PURIFICATION AND STUDIES OF METHYLGLYOXAL REDUCTASE FROM SHEEP LIVER

THESIS

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

For the Degree of

MASTER OF SCIENCE

By

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May, 1983

Lambert, Patricia A., Purification and Studies of Methylglyoxal Reductase from Sheep Liver. Master of Science (Biochemistry), May, 1983, 44 pp., 2 tables, 8 illustrations, 21 titles

The objectives of these investigations were (1) the purification of MG reductase from sheep liver and (2) studies of some of its characteristics.

MG reductase was purified 40 fold and showed a single band on SDS-PAGE. Molecular weight estimations with SDS-PAGE showed a molecular weight of 44,000; although gel filtration with Sephadex G-150 gave a molecular weight of 87,000 indicating that the enzyme might be a dimer. The Km for MG is 1.42 mM and for NADH it is 0.04 mM. The pH optimum for the purified enzyme is pH 7.0. Isoelectric focusing experiments showed a pI of 9.3.

In vivo experiments involving rats treated with 3,3',5-triiodothyronine (T_3) and 6-n-propyl-2-thiouracil (PTU) indicated that MG reductase was depressed by T_3 and elevated by PTU.

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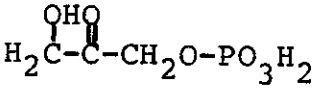
CHAPTER I

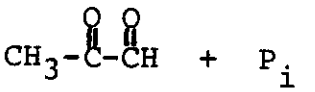
INTRODUCTION

The simplest α-ketoaldehyde, methylglyoxal (CH_3COCHO); (MG), has been shown to be a natural product of metabolism of bacteria, yeast and mammals (1-6). It was originally thought to be formed by glycolysing tissue, but this hypothesis has since been dismissed. Recent reports have verified the formation of MG as a reaction intermediate in the triosephosphate isomerase catalyzed reaction (7) and aldolase has been shown to be capable of the slow formation of MG when incubated with dihydroxyacetone phosphate (8).

An enzyme catalyzing the formation of MG from dihydroxyacetone phosphate in Escherichia coli was purified and studied by Hopper and Cooper in 1971 (1). The reaction catalyzed by MG synthase is as follows:



dihydroxyacetone phosphate



methylglyoxal

Among the compounds tested, dihydroxyacetone phosphate was shown to be the only substrate for the purified enzyme (1). It was also shown that 1 mM phosphate protected the enzyme against inactivation during gel filtration (1). However,

concentrations of phosphate that are close to the K_m for phosphate as a substrate for glyceraldehyde 3-phosphate dehydrogenase strongly inhibited MG synthase. Thus it was proposed that this enzyme might provide an alternate catabolic fate for triose phosphates formed during glycolysis. At high concentrations of phosphate, glyceraldehyde 3-phosphate dehydrogenase would be active but if the phosphate concentration fell sufficiently, the inhibition of MG synthase would be released and triose phosphates could be catabolyzed via MG. This would have the effect of increasing phosphate concentration and providing lactate, an energy source under aerobic conditions. As will be discussed later, there are several alternate fates for MG as well.

Although Yuan et al. (2) studied human, rat, and several invertebrate tissues, no evidence of an enzyme catalyzing the formation of MG from dihydroxyacetone phosphate in mammals was found. Recently, however, at least two investigators (3,4) have reported the isolation of MG synthase from mammalian sources. Sato et al. (3) reported the enzymatic formation of MG from glyceraldehyde 3-phosphate and dihydroxyacetone phosphate in whole cell suspensions of rat liver, and Ray and Ray (4) purportedly have isolated MG synthase from goat liver although this laboratory has not been able to reproduce their results.

In addition, MG synthase has been found in methylotropic yeasts by Babel and Hofmann (5).

