelectric focusing experiments were conducted using 1% LKB ampholines and a sucrose density gradient in 110 ml LKB columns. All solutions and gradients were prepared according to the supplier. Electrofocusing was performed in pH 4-6 ampholines at 500 volts for 48 hours.

Amino Acid Analyses

Samples were hydrolyzed *in vacuo* at 110° for 24, 48 and 72 hours in 6 N HCl containing 0.02% v/v 2-mercapto-ethanol. Amino acid analyses were carried out on a Durrum automatic protein/peptide analyzer equipped with Gilson Spectra/Glo Fluorometer and linear recorder. Values for serine and threonine were extrapolated to "zero time" hydrolysis. Cysteine and tryptophan were not quantified due to the destruction by acid hydrolysis. The determination of proline was precluded, since this imino acid fails to react with *o*-phthalaldehyde (Benson, 1975).

Iodoform Reaction

Carbon 1- or carbon 3-labeled [^{14}C]dihydroxyacetone phosphate was diluted with carrier dihydroxyacetone phosphate to a final concentration of 25 mM and a specific radioactivity of 2 - 3 x 10^3 cpm/μmole. Aliquots of 0.5 ml of each of the above solutions were incubated at 30° with 50 μl of enzyme solution (0.5 unit). After one hour, 1.0 ml of 10% NaOH was added to the reaction mixture, followed by 1.5 ml of iodine-potassium iodide solution (5 g potassium iodide, 2.5 g iodine in 20 ml of water). The reaction mixtures were heated at 60° in a water bath for two minutes, and the excess iodine was removed by the addition of a few drops of 10% NaOH and shaking. The reaction mixture was allowed to stand for 15 minutes, while the yellowish CHI_3 precipitate formed. Precipitates were collected by centrifugation, washed twice with 10-ml aliquots of water and dissolved in scintillation fluid. Controls were subjected to the same procedure but without the addition of methylglyoxal synthase.

Haloacetol Phosphate Assay

3-Bromoacetol phosphate (BAP) and 3-chloroacetol phosphate (CAP) derivatives (dimethylketal biscyclohexylammonium salts) were hydrolyzed to the ketones by treating with Dowex 50 (H^+) [50 mg of BAP or CAP and 1 g of Dowex 50 (H^+), mixing for 1 minute, followed by filtration

through a medium pore sintered glass frit] and incubating at 40° for 40 hours. The 3-iodo derivative (IAP) was treated similarly except with only a 30-minute incubation period. The concentrations of haloacetol phosphates were estimated as base-labile phosphates, and corrections were made for inorganic phosphate and DHAP as described by Hartman (1970b).

Kinetic Studies

All kinetic parameters were determined at 30.0°, and assays were performed as described in "Enzyme Assays". K_m and K_i values were evaluated using the weighted least squares method of Wilkinson (1961) as adapted to the IBM 360 computer (Cleland, 1967).

RESULTS

Synthesis of [^{14}C]Dihydroxyacetone Phosphate

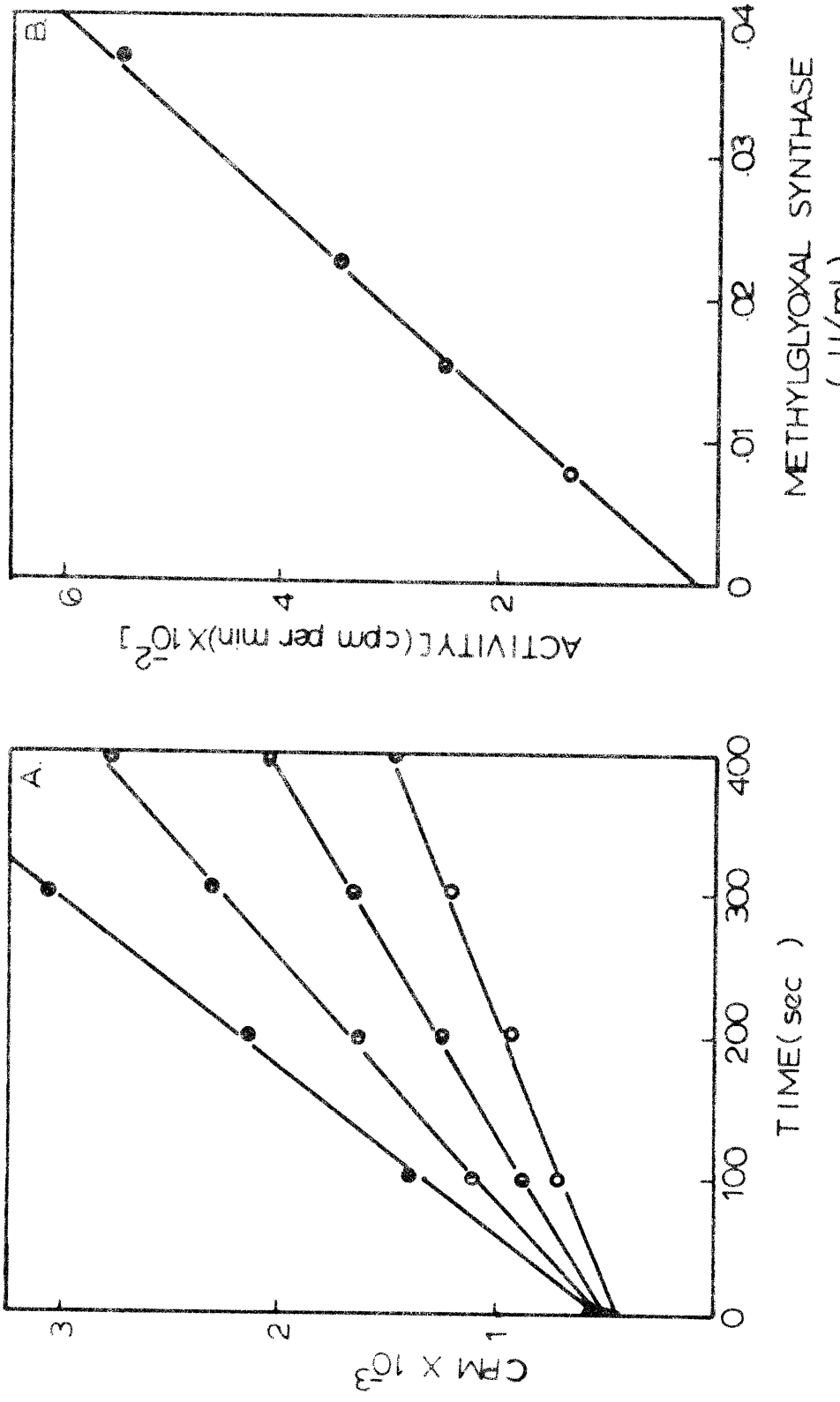
Approximately 50% of the total [^{14}C]glucose was converted to [^{14}C]dihydroxyacetone phosphate by the enzymatic system. The recovery of radiolabeled dihydroxyacetone phosphate from the ion exchange chromatography ranged from 40 to 50%. Thus, the overall yield of [^{14}C]dihydroxyacetone phosphate was from 20 to 30%. The most likely contaminant, glyceraldehyde 3-phosphate, was established by enzymatic assay to be less than 1%. The dihydroxyacetone phosphate appeared as a single spot following thin-layer chromatography in two solvent systems and cochromatographed with authentic dihydroxyacetone phosphate.

Evaluation of Validity of Isotope Assay And Other Assay Methods

Figure 2-A shows the linearity of methylglyoxal formation with respect to time when measured by the direct isotope method. Initial velocities were linear for at least six minutes, but after this time (and especially at higher levels of enzyme), zero order kinetics were no longer followed. When initial velocities were plotted as a function of enzyme concentration, a linear relationship was observed (Figure 2B), indicating that the rate of

Figure 2A. Initial rates of methylglyoxal formation using the direct isotope assay. Methylglyoxal synthase was assayed by incubating [^{14}C]dihydroxyacetone phosphate with the enzyme and measuring the [^{14}C]methylglyoxal formed as described in Methods. Aliquots (0.4 ml) from the Dowex microcolumn effluent were counted in 10 ml of Aquasol (New England Nuclear). The concentrations of methylglyoxal synthase for the incubations were 0.0075 units/ml (○); 0.015 units/ml (◐); 0.225 units/ml (◑); and 0.376 units/ml (●).

Figure 2B. Effect of enzyme concentration on initial rates. Initial velocities (from Figure 2A) are plotted as a function of the concentration of methylglyoxal synthase.


A
B
CPM X 10⁻³
TIME(sec)
ACTIVITY [(cpm per min)X 10⁻²]
METHYLGLYOXAL SYNTHASE
(U/ml)

formation of [^{14}C]methylglyoxal is a valid indicator of the methylglyoxal synthase concentration.

The rates of methylglyoxal synthesis as assessed by the direct assay compared favorably with the rates determined by the coupled enzyme spectrophotometric assay or the colorimetric method (Table II). However, the variability of the isotope assay method was higher than that of coupled enzyme spectrophotometric assays. In general, the colorimetric assay proved to be the most variable, and the least desirable of the three methods.

Distribution of the Enzyme

Tissue homogenates and cell-free extracts of rat liver, skeletal muscle and brain were assayed for methylglyoxal synthase by the coupled enzyme method. In all cases, no traces of the synthase was observed. However, it was realized that due to the competing activities of triosephosphate isomerase, aldolase and α-glycerophosphate dehydrogenase, and perhaps other enzymes such as acid and alkaline phosphatase, it might not be possible to accurately determine methylglyoxal synthase in crude cell homogenates.

In view of the fact that the levels of triosephosphate isomerase are so high in cell extracts, this enzyme appeared to be a likely candidate which could deplete the substrate. 1-Chloroacetol phosphate has been shown to irreversibly inactivate triosephosphate isomerase by covalently binding

TABLE II

COMPARISON OF THE THREE ASSAY METHODS FOR MEASURING METHYLGLYOXAL SYNTHASE

Assay Method	Pure MGS*	Crude MGS**
	Activity (units/ml)	
Direct Isotope Assay	11.2±0.6	4.0±0.3
Spectrophotometric Assay	10.8±0.1	3.3±0.1
Colorimetric Assay	9.7±1.0	2.1±0.3

*Methylglyoxal synthase isolated from P. vulgaris.

**Crude cell extracts from P. vulgaris.

to the catalytic center (Hartman, 1971). On the other hand, this reagent was found to have no inactivation effect on methylglyoxal synthase. When tissue extracts were incubated with 0.01 mM chloroacetol phosphate at 0° for 10 minutes, essentially all the triosephosphate isomerase was inactivated. As seen in Table III, inactivation of triosephosphate isomerase had no effect on the measured levels of methylglyoxal synthase. Tissue extracts of the rat showed no detectable methylglyoxal synthase even in extracts in which the isomerase had been inactivated. Thus, it appeared that triosephosphate isomerase was not interfering with the detection of methylglyoxal synthase.

In order to further test the possibility of interfering enzymes or inhibitors in cell extracts, known quantities of pure crystalline methylglyoxal synthase were added to tissue extracts, followed by reassaying the mixture. In all cases, the amount of enzyme measured in the crude extract was equal to that added, and thus, no interference by competing enzymes or inhibitors was implicated. It thus appears that valid enzyme assays for methylglyoxal synthase can be conducted on tissue and cell extracts.