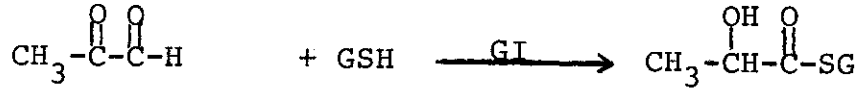
The biological activity of MG in rat hepatocytes and rat liver homogenates has been extensively studied by Danzani (6). Lipid peroxidation, a basic mechanism in cell pathology, was shown to lead to the production of large amounts of aldehydes including MG. These effects were studied by treating cells with CCl_4. Carbon tetrachloride is metabolized to $Cl\cdot$ and $CCl_3\cdot$ resulting in lipid peroxidation which causes severe damage to cell membranes. The effects on the cell were attributed to the aldehydes derived from the peroxidative degradation of unsaturated fatty acids. Methylglyoxal was shown to inhibit amino acid incorporation into post mitochondrial proteins as much as 91% at a concentration of 1 mM (6). It was shown that thiol compounds such as glutathione, dithiothreitol and cysteine protected against this inhibition suggesting that sulfhydryl groups are the target (6).

Methylglyoxal has been shown to inhibit the secretion of presynthesized proteins by rat liver cells. Furthermore, pre-incubation with MG produced a decrease in colchicine binding to rat liver supernatant tubulin (6). Since sulfhydryl groups are required for tubulin polymerization, it seemed that aldehydes such as MG could mimic the action of colchicine which is known to prevent polymerization of

tubulin. In other studies, MG and 4-hydroxypentenal produced a decrease in the mitotic index in bone marrow and small intestine epithelial cells (6). The effect of MG on several enzymes has been studied and it was found that cytochrome P-450 activity, for example, was significantly depressed when MG was incubated with liver microsomes.

The function of MG in the cell has been investigated extensively but a clear role has so far not been found. Albert Szent-Gyorgi states, "the energy driving life is derived from the transfer of an electron from hydrogen to oxygen" (9). He proposed that when life originated there was no free oxygen and the MG enabled the bound O_2 to be used as an electron acceptor. MG interacts with amino groups on protein molecules forming a Schiff base. This Schiff base was found to interact with its second neighbor peptide-bond entering a charge transfer reaction with it and taking electrons from it (9). In this regard, Fodor et al. (10) found MG bound to structural proteins of beef liver. According to Szent-Gyorgi, the living state of protein molecules is the electronically desaturated state. MG enables proteins to be desaturated with the Schiff base being negatively charged and the peptide chain positively charged (9).

The glyoxalase system which is ubiquitous in nature catalyzes the following reactions:



Methylglyoxal Glutathione S-lactoyl glutathione

S-lactoyl glutathione D-lactate glutathione

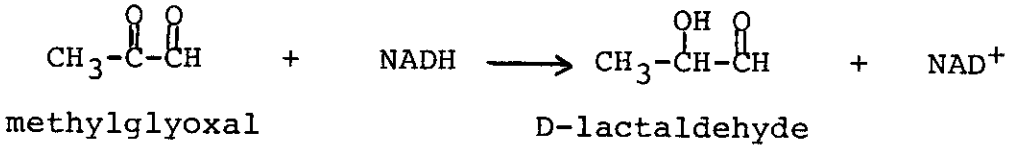
Inhibitors of the glyoxalase system have been studied as a possible approach to anticancer agents. Vince and Daluge suggest that inhibitors of glyoxalase I may provide carcinostatic activity by preventing the metabolism of MG in tumor cells (11). They concluded that the high concentration of lactic acid and the deficiency of MG in cancer cells suggest that these cells have lost the ability to maintain a proper balance of MG and continue to grow at an uncontrolled rate. Thus, an inhibitor of glyoxalase I would result in an accumulation of MG in cancer cells. It has also been suggested that MG may inhibit protein synthesis by binding reversibly with tRNA molecules (11). However, the glyoxalase system is not the only pathway of MG degradation as discussed below.

MG is also an intermediate in the proposed amino-acetone pathway (Figure 1) in working muscle tissue (12).