Human Tissues

Human erythrocytes, lymphocytes, skeletal muscle, kidney, liver, brain and cardiac muscle were tested for methylglyoxal synthase by all three assay methods

TABLE III

EVALUATION OF POSSIBLE INTERFERENCE OF TRIOSEPHOSPHATE ISOMERASE ON THE MEASUREMENT OF METHYLGLYOXAL SYNTHASE

Enzyme Mixture*	Enzyme Activity (units/ml) MGS	TPI
Pure MGS from P. vulgaris	4.7	0
Rat liver extract (31.2 mg/ml)	0	157
Pure MGS + liver extract	4.6	157
Pure MGS + liver extract + chloroacetol phosphate	4.6	0

*Purified methylglyoxal synthase (MGS) was assayed alone or in the presence of triosephosphate isomerase (TPI) in a rat liver extract. Treatment of the mixture with chloroacetol phosphate (0.01 mM) was at 0° for 10 minutes.

discussed above. In no case was any indication of methylglyoxal synthase observed. Tests were carried out on entire cell homogenates, as well as centrifuged extracts. Cells were extracted in a variety of buffers from pH 6.5 to pH 8.5. Dialysis, the addition of 10% v/v glycerol, or the addition of numerous coenzymes or metal ions had no effect. In all cases, the addition of purified P. vulgaris methylglyoxal synthase to the tissue extracts revealed no inhibitors or interfering enzymes present in the cell extracts. Thus, it appears that methylglyoxal synthase is not present in human tissues. The methods employed would have been able to detect as little as 0.01 International Units of the enzyme per gram of tissue.

Other Eukaryotes

In addition to human and rat tissues, other species including crayfish, crab, shrimp, ascarid, snail, lizard, frog, clam, starfish, jellyfish and sponge were examined and showed no traces of methylglyoxal synthase.

Extracts from lizard (Anolis carolinensis), frog (Rana catesbeiana), or starfish (Asterias forbesi) showed a transient increase in absorbance at 240 nm when assayed by the coupled enzyme assay system. However, the absorbance increased only 0.01 to 0.03 over a two to three minute period, then leveled off. The basis of the interference is not certain, but could represent some alpha ketoaldehyde

in these extracts which may be enzymatically condensed with glutathione by glyoxalase I. It is interesting that of all of the organisms examined, only these three species exhibited this behavior.

Yeast, Saccharomyces cerevisiae and Candida albicans, algae, (Chlorella sp.), fungi (Aspergillus sp., Trichophyton sp., and Penicillium sp.) and Euglena gracilis (both heterotropically and photoautotropically grown) all failed to exhibit the synthase.

Bacteria

Table IV summarizes a variety of bacteria which were found to contain methylglyoxal synthase activity. Methylglyoxal synthase were observed in aerobic, anaerobic and facultative anaerobes. In addition to the bacteria listed in Table IV, the following organisms were screened, but exhibited negligible activity (i.e., less than 5×10^{-5} units/mg); Bacillus megaterium, Bacillus subtilis, Clostridium butyricum, Clostridium sporogenes, Corynebacterium diphtheriae, Erwinia carotovora, Lactobacillus bulgaricus, Lactobacillus casei, Leuconostoc sp., Pediococcus cerevisiae, Pseudomonas aeruginosa, Pseudomonas ovalis, Staphylococcus citres, epidermis, and lactis; and Streptococcus thermophilus. Azotobacter vinlandii and Streptococcus faecalis have been reported to not contain the enzyme (Cooper et al., 1971), and were not reassayed. It is of further interest that the levels of

TABLE IV

COMPARISON OF METHYLGLYOXAL SYNTHASE LEVELS IN BACTERIA

Bacterium	Methylglyoxal Synthase* (Specific Activity)
Aerobacter aerogenes	0.37
Erwinia uredora	(0.055)
Escherichia coli K_{10} (glucose grown)	(0.174)
Escherichia coli K_{10} (pyruvate grown)	(0.190)
Escherichia coli K_{12}	0.50
Escherichia coli B/r	0.14
*Klebsiella pneumonae**	0.26
Proteus vulgaris	0.045 (0.064)
Proteus rettgeri	(0.020)
Salmonella parathyphus	0.47
Serratia marcescens	(0.043)
Shigella boydii	0.39

*Values in parentheses are from Cooper et al. (1971). All values are expressed as micromoles of methylglyoxal formed per minute per mg of protein at 30°C.

methylglyoxal synthase varied widely (0.02 to 0.50 units/mg) from the various bacteria.

Since it has been postulated that the methylglyoxal shunt is regulated by levels of inorganic phosphate, the effect of phosphate concentration in the growth media was examined. Varying the phosphate concentration from 2 μM to 2 mM had no effect on the specific activity of the enzyme in P. vulgaris. While the enzyme is synthesized constitutively in the case of facultative anaerobes, the specific activity of the enzyme was lower when grown under anaerobic conditions.

Isolation of Methylglyoxal Synthase from Proteus vulgaris

The enzyme was isolated by using two consecutive anion exchange chromatographic separations followed by preparative isoelectric focusing. The procedure utilized is a modification of that of Tsai and Gracy (1976). Since a large scale preparation was required, a batch procedure using DEAE cellulose, followed by elution with a linear salt gradient (0.0 - 0.4 M KCl in a total of 2 liters, was adopted instead of the DEAE-Sephadex column described by Tsai and Gracy (1976). Fractions containing enzyme activity were pooled and dialyzed overnight against 10 mM imidazole buffer, pH 7.6, containing 1 mM EDTA, 1 mM inorganic phosphate, and 0.05% v/v 2-mercaptoethanol. The dialysate was then loaded onto a second, smaller column

(1.5 cm x 60 cm), packed with DEAE-Biogel, which had been equilibrated with the dialysis buffer. After the sample had been applied to the column, 50 ml of column buffer containing 0.1 M KCl was passed through the column. After this initial wash, a linear gradient consisting of 300 ml of column buffer with 0.1 M KCl in the mixing chamber and 300 ml of column buffer containing 0.3 M KCl in the reservoir was applied. Fractions containing methylglyoxal synthase activity were collected and dialyzed overnight. Following this step 20 to 30 mg of protein from this partially purified fraction were electrofocused in a preparative isoelectric focusing column with pH 4-6 narrow range ampholines. A summary of the purification of methylglyoxal synthase is shown on Table V. Since the enzyme was very unstable in the range pH 4-6, the overall yield was only 5 to 10%.

Homogeneity Studies

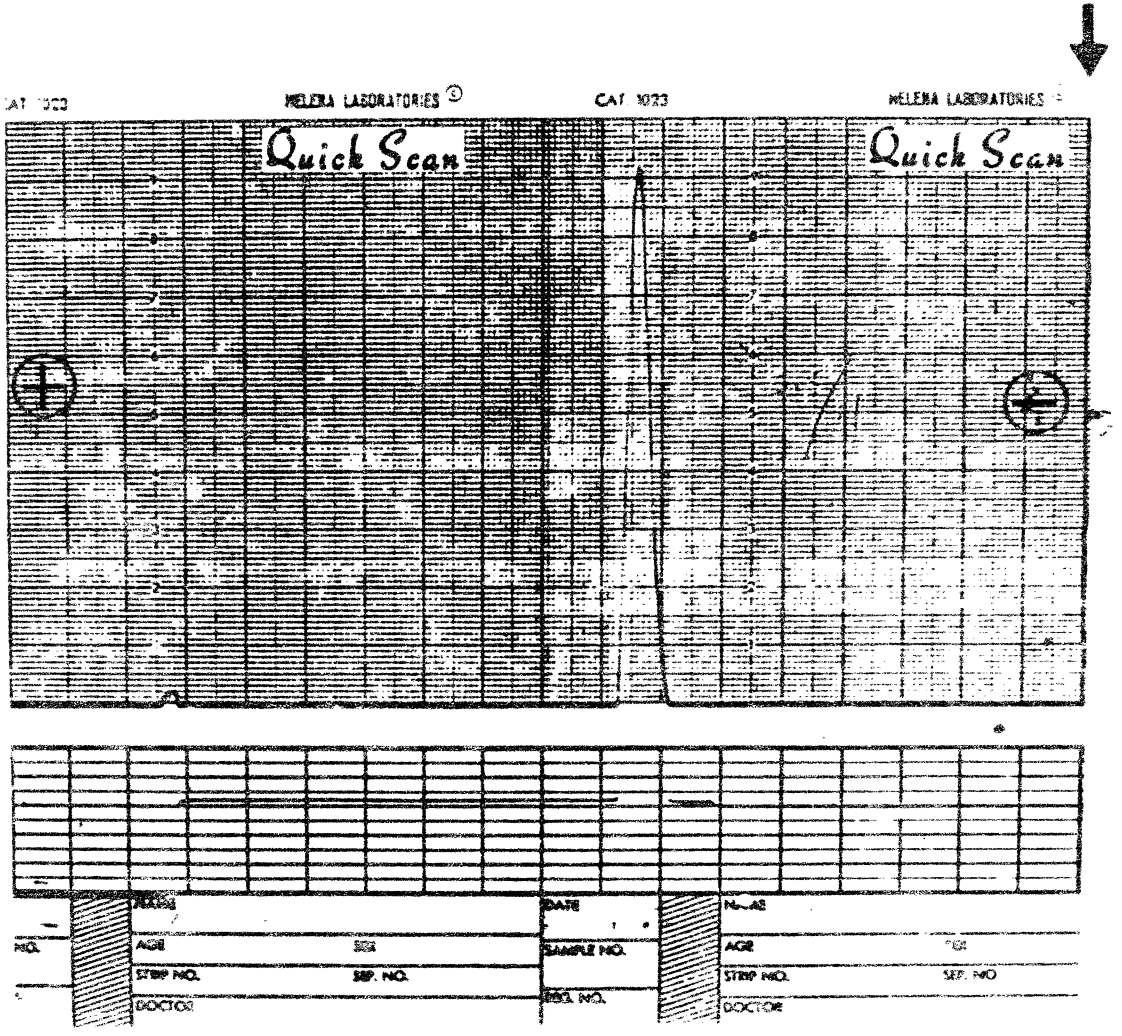
The homogeneity of the enzyme was evaluated for samples with specific activities of 9 to 11 units/mg. Figure 3 shows a densitometer tracing of a sample with specific activity 10.5 units/mg after electrophoresis on standard SDS-polyacrylamide gels. Only a slight contaminant protein was found, indicating that the enzyme was essentially homogeneous.

TABLE V

ISOLATION OF METHYLGLYOXAL SYNTHASE FROM *P. VULGARIS*

Stage of Purification	Volume	Total Protein	Total Activity	Specific Activity	Recovery	Purification Factor
	ml	mg	units	units/mg	%	fold
1. Crude extract	264	16,900	755	0.043	100	
2. DEAE cellulose	360	468	360	0.76	47	17
3. DEAE Biogel	50	70	252	3.6	33	84
4. Isoelectric focusing	11	4.2	49	11	5.6	270

Figure 3. Densitometer tracing of SDS polyacrylamide gel electrophoresis of isolated methylglyoxal synthase from Proteus vulgaris. Approximately 3 μg of enzyme (specific activity 10.5 units/mg) was applied to the 5.0% gel which was prepared and run as described by Fairbanks et al. (1971). The position of the main peak was essentially the same as the marker protein (bovine serum albumin, molecular weight - 67,000 daltons) run in an identical tube. The anode is to the left, and the cathode the right. The arrow (⬇) indicates the top of the gel.


HELENA LABORATORIES
Quick Scan
HELENA LABORATORIES
Quick Scan
DATE
SAMPLE NO.
AGE
STRIP NO.
DOCTOR

Amino Acid Composition of Methylglyoxal Synthase from P. vulgaris

Due to the limited amount of protein available, the complete amino acid analysis for methylglyoxal synthase was not possible. However, on the basis of composition calculated from chromatograms from 24, 48 and 72 hour hydrolysates, a minimum composition with minimum molecular weight was calculated and is presented in Table VI.

Stability of the Enzyme

When the enzyme was stored at 4° in 10 mM imidazole buffer, pH 7.0, containing 1 mM EDTA, 1 mM KH_2PO_4, 0.05% (v/v) 2-mercaptoethanol at a protein concentration of 0.1 to 0.5 mg per ml, no significant loss of activity was observed for a month. However, upon removal of inorganic phosphate, the enzyme rapidly lost activity. For example, a 30-hour dialysis against the above buffer without phosphate resulted in 50% loss of activity.

The isolated enzyme proved to be inactivated by freezing. Even in the presence of phosphate, freezing for 24 hours resulted in 90% loss of catalytic activity. During this inactivation, a precipitate formed. Activity could not be recovered either by dialysis against buffer containing phosphate or by warming the solution or the precipitate at 37°. As shown in Figure 4, the presence of 1 mM inorganic phosphate not only reduced the rate of

TABLE VI

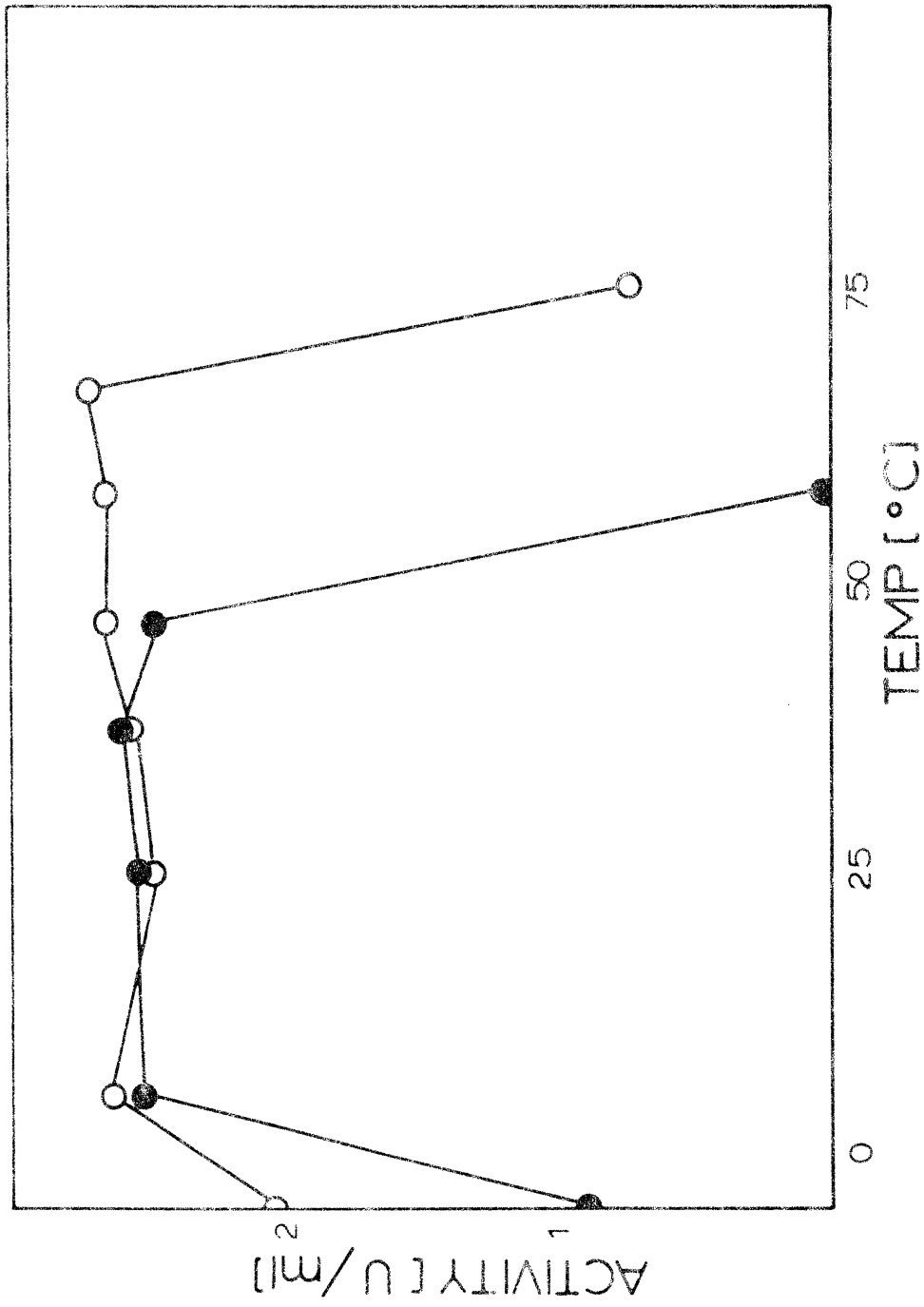
MINIMUM AMINO ACID COMPOSITION OF METHYLGLYOXAL SYNTHASE FROM *P. VULGARIS*

Amino Acid**	Residues/Isoleucine*	Nearest Integer
Lysine	5.0	5
Histidine	8.4	8
Arginine	9.6	10
Aspartic acid	14.6	15
Threonine	12.8	13
Serine	24.4	24
Glutamic acid	22.8	23
Glycine	28.4	28
Alanine	8.6	9
Valine	<1	0
Methionine	<1	0
Isoleucine	1.0	1
Leucine	5.0	5
Phenylalanine	2.2	2

*Minimum molecular weight = 14,600.

**Tryptophan, cysteine, proline and tyrosine were not included by this analysis.

Figure 4. Effect of temperature on the stability of methylglyoxal synthase. Methylglyoxal synthase (0.2 mg/ml) was incubated in 10 mM imidazole buffer, pH 7.6, containing 10 mM 2-mercaptoethanol for one hour in the presence (○) and absence (●) of 1 mM potassium phosphate. Aliquots were removed after one hour and assayed at 30° by the coupled spectrophotometric method.


ACTIVITY [U /ml]
2
1
0
25
50
75
TEMP [°C]

freeze-inactivation, but also markedly increased the stability of the enzyme toward higher temperatures. Incubation of the enzyme with methylglyoxal had no effect on the thermostability of the enzyme.

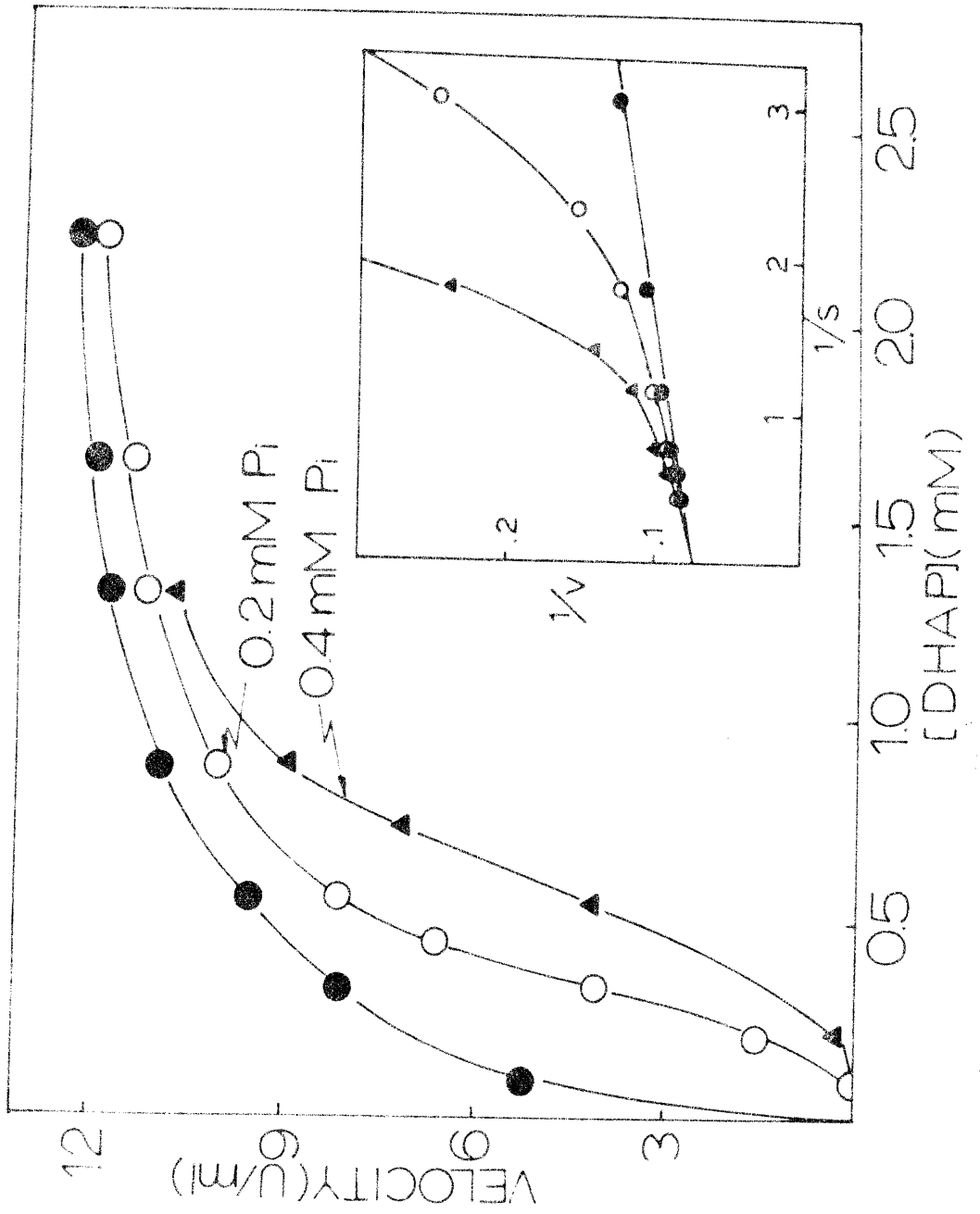
Product Inhibition

One of the primary objectives in elucidating any enzyme-catalyzed reaction mechanism is the establishment of the sequence of events involved in substrate binding and product release. It was difficult to measure the methylglyoxal synthase reaction in the reverse direction. Thus, the problem was approached by product inhibition methods.

The inhibition by inorganic phosphate is complex and transforms the hyperbolic saturation kinetics with respect to dihydroxyacetone phosphate into a sigmoidal response (Figure 5). Nonetheless, this nonlinear inhibition by inorganic phosphate is competitively overcome at high levels of dihydroxyacetone phosphate. In the absence of additional physical information related to the number of phosphate binding sites, and assuming the simplest case, these data suggest that inorganic phosphate binds to the same form of enzyme as does dihydroxyacetone phosphate (Boyer, 1970).

It was not possible to assess product inhibition by methylglyoxal utilizing the coupled assay, but it was possible to measure this inhibition by using both the

Figure 5. Effect of inorganic phosphate on initial velocity. Methylglyoxal synthase was assayed at 30° at varying concentrations of dihydroxyacetone phosphate and fixed levels of inorganic phosphate. Reactions were performed in the absence (●) of inorganic phosphate, as well as in the presence of 0.2 mM (○) and 0.4 mM (▲) inorganic phosphate. The insert double reciprocal plot indicates that the nonlinear inhibition is competitively prevented at high levels of substrate.