Several amino acids are metabolized during exercise in animals (12). The aminoacetone pathway is a proposed mechanism of amino acid degradation during short-duration exercise. The physiological functions of this pathway may be regarded as (1) the production of a free coenzyme A for the breakdown of pyruvate, free fatty acids and ketone bodies, (2) the reduction of MG with formation of NAD^+ and (3) the introduction of carbon skeletons from amino acids for energy production or gluconeogenesis.

It is obvious from the previous discussion that several enzymes are involved in MG degradation. The primary and most active of these is apparently the glyoxalase system. However, at least three other enzymes are capable of catabolyzing MG. They are α-ketoaldehyde dehydrogenase (MG dehydrogenase) (13), D-lactaldehyde dehydrogenase (MG reductase) (14) and L-glycol dehydrogenase (15). This reaction scheme is illustrated in Figure 2.

In 1964, Ting et al. reported an enzyme in rat liver capable of the preferred oxidation of the D-isomer of lactaldehyde with MG as the reaction product (14). The overall reaction was reported as



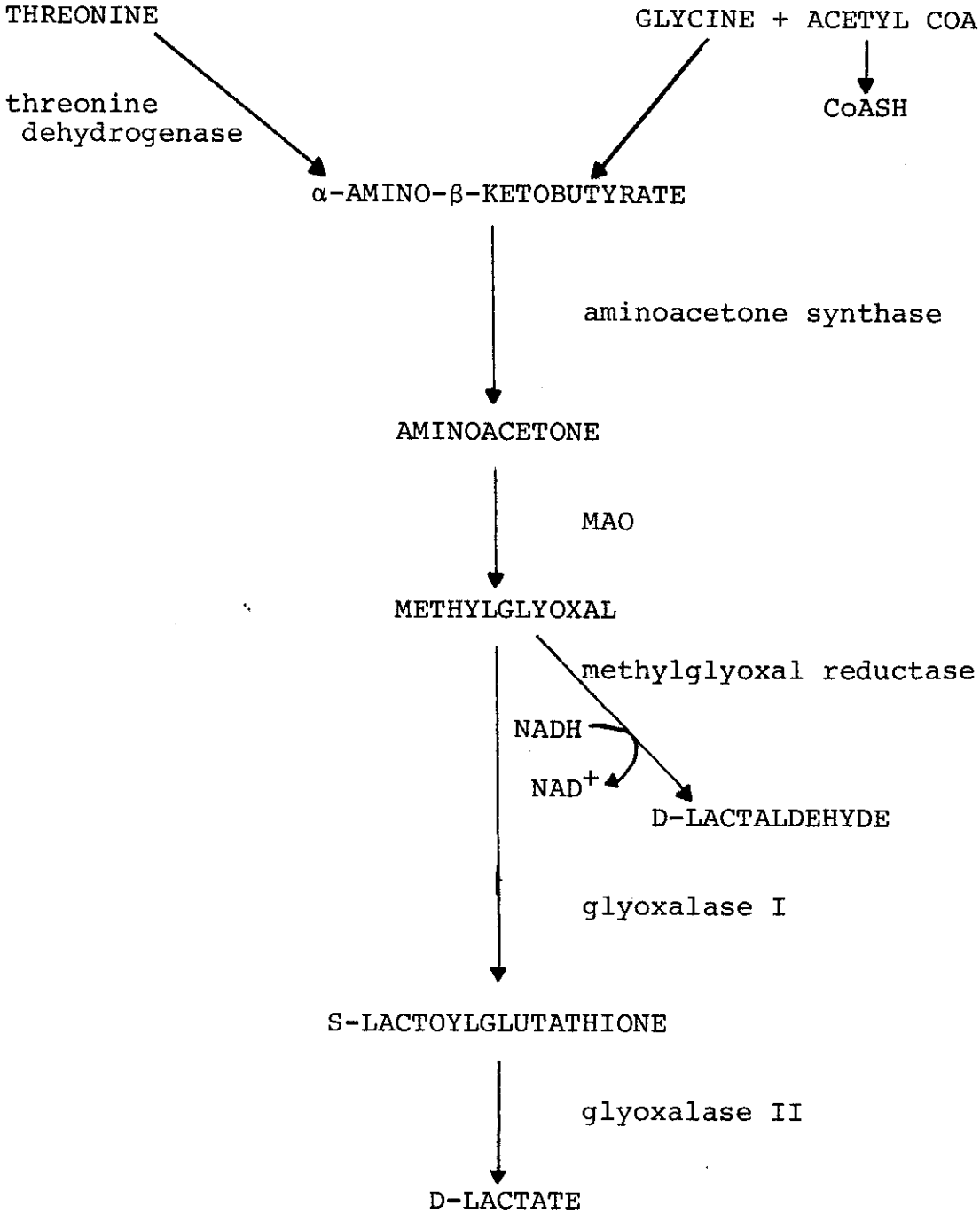


Figure 1

Aminoacetone Pathway

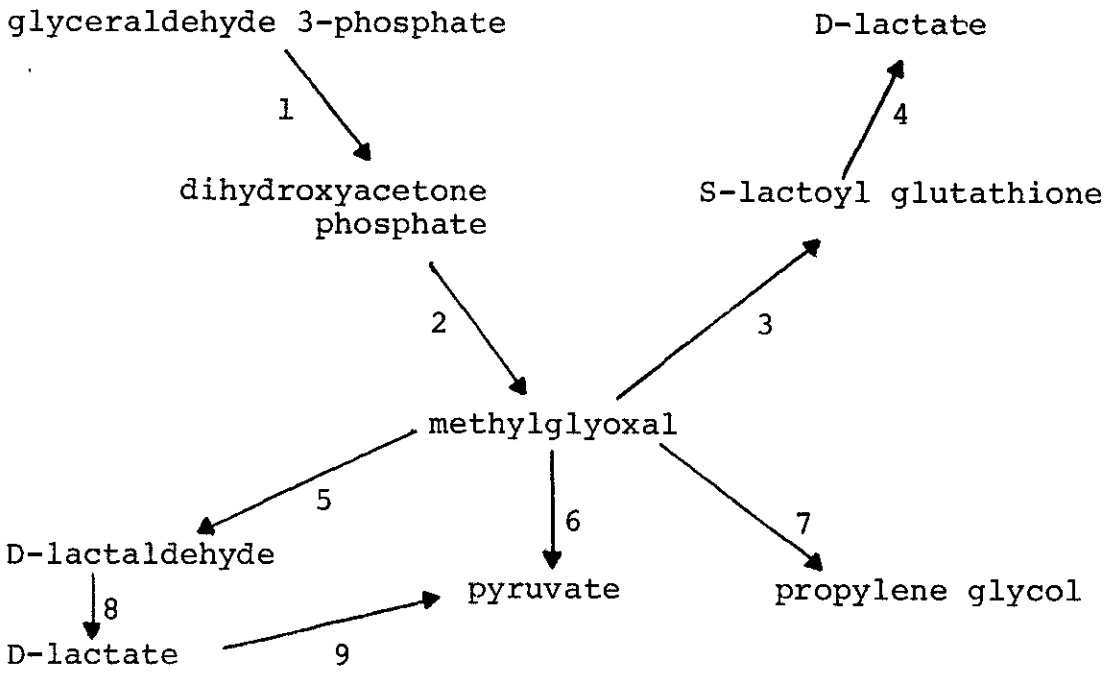


Figure 2

Some Enzymes of Methylglyoxal Metabolism

1. glyceraldehyde 3-phosphate dehydrogenase
2. methylglyoxal synthase
3. glyoxalase I
4. glyoxalase II
5. methylglyoxal reductase
6. methylglyoxal dehydrogenase
7. L-glycol dehydrogenase
8. DL-lactaldehyde dehydrogenase
9. D-lactate dehydrogenase