VELOCITY(U/ml)
12
9
6
3
0.5
1.0
1.5
2.0
2.5
[DHAP](mM)
0.2mM Pi
0.4mM Pi
1/v
.2
.1
1/s
1
2
3

colorimetric and direct radioisotope assay. With either assay system, methylglyoxal was found to behave as a linear noncompetitive inhibitor (Figure 6). This noncompetitive product inhibition by methylglyoxal, when viewed with the competitive product inhibition by inorganic phosphate, indicates an ordered release of products with methylglyoxal being released prior to inorganic phosphate. Secondary replots of slopes and intercepts vs. methylglyoxal concentrations were linear and yielded K_{ii} and K_{is} values for methylglyoxal of 0.64 mM and 0.65 mM, respectively. For an ordered Uni-bi reaction of this type,

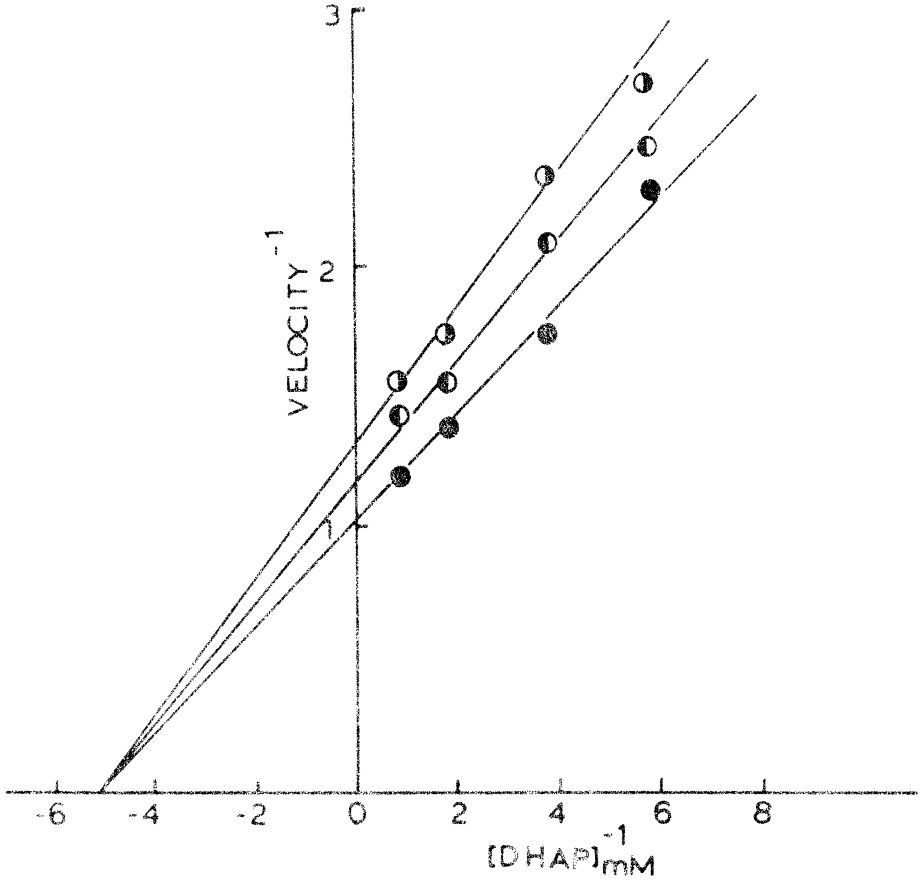
$$\frac{(K_{ii})\ (K_{m\ DHAP})}{(K_{i\ DHAP})} = K_{is}$$

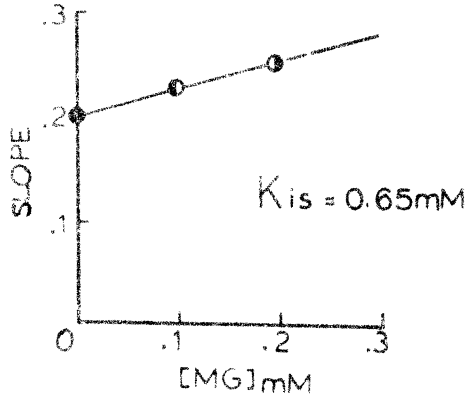
where K_{ii} and K_{is} are the apparent dissociation constants determined from intercept and slope replots, respectively (Segel, 1977). A true dissociation constant for dihydroxyacetone phosphate ($K_{i\ DHAP}$) of 0.21 mM was thus calculated. Therefore, it appears that the apparent K_m for dihydroxyacetone phosphate obtained from standard initial velocity studies (0.2 mM) represents the actual dissociation constant for the dihydroxyacetone phosphate - enzyme complex.

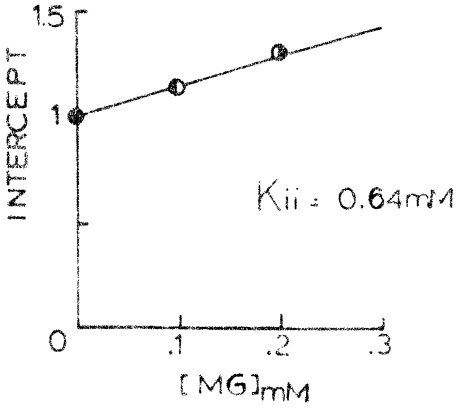
The Overall Course of the Reaction

The overall reaction catalyzed by methylglyoxal synthase may be viewed as a dephosphorylation and

Figure 6. Effect of methylglyoxal on initial velocity. Initial velocities were measured by the direct isotope assay at pH 7.0, 30°. The results were plotted in the standard double reciprocal fashion. The velocities are expressed on a relative scale. Reactions were carried out in the absence of methylglyoxal (●), as well as in the presence (◐) 0.1 mM, and (◑) 0.2 mM methylglyoxal. The secondary replots below are for slope and intercept changes in the primary double reciprocal plot.


VELOCITY^{-1}
3
2
1
-6
-4
-2
0
2
4
6
8
$[DHAP]^{-1}_{mM}$


SLOPE
.3
.2
.1
0
.1
.2
.3
[MG]mM
Kis = 0.65mM


INTERCEPT
1.5
1
0
.1
.2
.3
[MG]mM
Kii = 0.64mM

intramolecular oxidation-reduction process. However, it is not clear a priori which carbon atom, i.e., C-1 (the phosphorylated carbon) or C-3 (the carbon bearing the free hydroxyl) is oxidized and which is reduced. In order to answer this question, C-1 and C-3 radiolabeled dihydroxyacetone phosphates were prepared and utilized as substrates. The structural relationship of substrate and product was determined by examining the radioactivity of the methyl carbon of methylglyoxal after precipitating it as CHI_3, as indicated below:

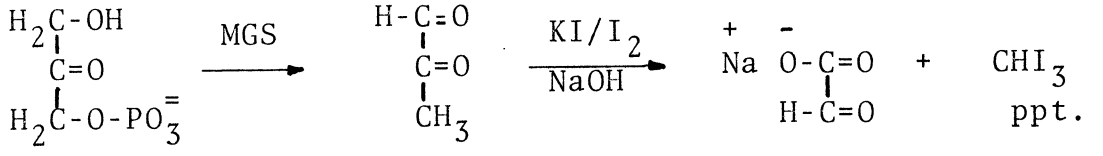


Table VII shows that formation of radioactive CHI_3 occurred only when the phosphorylated carbon of dihydroxyacetone phosphate was radiolabeled. When the nonphosphorylated carbon of dihydroxyacetone phosphate was radioactive, treatment of the methylglyoxal with alkaline KI/I_2 resulted in a nonradioactive precipitate of CHI_3. In both cases, the overall extent of the reactions (dihydroxyacetone phosphate to methylglyoxal) was essentially the same. These results clearly indicate that it is carbon 3 of dihydroxyacetone phosphate which is oxidized, while carbon 1, bearing the phosphate ester, is reduced to the methyl group of methylglyoxal.

TABLE VII

OVERALL COURSE OF THE REACTION

Substrate	Radioactivity of CHI_3 Precipitate (cpm)	Percent Reaction Completion**
H_2-C-OH C=O H_2*C-O-$PO_3^=$	7900	50
H_2*C-OH C=O H_2C-O-$PO_3^=$	170	50

Reaction condition was presented in "Methods" section.

*The position of the ^{14}C label is noted by the asterisk.

**Percent reaction completion was estimated from the amount of substrate (dihydroxyacetone phosphate) remaining in the reaction mixture at the end of the incubation period by coupling with α-glycerophosphate dehydrogenase and measuring the oxidation of NADH at 340 nm.

Effects of the Sulfhydryl Reagents

Sulfhydryl reagents have been widely utilized to probe "essential SH groups" required for enzyme activity of a variety of enzymes. Two of the most commonly used reagents, p-mercuribenzoate and iodoacetate, were tested with methylglyoxal synthase.

p-Mercuribenzoate (PMB)

Methylglyoxal synthase (0.1 mg) was added to 1 ml of imidazole buffer (50 mM, pH 7.0) containing 0.5 mM PMB. Aliquots were withdrawn at 30-minute intervals. Enzyme activity and the change of absorbance at 250 nm were measured against both enzyme and reagent control blanks. At the end of a three-hour period, the reaction essentially reached completion as indicated by the change in absorbance at 250 nm. However, the enzyme remained 100% active under these conditions.

Iodoacetate (IAA)

Incubation of the enzyme (0.1 mg/ml) for four hours with 5 mM IAA in 50 mM imidazole, pH 7.0, or 50 mM triethanolamine, pH 8.0, at 37°, resulted in no loss of enzyme activity when assays were conducted through the entire incubation period. These results and those described above utilizing p-mercuribenzoate strongly suggest that a sulfhydrl group is not intimately involved in the catalytic mechanism.

Effect of $NaBH_4$ on the Enzyme-Substrate Complex

Since Bonsignore et al. (1970) showed that free lysine catalyzes methylglyoxal formation from glyceraldehyde via the formation of a Schiff-base intermediate, it was possible that the methylglyoxal synthase reaction also involved the formation of a Schiff-base intermediate. To test this possibility, methylglyoxal synthase (0.1 mg/ml) was treated with sodium borohydride (5 mM) in the presence and absence fo dihydroxyacetone phosphate (1 mM) in 50 mM imidazole buffer, pH 7.0, at 37°. Aliquots were withdrawn and assayed at different times throughout a 30-minute incubation period. No evidence for the formation of a Schiff base was detected, since methylglyoxal synthase treated with sodium borohydride in the presence of dihydroxyacetone phosphate had the same activity as enzyme treated in the absence of dihydroxyacetone phosphate. This was about 65% of the original activity.

Attempts to Desensitize the Enzyme

Since inorganic phosphate markedly alters the saturation kinetics of dihydroxyacetone phosphate from hyperbolic to sigmoidal, attempts to desensitize the enzyme were carried out. However, incubation of enzyme (0.1 mg/ml) at 45°, 50°, and 55° for 30 minutes, followed by saturation kinetic studies on the enzyme in the presence and absence of 0.1 mM inorganic phosphate, gave no evidence

of desensitization. Moreover, in the absence of inorganic phosphate, K_m and V_{max} values were essentially unchanged from the untreated enzyme.

Metal Ions and Coenzymes

In view of the unique reaction catalyzed by methylglyoxal synthase, it was suspected that catalysis might involve either an essential metal ion or a coenzyme. However, exhaustive treatment of the enzyme with chelating agents such as EDTA or dithizone did not alter the rates of catalysis.

Incubation of the enzyme (0.1 mg/ml) with a variety of metals (as chlorides at 1 mM concentration) for four hours resulted in precipitates for the mixtures containing Fe^{++}, Fe^{+++}, and Cd^{++}. Other metal ions resulted in only slight changes in activity and are summarized in Table VIII. None of the metal ions provided implication of the involvement of an essential metal atom in the catalytic mechanism.

Cofactors including NADH, NAD^+, $NADP^+$, NADPH, FAD, FMN, Vitamin B_6, and Vitamin B_{12} were tested with enzyme. In all cases, no evidence of involvement was observed, and the presence of these coenzymes had no effect on catalysis.

TABLE VIII

EFFECTS OF METAL CHLORIDES ON METHYLGLYOXAL SYNTHASE

Metal Chlorides (1 mM)	V_m*/V_c** (%)
Ni^{++}	83.0
Ca^{++}	91.6
Fe^{++}	58.3 (ppt)
Fe^{+++}	8.3 (ppt)
Cd^{++}	10.4 (ppt)
Co^{++}	67.0
Mg^{++}	102
Zn^{++}	96.0
Mn^{++}	100

*Initial velocity after incubation in the presence of metal chlorides.

**Initial velocity of the control.

Labeling the Catalytic Center

Insights were sought as to the nature of the catalytic center of the enzyme. Hartman and coworkers (1971) have shown that the haloacetol phosphates are effective affinity labels for triosephosphate isomerase and aldolase. Since the haloacetol phosphates are analogs of dihydroxyacetone phosphate, it was reasoned that they might bind in the catalytic center of the synthase. Furthermore, it was reasoned that an active nucleophilic residue in the catalytic center might be close enough to displace the halogen and bring about a covalent labeling of the active center of the enzyme.