This enzyme was called D-lactaldehyde dehydrogenase (E.C. 1.1.1.78). Although most of their experiments were done in the direction D-lactaldehyde → MG, it was stated that the reaction was reversible. Their isolation scheme involved only an $(NH_4)_2SO_4$ precipitation and was basically concerned with removing lactaldehyde reducing activity. DL-glyceraldehyde and DL-lactaldehyde could both serve as substrates. The apparent K_m value for DL-lactaldehyde was 0.065 M which seems unreasonably high for physiological significance. The K_m value for NAD^+ was 0.34 mM and $NADP^+$ was found to be inactive. It was also found that the rate of reaction continued to increase as the pH was raised, up to pH 11. Rat muscle, brain, kidney, heart, liver, spleen and lung were studied with the highest activity found in skeletal muscle and lowest in lung (14).

In 1970, Willets and Turner (16) studied threonine metabolism in Bacillus subtilis. It was stated that this bacterium was the first microorganism found to contain an NADPH linked MG reductase. This enzyme was essentially irreversible (MG to D-lactaldehyde), in contrast to the rat liver enzyme, and showed no activity with NADH. These investigators also found that activity was enhanced by ATP up to three fold at 1.6 mM. Also, enzyme formation could be induced by growth on threonine (16).

Recently, Smits and Johnson (17) studied MG and enzymes of its metabolism in Douglas-fir needles and needle callus. Glyoxalase I and glyoxalase II were found only in the proliferative callus tissue and MG synthase was found only in the non-proliferative needles. MG reductase was found in both tissues but to a much greater extent in callus tissue. MG itself was found only in the non-proliferative needle tissue (17). It was postulated that MG degrading enzymes such as MG reductase found in the proliferative callus tissue were there to reverse the inhibition of growth by MG, and that MG synthase found in the non-proliferative needles functioned to inhibit growth (17).

In view of the previously discussed proposed functions of MG in cell growth and regulation, further insights into its metabolism would seem desirable. The objectives of the investigation herein reported were to purify one of the enzymes of MG metabolism, MG reductase, and to study some of its characteristics. Up to this point, this enzyme has not been purified to homogeneity and studies done on it were performed at conditions that were far from physiological (14). Since some initial studies such as molecular weight and K_m estimations for this enzyme had been done on enzyme from bacterial sources (16), it was decided to investigate the characteristics of the enzyme

from a mammalian source. Sheep liver was chosen as a suitable source for several reasons. It was easily obtainable, could be obtained in large quantities and demonstrated a high activity of MG reductase. An obvious disadvantage, however, was that this source did not lend itself to studies of the in vivo production of this enzyme. Due to the numerous reports (18-23) of the effects of thyroid hormones on various enzymes associated with energy metabolism, it was decided to investigate the effect of 3,3',5-triiodothyronine (T_3) on MG reductase. The animal model for this study was the albino rat. In conjunction with the T_3 treated animals, 6-n-propyl-2-thiouracil (PTU) was administered to a separate group since this substance is known to be an inhibitor of thyroid hormone binding to receptors (29).

It seems apparent that MG reductase is an important enzyme in MG metabolism. The purification, characterization, kinetic and regulatory studies of this enzyme may lead to a better understanding of methylglyoxal metabolism.

CHAPTER II

EXPERIMENTAL PROCEDURE

General

Materials. Sheep liver was generously donated by Fischer's Meat Market in Munster, Texas. The livers were obtained immediately after slaughter and frozen. Methylglyoxal was prepared according to the procedure of Kellum et al. (19) and lactaldehyde was synthesized by the method of Zagalek (20). Pyruvaldehyde dimethyl acetal was purchased from Aldrich Chemical Co., Milwaukee, WI. 3,3',5-triiodothyronine (T_3), NADH, NAD^+, threonine, ninhydrin, Dowex resin, 6-n-propyl-2-thiouracil and other chemicals were purchased from Sigma Chemical Co., St. Louis, MO. Pharmalyte carrier ampholytes and Sephadex IEF were from Pharmacia Fine Chemicals, Piscateway, NJ. Fractogel TSK HW55-S was obtained from Pierce Chemical Co., Rockford, IL.

Routine MG reductase assay. MG reductase activity was determined by a modification of the procedure of Willetts et al. (16). The reaction mixture contained 3.3 mM MG, 0.25 mM NADH and 100 mM potassium phosphate buffer pH 7.0 plus the enzyme preparation in a total

volume of 3 ml. Enzyme activity was determined by measuring the decrease in absorbance at 340 nm due to the formation of NAD^+ from NADH (ϵ_{340} = 6.22 $mM^{-1}cm^{-1}$). Initial rates were measured using a Beckman DU-8 spectrophotometer. The reaction was initiated by addition of the enzyme and the blank cuvette contained all components of the reaction mixture except enzyme. A unit of MG reductase activity is defined as the amount of enzyme catalyzing the formation of 1 μ mol of NAD^+ per minute in the routine assay system. Specific activity is expressed as units per mg protein.

Determination of protein concentration. Protein concentrations were determined by the colorimetric Coomassie Blue procedure of Bradford (21). Crystalline bovine serum albumin was used as the protein standard.

Experiments Involving Injection of Rats with 3,3',5-Triiodothyronine and Feeding with 6-n-Propyl-2-thiouracil

3,3',5-Triiodothyronine (T_3) injection. T_3 (1 mg in 1 ml 0.05 N NaOH) was injected intraperitoneally daily for five days. At the end of five days rats were sacrificed by guillotining and livers were removed. These livers were homogenized individually in 50 mM imidazole-HCl buffer pH 7.0 containing 5 mM $MgSO_4$ at a ratio of 3 ml

buffer to one gram tissue. The resulting homogenate was centrifuged at 100,000 x g for 30 minutes on a Beckman model L ultracentrifuge and the supernatant was reserved for analysis.

6-n-propyl-2-thiouracil administration to rats. Rats were given a solution of 0.1% 6-n-propyl-2-thiouracil (PTU) in place of their regular drinking water. This treatment was carried out for a period of three weeks prior to sacrificing the animals. Livers were treated as described for T_3 rats. In each experiment control rats were maintained in the same environment as T_3 and PTU rats and their livers were processed in the manner described above.

Other enzyme assays. Glyoxalase I was assayed as previously described (19).

Malic enzyme assay. The reaction mixture for malic enzyme contained 30 mM L-malic acid adjusted to pH 7.0 with 5 N KOH, 1 mM $NADP^+$, 5 mM $MnSO_4$, and 50 mM TRIS/SO_4 pH 7.5 buffer. The reaction was initiated by addition of the enzyme to the sample cuvette. Enzyme activity was determined by measuring the increase in absorbance at 340 nm due to the formation of NADPH from $NADP^+$. A unit of malic enzyme activity is defined as the amount of enzyme

catalyzing the formation of one μ mol NADPH per minute in the routine assay.

Purification of Methylglyoxal Reductase

Preparation of sheep liver extract. Sheep liver extract was prepared by homogenizing sheep liver in three volumes of cold 10 mM potassium phosphate buffer pH 7 containing 20% glycerol and 0.1% β mercaptoethanol (BME) for approximately one minute in a Virtis "45" homogenizer. The resulting homogenate was filtered through cheesecloth to remove connective tissue and centrifuged for 30 minutes at 100,000 x g. The supernatant was removed and utilized for purification. All purification steps were conducted at 4°C.

Ammonium sulfate fractionation. Ammonium sulfate in a ratio of 0.34 g per ml of sheep liver extract was added slowly with constant stirring to the cold liver extract. The pH was maintained at 6.5 to 7.0 during addition. After addition, the solution was allowed to stir an additional 15 minutes then centrifuged at 2800 x g for 30 minutes. The supernatant from this step was treated with an additional 0.16 g per ml of ammonium sulfate following the same procedure. The pellet from this step was dissolved in a minimum volume of 20 mM TRIS/HCl pH 7.5,

10% glycerol, 0.1% BME (20 mM medium A). This solution was saved for further purification steps and is referred to as ammonium sulfate fraction 2.