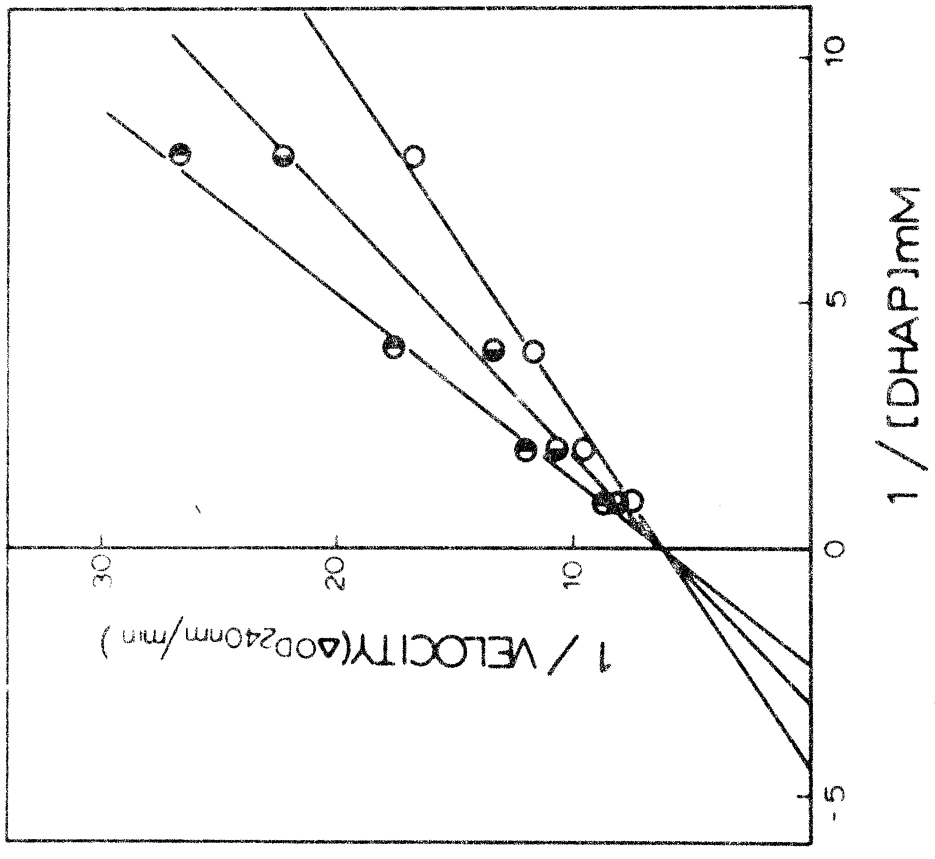
Chloroacetol Phosphate

Reaction of 5 mM chloroacetol phosphate with 0.1 mg/ml of methylglyoxal synthase at 37° for 4 hours resulted in no detectable inactivation of the enzyme. However, when enzyme assays were carried out in the presence of chloroacetol phosphate, a typical competitive inhibition pattern was observed (Figure 7). A dissociation constant (K_i) value of 0.93 mM was obtained. This compared with a K_m value of 0.22 mM for dihydroxyacetone phosphate.

Bromo- and Iodoacetol Phosphates

Incubation of methylglyoxal synthase with bromo- or iodoacetol phosphate at 37° resulted in irreversible

Figure 7. Inhibition of methylglyoxal synthase by chloroacetol phosphate. Initial rates were measured at various dihydroxyacetone phosphate concentrations in the presence and absence of the analogue. Results are plotted in the double-reciprocal form: ○ = no inhibitor; ◐ = 0.35 mM chloroacetol phosphate; ◑ = 0.70 mM chloroacetol phosphate.


1 / VELOCITY(ΔOD240nm/min)
30
20
10
0
-5
5
10
1 / [DHAP]mM

inactivation of methylglyoxal synthase. The initial loss of 40-50% of activity followed pseudo first-order kinetics (Figure 8). Thereafter, the decreased rate was probably attributable to the hydrolysis of the haloacetol phosphates during longer periods of incubation (Hartman, 1970). In order to gain further insight into the mechanism of this inactivation, two schemes may be represented as follows:

$$\text{XAP} + \text{MGS} \xrightarrow{k_{2nd}} \text{XAP-MGS} \qquad (1)$$

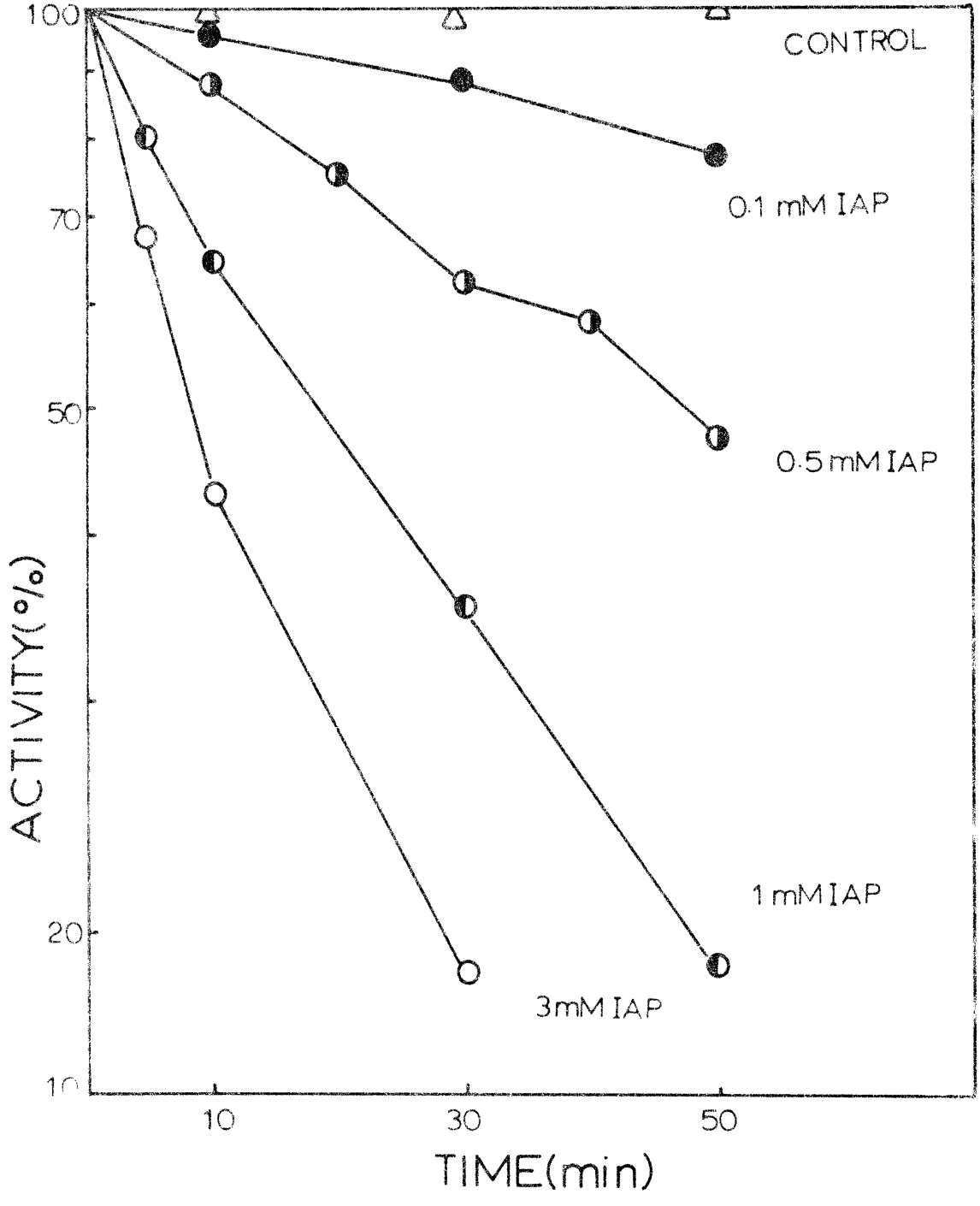
$$\text{XAP} + \text{MGS} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{XAP}\cdots\text{MGS} \xrightarrow{k_2} \text{XAP-MGS} \qquad (2)$$

If the inactivation is a simple bimolecular reaction as represented in equation (1), the second order rate constant can be thus obtained by:

$$\tau = \frac{\ln 2}{[I]k_{2nd}}$$

in which τ is the half-time of inactivation, and [I] is the molar concentration of the inhibitor (Aldridge, 1950). On the other hand, if the inhibitor combines reversibly with the active site before covalent modification as shown in equation (2), applying steady-state kinetics, Meloche (1967) has derived a linear rate expression corresponding to equation (2) which clearly shows the dependence of the inactivation rate on inhibitor concentration:

Figure 8. Time course inactivation of methylglyoxal synthase by iodoacetol phosphate (IAP). Methylglyoxal synthase (0.1 mg/ml) was incubated with 0.1 mM IAP (●), 0.5 mM IAP (◑), 1 mM IAP (◐), 3 mM IAP (○), and 0.0 mM IAP (△) at 37°, pH 7.0 (50 mM imidazole). Periodically, aliquots of the reaction mixture were withdrawn and assayed immediately by the coupled enzyme spectrophotometric method.


100
70
50
20
10
ACTIVITY(%)
CONTROL
0.1 mM IAP
0.5 mM IAP
1 mM IAP
3mM IAP
10
30
50
TIME(min)

$$\tau = \frac{1}{[I]} (T\ K_{inact}) + T$$

K_{inact} is $\frac{k_{-1} + k_2}{k_1}$ and is comparable to K_m in the Briggs Haldane treatment of the Michaelis-Menten expression, and T is the minimal inactivation half-time. Since a plot of τ vs. $\frac{1}{[I]}$ should intercept the τ axis at zero if equation (1) is operative, and at a finite value (T) if equation (2) is operative, the two schemes are kinetically distinguishable. Figure 9 shows the plot of τ vs. $\frac{1}{[XAP]}$. Limiting half-lives of inactivation of 52 and 13 minutes were obtained for the bromo- and iodoacetol phosphates, respectively.

These results suggest that the inactivation mechanism follows the second scheme [equation (2)] which is a typical course of inactivation mechanism for competitive type of inhibitors. Furthermore, apparent dissociation constants of 0.25 and 1.1 mM were obtained for the bromo- and iodoacetol phosphates, respectively (Figure 9).

Figure 10 shows the substrate, dihydroxyacetone phosphate, and the product, inorganic phosphate, both protect against the haloacetol phosphate-induced inactivations. These observations further support the idea that the haloacetol phosphates are binding in the catalytic center. The inactivated enzyme could not be reactivated by reducing agents such as cysteine or by exhaustive

Figure 9. Inhibition half-life ($\tau_{1/2}$) determined from initial rates of inactivation as a function of the reciprocal of concentrations of bromoacetol phosphate (○) or iodoacetol phosphate (●). Reaction mixtures, buffered with 50 mM imidazole (pH 7.0), contained methylglyoxal synthase (0.1 mg/ml), and various concentrations of bromo- and iodoacetol phosphate at 37°C.