Sephadex G-100 gel filtration. The column (2.5 X 70 cm) was equilibrated in 20 mM medium A and a maximum of 10 ml of ammonium sulfate fraction 2 was applied. Fractions (3.8 ml) were collected and analyzed for MG reductase activity and for protein content. Fractions which showed at least a two-fold increase in specific activity were pooled to be used in the next purification step.

Second ammonium sulfate fractionation. The most active fractions from the Sephadex G-100 column were treated with 0.6 g per ml of ammonium sulfate as previously described and centrifuged at 2800 x g for 20 minutes to remove precipitated protein. The resulting pellet was dissolved in a minimal volume of 20 mM medium A.

Fractogel TSK gel filtration. A 2.5 X 45 cm column was packed according to the manufacturers directions with Fractogel TSK HW55-S. The column contained approximately 200 ml packed gel. The sample applied to the column was from the second ammonium sulfate step. The buffer used to equilibrate and elute the column was 20 mM medium A.

Fractions (1.5 ml) representing at least a 3-fold purification or greater were pooled and used for the next step.

Blue Dextran affinity chromatography. Preparation of Blue Dextran Sepharose 4B was done according to the procedure outlined by Ryan and Vestling (18). The procedure was modified, however, by employing two activation-coupling steps in order to insure maximum ligand coupling. A 1.5 X 30 cm column was packed with the Blue Dextran Sepharose and equilibrated with 20 mM medium A. The sample consisted of pooled fractions from the Fractogel TSK column or pooled fractions directly from the G-100 column. Elution of MG reductase was accomplished by treating the column with approximately 80 ml of 15 mM NADH in 20 mM medium A. Fraction volumes were 2.8 ml.

Final purification steps. The presence of carrier ampholytes in the enzyme fractions from the isoelectric focusing column interfered with enzyme assays and polyacrylamide gel electrophoresis. Before enzyme assays were conducted the purified enzyme was applied to a Sephadex G-25 column (1.5 X 30 cm) to remove the contaminating ampholytes. For SDS polyacrylamide gel electrophoresis, the enzyme was dialyzed overnight in water, placed on a G-25 column and then lyophilized.

SDS polyacrylamide gel electrophoresis. Sodium dodecyl sulfate polyacrylamide gels were prepared according to the method of Maizel (24) except that gels were made 10% in acrylamide and 100 mM sodium phosphate buffer pH 7.0 was used plus 0.2% SDS. The samples for electrophoresis each contained 1% SDS, 20% glycerol and 2 to 4% BME. Approximately 2.5 microliters of bromphenol blue tracking dye were added to each sample in order to observe the progress of the electrophoresis. Samples were boiled for 2 minutes before application to gels. The gels were electrophoresed at 5 mA per tube until the samples had run onto the gels, then they were run at 8 mA per tube for the rest of the electrophoresis.

Polyacrylamide gel isoelectric focusing. Polyacrylamide gels (5%) were prepared according to the procedure in the Pharmacia Flat Bed Apparatus FBE 3000 instruction manual. The gels contained 1.9 ml Pharmalyte carrier ampholytes pH 8 to 10.5 for a 115 X 230 mm plate. The gels were prefocused for 30 minutes before sample application. Samples (20 to 40 microliter) were applied to filter paper strips or with the sample applicator device to the center of the gel and after 20 minutes of focusing, paper strips or sample applicator were removed. Running

conditions typically were 30 W constant power with a voltage limit of 2000 V for two hours. Immediately after focusing, pH readings were taken at one cm intervals along the direction of the gradient. Gels were fixed in 5% sulfosalicylic acid plus 10% TCA for one hour, placed in destaining solution for 30 minutes and stained for three to six hours in 0.2% Coomassie Brilliant Blue R-250 staining solution. Gels were destained until the background was clear.