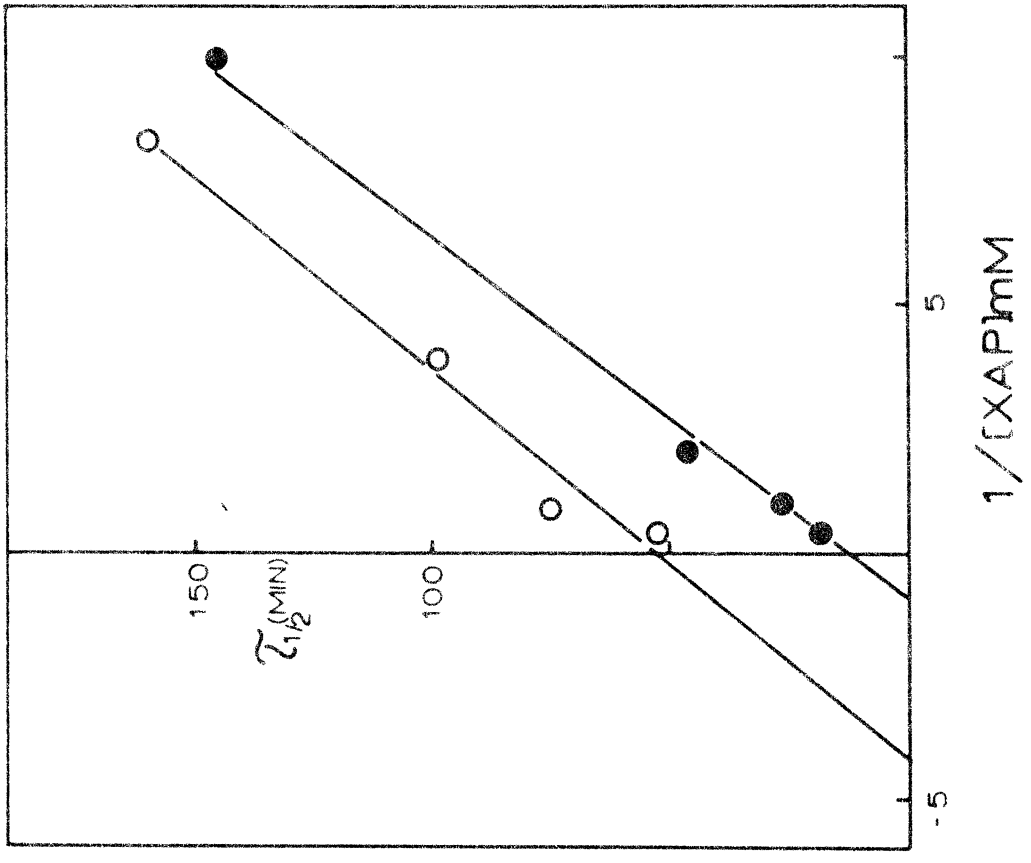
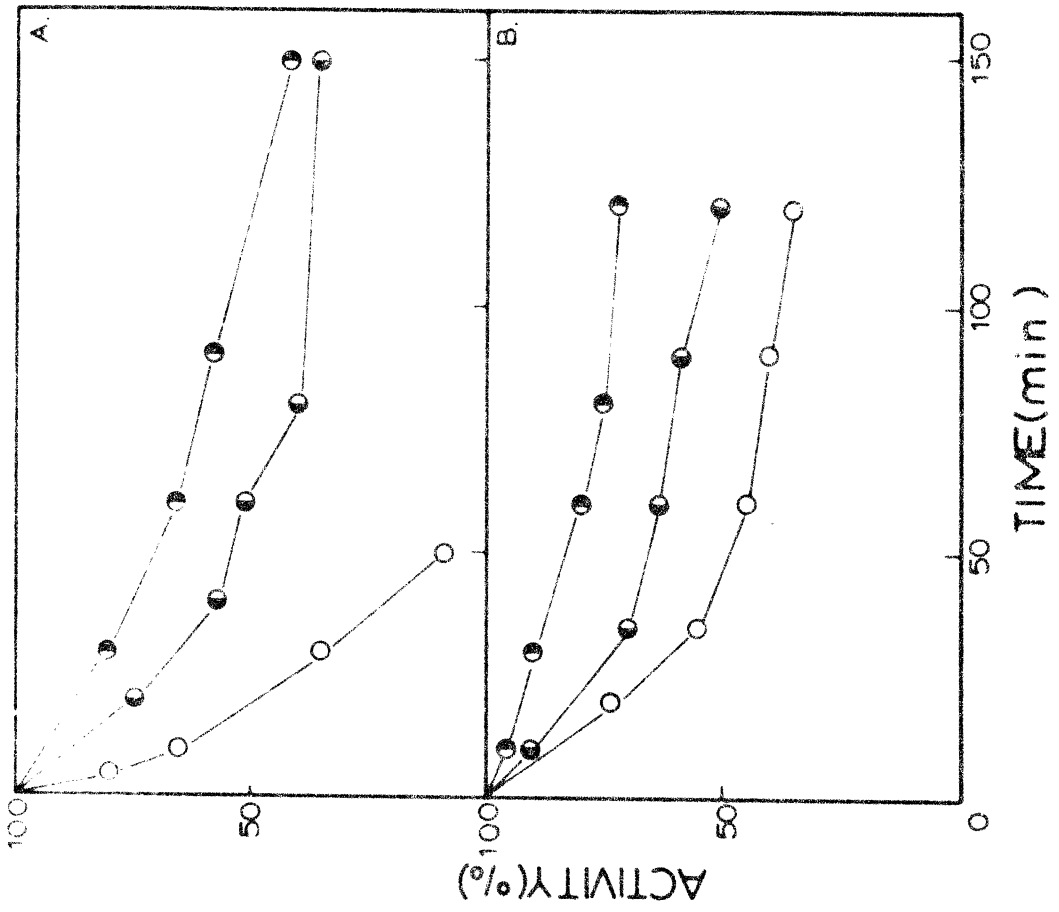

150
100
$\tau_{1/2}^{(MIN)}$
5
-5
1/[XAP]mM

Figure 10. Protective effects of dihydroxyacetone phosphate and inorganic phosphate on the inactivation of methylglyoxal synthase by haloacetol phosphates.

A. Reaction mixtures contained methylglyoxal synthase (0.05 mg/ml) and 1 mM iodoacetol phosphate with either no further addition (○), 0.15 mM dihydroxyacetone phosphate (◐), or 0.1 mM inorganic phosphate (◑). Aliquots were incubated as in Figure 8 and assayed as a function of time for remaining methylglyoxal synthase activity.

B. Reaction mixtures contained methylglyoxal synthase (0.05 mg/ml) and 1.2 mM bromoacetol phosphate with either no other additions (○), 0.15 mM dihydroxyacetone phosphate (◐), or 0.1 mM inorganic phosphate (◑). All reactions were carried out at 37°C, pH 7.0.

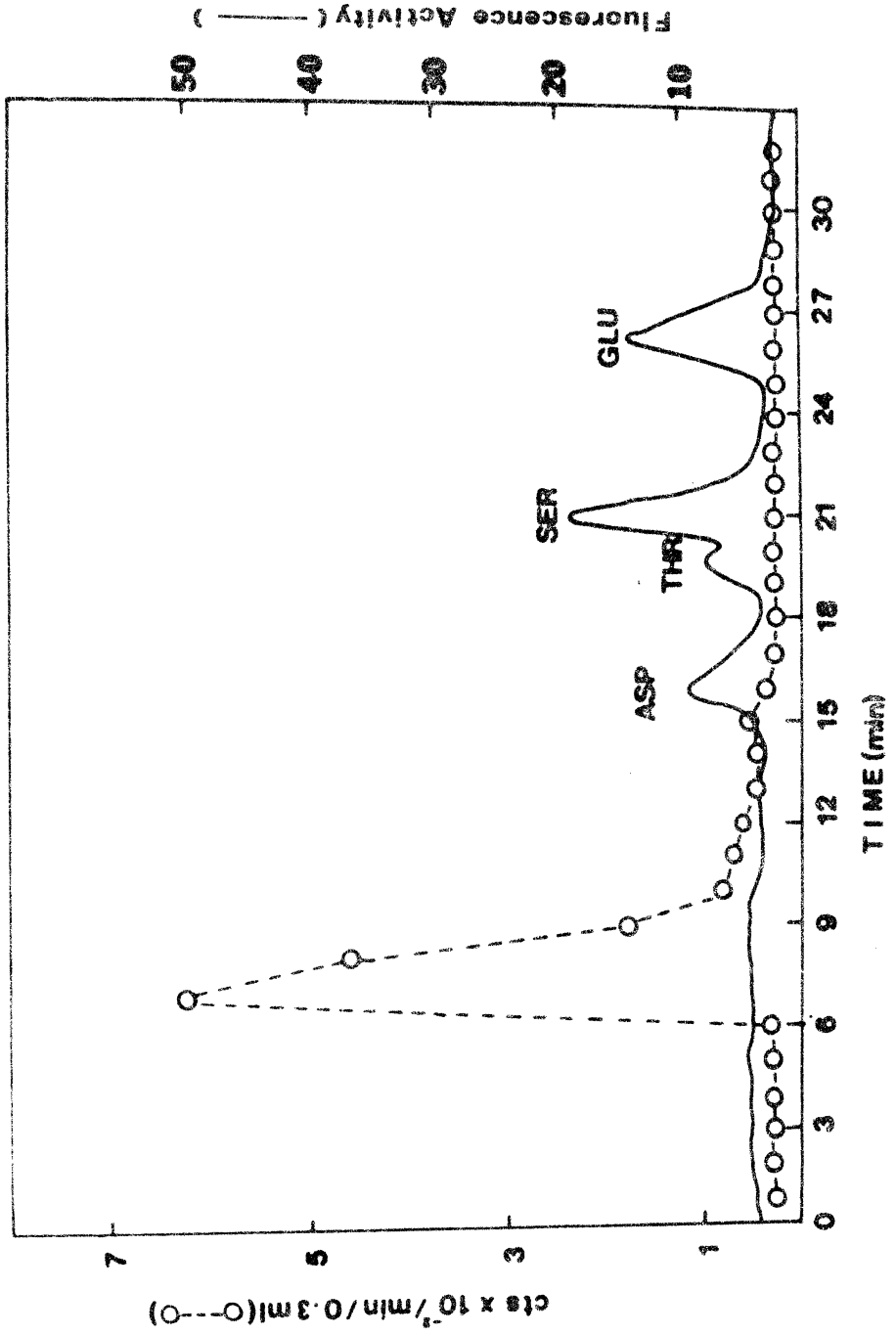

A.
B.
100
50
100
50
0
50
100
150
ACTIVITY(%)
TIME(min)

dialysis against buffer containing 0.1% v/v 2-mercapto-ethanol.

Identification of the Haloacetol Phosphate-Modified Residue

The enzyme (0.1 mg) was inactivated with iodoacetol phosphate, then treated with [^{3}H]$NaBH_4$ (706 mCi/mmole) for 30 minutes, at 30°, pH 7.8 (0.1 mM $NaHCO_3$ buffer), thereby reducing the carbonyl of the incorporated reagent and introducing a tritium label (Hartman, 1971). The reaction mixture was then made 0.1 M in β-mercaptoethanol and dialyzed exhaustively against water for 7 days. A net radioactivity incorporated into the protein was 6×10^5 cpm (after subtracting a "control" in which native methylglyoxal synthase was treated under the same condition but without haloacetol phosphate). This tritium labeled sample was divided into three aliquots and subjected to acid hydrolysis for 24, 48 and 72 hours, followed by automatic amino acid analysis. The column eluant was collected with a fraction collector at a rate of 1 minute per fraction from the outlet of a fluorometer. A single radioactive peak which was fluorescence-negative appeared 9 minutes ahead of the first fluorescence-positive amino acid residue — aspartic acid (Figure 11). The identity of this radioactive derivative is not certain but would appear to be the result of a modification of either an

Figure 11. Partial chromatogram of the acid hydrolysate of methylglyoxal synthase after modification with iodoacetol phosphate, followed by reduction with [^{3}H]$NaBH_4$. The fluorescence pattern (solid line) was directly traced from the recording chart paper. Radioactivity of eluant fractions (open symbols) were counted in a liquid scintillation spectrophotometer. Only the first portion of the chromatogram is shown, although the entire eluate was analyzed. No additional radioactive peaks were detected.


cts x 10⁻³/min/0.3ml(O---O)
Fluorescence Activity (——)
TIME (min)
ASP
THR
SER
GLU

aspartic or glutamic acid residue in the active center (See DISCUSSION).

DISCUSSION

Assaying the Enzyme

The lethal effects of methylglyoxal on macromolecular synthesis have been demonstrated by several investigators (Szent-Gyorgy, 1967; Zwaig and Dieguez, 1970; Krymkiewicz et al., 1971; Freedberg et al., 1971; Rekate et al., 1973). These observations led to the concept that this compound might be a universal regulator of cell proliferation (Szent-Gyorgy, 1967). Subsequently, chemotherapy studies showed that methylglyoxal could indeed inhibit the growth of ascites tumors (Apple et al., 1967, 1968), and although a vast literature has evolved regarding the use of alpha ketoaldehydes as antitumor agents, surprisingly little is understood regarding the molecular basis of these effects.

The biosynthesis of methylglyoxal from glycolytic intermediates has been pursued for many years. It was long postulated as a normal intermediate of glycolysis, until the phosphorylated pathway was elucidated by Embden, Meyerhoff and others. As a consequence, the significance of nonphosphorylated derivatives such as methylglyoxal has remained a puzzle. Although methylglyoxal has been reported in numerous tissues under normal and abnormal

conditions, there has been no explanation for its synthesis and catabolism. In recent years it has been assumed that the compound is generated largely, if not entirely, by nonenzymatic reactions from various trioses and triose derivatives. Therefore, the discovery of methylglyoxal synthase in E. coli (Cooper et al., 1970) was an exciting event which rasied new possibilities relating to the significance of this compound and its effects in vivo.

The physiological significance of this finding can also be viewed from the fact that this enzyme constituted the "missing link" in a possible glycolytic bypass which circumvents the last half of glycolysis (i.e., from dihydroxyacetone phosphate to pyruvate). Since it would seem to be energetically unfavorable for glucose catabolism to follow this shunt pathway, an extensive survey for the occurrence of methylglyoxal synthase was initiated in this investigation.

Due to the toxicity of methylglyoxal, an enzymatic pathway leading to its formation might be expected to be highly controlled in vivo. Therefore, it was reasoned that the level of the enzyme might be very low or difficult to be detected in normal tissue of higher organisms.

Two enzymatic assay methods (glyoxalase I-coupled spectrophotometric assay and the colorimetric stop assay) have been utilized in previous studies of enzymes from E. coli (Cooper et al., 1970, 1971, 1972), P. saccharophila

(Cooper, 1974) and P. vulgaris (Tsai et al., 1976). The glyoxalase I-coupled enzyme assay method is convenient, reproducible, fast and economical. However, it is not possible to use this assay in the studies on the kinetic mechanism such as product inhibition, metal ion or cofactor involvement. The colorimetric assay is based on the quantification of the 2,4-dinitrophenylhydrazine derivative of methylglyoxal. Unfortunately, this derivatization process is highly nonspecific, in that any carbonyl compound can be derivatized in the reaction, and thus interfere with the colorimetric determination. Furthermore, the color developed in the assay mixture is not stable, and the gradual decline in absorbance causes inconsistent measurements. Thus, the reproducibility of the method is poor.

In view of the limitations of the two previous assay methods, a direct assay was developed by using [^{14}C]-labeled substrate, dihydroxyacetone phosphate. This isotope assay has several advantages over the other assay methods. First, the sensitivity of the method is limited only by the specific radioactivity of the substrate, and it is thus inherently more sensitive than any existing method for detecting the enzyme. Secondly, the method does not require the addition of coupling enzymes, cosubstrates or trapping reagents which may interfere with the reaction. The direct assay

is, therefore, not subject to artifacts which can be due to conditions affecting coupling enzymes, coenzymes or color development. Although phosphatases which convert dihydroxyacetone phosphate to dihydroxyacetone and inorganic phosphate potentially could interfere with the reaction, no evidence of such interference was observed within the time limits and under the conditions used in these studies. Some variations of results were observed in this system due to the slight difference in recoveries of methylglyoxal from the ion exchange resin. However, the degree of variation was acceptable within the limits of the assay.

Distribution of the Enzyme

Based on the above mentioned considerations that potential problems may exist in the detection and measurement of methylglyoxal synthase in tissue and cell extracts, a careful analysis of the effects of (a) competing enzymes, (b) methods of preparation of cell extracts, and (c) possible interfering factors was conducted. Incubation of pure methylglyoxal synthase isolated from P. vulgaris with extracts from higher organisms suggested no interference with the enzyme or inhibition, and indicated the absence of the synthase in these tissues. Considering all

the species tested thus far, it appears that the enzyme is restricted to certain Enterobacteriaceae. [The exception of Klebsiella is not totally inconsistent in that this species, while not termed as Enterobacteria, is culturally and biochemically similar to the Enterobacter (Pelczar and Reid, 1972)].

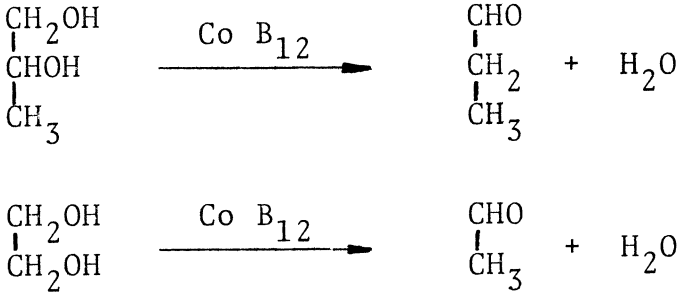
Physiological Function

The physiological role of this enzyme in bacterial systems is still unclear. The enzyme appears to be regulated by inorganic phosphate, and this sigmoidal inhibition pattern was observed in vitro with enzymes from three different bacterial species. Thus, maximal methylglyoxal synthase activity would occur at high dihydroxyacetone phosphate concentration and low inorganic phosphate concentration. It has been proposed (Cooper, 1974) that perhaps a physiological function of the shunt is to provide a route to pyruvate when levels of inorganic phosphate are low enough to make glyceraldehyde 3-phosphate dehydrogenase rate-limiting. Since the methylglyoxal synthase reaction generates inorganic phosphate, the shunt would also increase the concentration of inorganic phosphate such that the normal glycolysis could resume. Hopper and Cooper (1972) have suggested that the pathway could also provide a mechanism to prevent the accumulation of high levels of phosphorylated glycolytic intermediates which

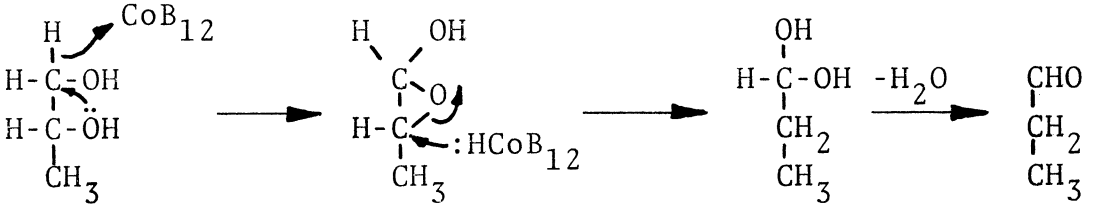
inhibit glycolysis. Moreover, the pathway could provide a synthetic route for D-lactate which is known to be involved in certain bacterial transport processes (Kaback, 1970). A comparison of the properties of the enzymes from E. coli, P. saccharophila, and P. vulgaris is tabulated in the Appendix.

Reaction Mechanism

This reaction appears to be complex involving a phosphate-dependent dehydration and internal oxidation-reduction. It bears some resemblance to the vitamin B_{12}-dependent dioldehydratases (Barker, 1967).



Retey and coworkers (1966) postulated a mechanism of the dioldehydratase reaction in which CoB_{12} served as a hydride carrier.



In the case of the methylglyoxal synthase, however, there was no evidence of participation of B_{12} or any other coenzyme in the reaction.

In view of the 3-carbon carbohydrate metabolism in avian spermatozoa proposed by Riddle (1973) (Figure 12), the overall course of this internal oxidation-reduction process in the conversion of dihydroxyacetone phosphate to methylglyoxal and inorganic phosphate would seem to be bidirectional. That is to say that perhaps either C-1 or C-3 could be oxidized to aldehyde. In order to investigate this, C-1 and C-3 [^{14}C]-labeled dihydroxyacetone phosphates were synthesized enzymatically from [^{14}C]-labeled glucose. Utilizing these labeled dihydroxyacetone phosphate substrates, it was clearly demonstrated that C-3 of dihydroxyacetone phosphate is oxidized to the aldehyde, while C-1, bearing the phosphoester, is dephosphorylated and reduced to the methyl group. It, therefore, appears that the mechanism must involve C-O bond scission rather than O-P cleavage. The overall scheme can thus be represented:

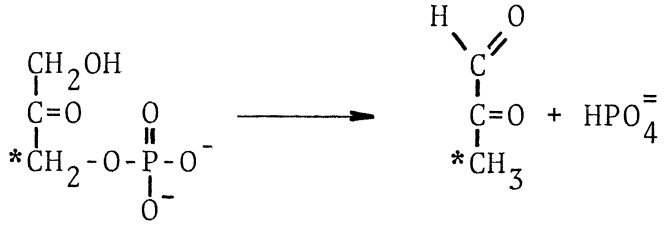
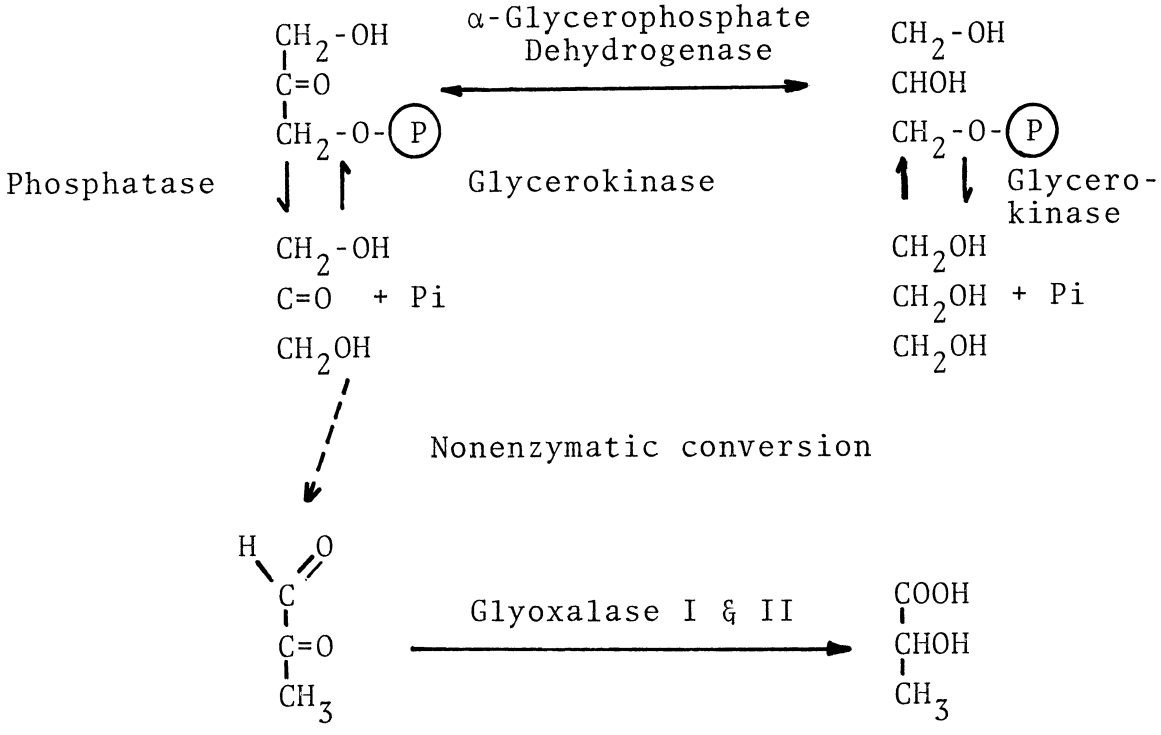


Figure 12. Carbohydrate metabolism of 3-carbon level in avian spermatozoa.


α-Glycerophosphate
Dehydrogenase
Phosphatase
Glycerokinase
Glycero-
kinase
+ Pi
+ Pi
Nonenzymatic conversion
Glyoxalase I & II

The nature of oxidation-reduction processes in enzyme-catalyzed reactions normally involves either protonation-deprotonation, as in the aldose-ketose isomerase reactions (Gracy et al., 1968; Rieder et al., 1959; Dysson et al., 1968), or hydride transfer as in dioldehydratase reactions (Barker, 1967). These transfer mechanisms can be assisted by the participation of metal ion or cofactor in the catalytic center or simply accomplished by general acid-base groups in the active site of the enzyme. Thus, a search for the possible involvement of metal ion or cofactor in methylglyoxal synthase catalyzed reaction was undertaken. However, from these studies there was no evidence for the participation of either a metal atom or a coenzyme.

The product inhibition studies suggest that this reaction proceeds by an ordered sequential release of products with methylglyoxal being released prior to inorganic phosphate. A variety of phosphorylated analogs and intermediates of glycolysis, including phosphoenolpyruvate, glyceraldehyde-3-phosphate, and pyrophosphate, are competitive inhibitors of the enzyme. On the other hand, nonphosphorylated trioses, such as dihydroxyacetone or glyceraldehyde, are not inhibitory (Tsai and Gracy, 1976). Thus, both the product inhibition and the effects of these competitive inhibitors suggest that the phosphate moiety of the substrate is required for binding, and that

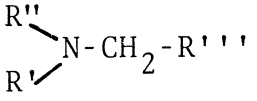
inorganic phosphate binds to the free enzyme. The effects of inorganic phosphate on the stability of the enzyme are also consistent with this interpretation.

Haloacetol phosphates were synthesized as potential affinity labels for enzymes involved in the metabolism of dihydroxyacetone phosphate. These substrate analogs have been shown to exhibit different activities with respect to the inactivation of triosephosphate isomerase (Hartman, 1971), rabbit muscle (Hartman, 1970b), or yeast aldolase (Lin et al., 1971). The inactivation of methylglyoxal synthase by these substrate analogs also exhibits a distinctive pattern, i.e., the activity of the iodoacetol phosphate is greater than that of the bromoacetol phosphate. Furthermore, no inactivation of methylglyoxal synthase was observed by chloroacetol phosphate (although it is a strict competitive inhibitor of the enzyme). These studies suggest that the bromoacetol and iodoacetol phosphates are indeed active-site reagents based on the following observations: (a) the loss of enzyme activity is a time-dependent pseudo-first order process; (b) substrates, dihydroxyacetone phosphate and inorganic phosphate, protect against the inactivation process; (c) the enzyme inactivation is irreversible, and does not appear to be caused by the oxidation of sulfhydryl groups; (d) the dissociation constant (K_i) values of the haloacetol phosphates are

comparable with K_m values for the substrates; and (e) the different potency of these analogs for inactivation can be explained according to the relative rates of displacement of halogen by Sn_2 mechanism, i.e., I>Br>Cl (Hine, 1962), suggesting a reactive nucleophile may be located near the haloacetol phosphate binding site.

The nature of the reactive nucleophile was approached by reduction of iodoacetol phosphate-inactivated methylglyoxal synthase with NaB^3H_4, thereby incorporating a tritium label into the modified residue(s). When the acid hydrolysate of [^{3}H]-labeled methylglyoxal synthase was analyzed by amino acid analysis, a single radioactive o-phthalaldehyde-negative peak was found nine minutes ahead of aspartic acid on the chromatogram. This finding is consistent with the results found in hydrolysates of triosephosphate isomerase which had been treated with chloroacetol phosphate and NaB^3H_4 (Hartman, 1971). This o-phthalaldehyde-negative species was found to be glycerol, which was formed during acid hydrolysis. This indicates that the original modification occurred through an acid labile linkage. Lin and cowkrers (1971) showed that the thioether linkage between cysteine and chloroacetol phosphate is stable during acid hydrolysis. Thus, an ether or thioether linkage is not likely to be the case for the modification of methylglyoxal synthase. Furthermore, the possibility that the

modification occurred between a basic amino acid residue such as histidine, arginine, or lysine can be also precluded, since similar derivatives of this type,

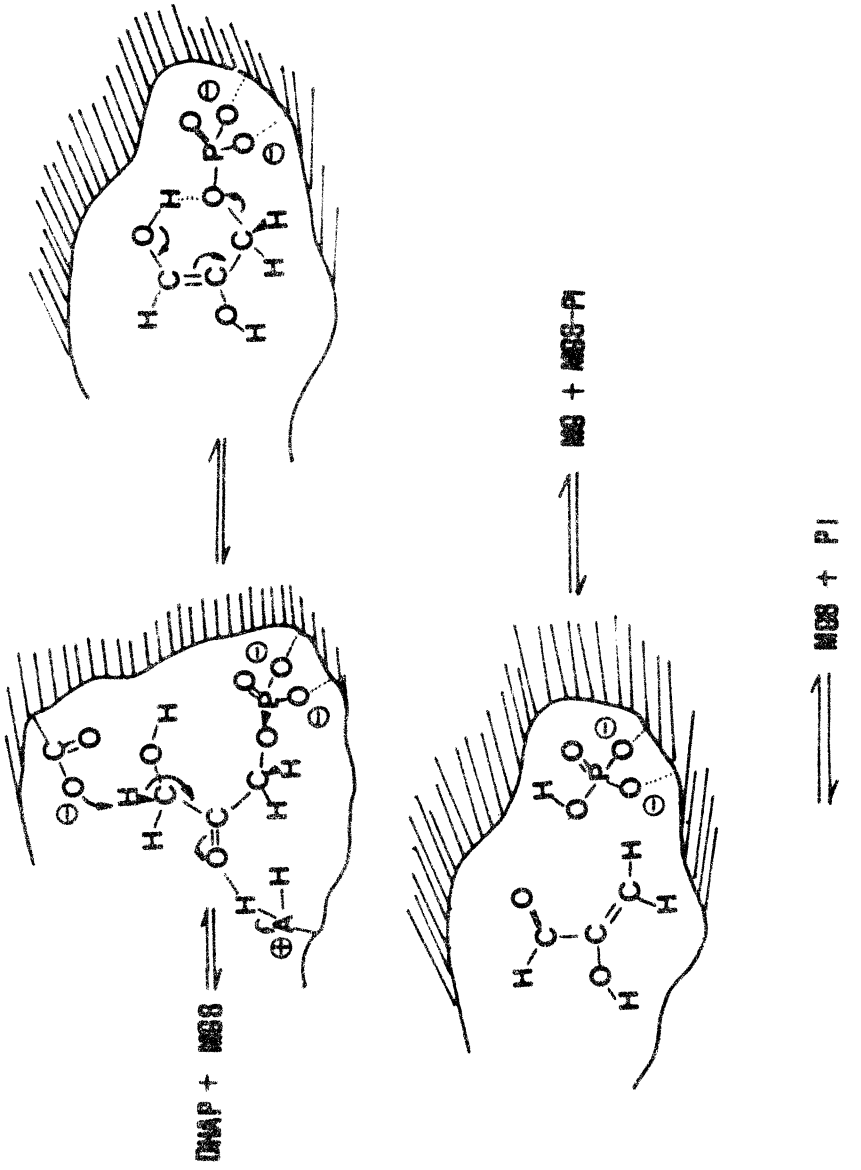


were shown to be stable during acid hydrolysis of modified phosphoglucose isomerase (Gibson et al., 1977). Therefore, the most likely modification would seem to be the formation of an ester derivative of glutamate or aspartate.

The substrate, dihydroxyacetone phosphate, exists in neutral aqueous solution at 20° as a mixture of keto, gem diol and enolic forms in the ratio 55:44:1 (Reynolds et al., 1971). While it is not known which of these forms of dihydroxyacetone phosphate bind, the enol is known to exist in relatively strong alkaline solution (Sellinger et al., 1958). Furthermore, the formation of the hydrogen-bonded enol form has been shown to facilitate the phosphoester hydrolysis (Sellinger et al., 1958).

From the above considerations and observations, a possible reaction mechanism is shown in Figure 13. In this scheme, methylglyoxal synthase, like triosephosphate isomerase, α-glycerophosphate dehydrogenase and aldolase, is shown as binding the keto form of dihydroxyacetone phosphate as the active substrate. However, this does not

Figure 13. Proposed mechanism of catalytic action of methylglyoxal synthase.



preclude the binding of other tautomeric forms of substrate. A carboxylate group from either glutamic or aspartic acids located in the active center, first catalyzes the keto-enol tautomerization to the hydrogen-bonded enol form. Since neither a metal nor Schiff's base appears to be involved in polarizing the carbonyl of dihydroxyacetone phosphate, it is likely that a proton derived from an electrophilic amino acid residue is also involved in the formation of the enediol. The hydrogen-bonded enediol intermediate then facilitates the oxidation-reduction and phosphoester cleavage with C-O bond breaking, followed by the ordered release of methylglyoxal prior to phosphate. The proposed mechanism also indicates a requisite phosphate binding domain.

Recently, Rose and Sommers (1977) have considered the stereochemistry of the deprotonation and protonation in this reaction by using partially purified methylglyoxal synthase from E. coli. Their studies indicate that the enzyme stereospecifically abstracts the pro-S hydrogen at C-3 of dihydroxyacetone phosphate and nonstereospecifically protonates the C-3 methyl group of methylglyoxal. Thus, the observations of Rose and Sommers corroborate the proposed mechanism.

The proposed reaction scheme incorporates and is consistent with all the presently known information on the enzyme. Future studies such as the identification of

the electrophoretic and nucleophilic groups in the active center, the composition and sequence of the active site region of the enzyme, will be necessary to further verify and extend our knowledge of this unusual enzyme. Perhaps only with a full understanding of the enzyme itself will we be able to fully appreciate the physiological significance of the enzyme and its role in the metabolism of methylglyoxal.

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APPENDIX

COMPARISON OF THE PROPERTIES OF METHYLGLYOXAL SYNTHASE FROM P. VULGARIS, E. COLI, AND PS. SACCHAROPHILA

Property	Methylglyoxal Synthase from		
	P. vulgaris	E. coli	Ps. saccharophila
Activity in cell extract (unit/mg of protein)	0.045	0.32	0.053
Purified enzyme activity (unit/mg of protein)	11	500	5.7
Stability	Cold labile	Cold labile	Cold labile
Molecular weight (dalton)	135,000	67,000	67,000
Subunit molecular weight (dalton)	66,000	?	?
pH optimum	7.7	7.5	8.2
K_m (mM) of DHAP (at pH 7.0)	0.2	0.47[a] 0.70[b]	0.09
Response to DHAP in presence of Pi, Hill coefficient	1.58[c] 2.6[d]	2.6[e] 3.0[f]	2.9[g]

APPENDIX--(Continued)

Type of inhibitor and K_i (mM) for:			
PPi	Competitive (0.075)	Competitive (0.095)	Competitive (0.048)
3-Phosphoglycerate	Competitive (>1.0)	Noncompetitive	Competitive (0.029)
Phosphoenolpyruvate	Competitive (>1.0)	Noncompetitive	Competitive (0.096)
Phosphoglycolate	Competitive (0.0075)	?	?
Glyceraldehyde 3-Phosphate	Competitive (0.65)	Not inhibitory	?
Methylglyoxal	Noncompetitive (0.65)	Not inhibitory	?

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[a]Value reported by Cooper et al. (1971).

[b]Value reported by Freedberg et al. (1971).

[c]Value at [Pi] = 0.2 mM.

[d]Value at [Pi] = 0.4 mM.

[e]Value at [Pi] = 0.3 mM (Cooper).

[f]Value at [Pi] = 1 mM (Freedberg).

[g]Value at [Pi] = 0.52 mM.