CHAPTER III

RESULTS AND DISCUSSION

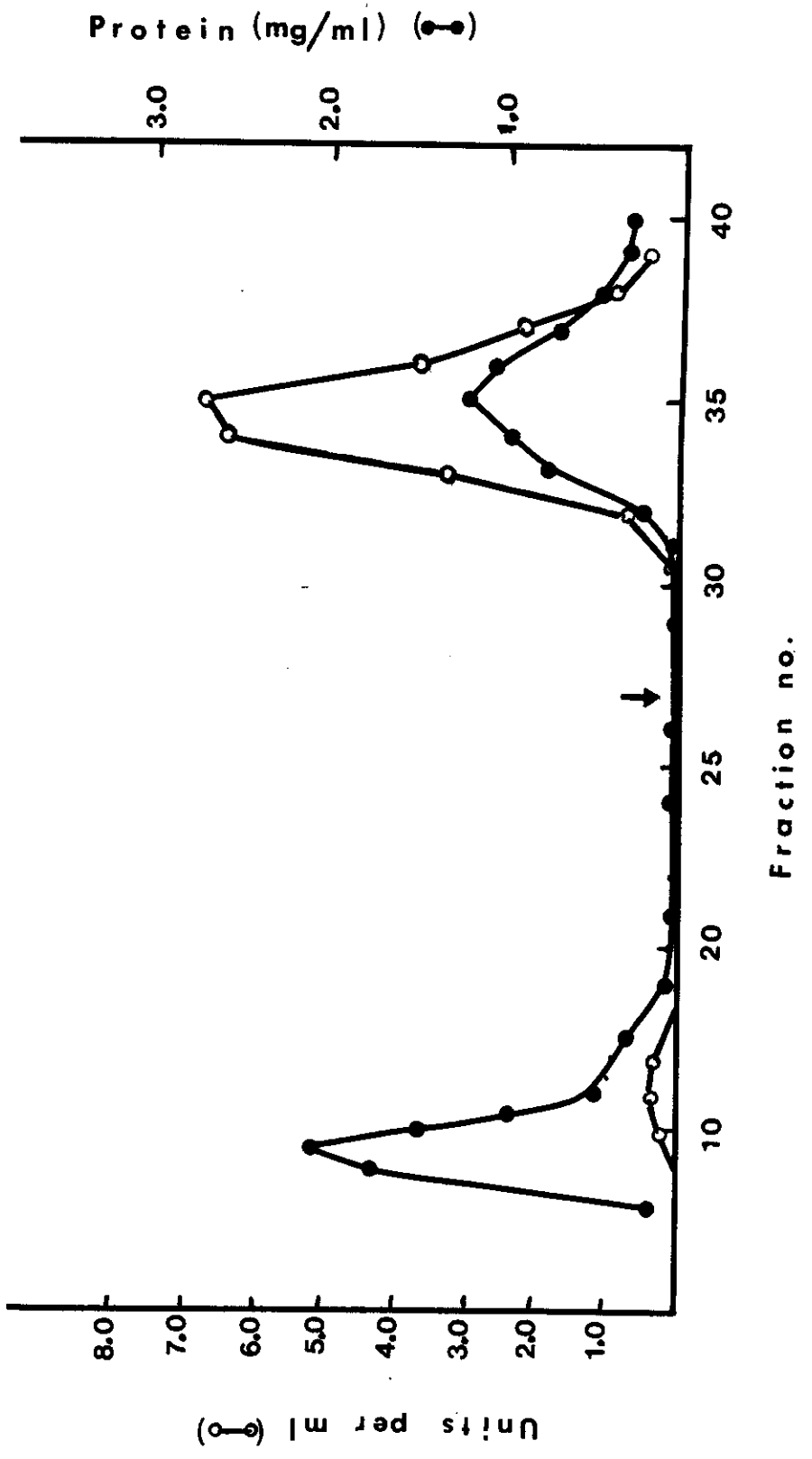
The purification of MG reductase was accomplished by employing a variety of methods. Among those techniques used were ammonium sulfate fractionation, gel filtration chromatography, affinity chromatography with Blue Dextran-Sepharose, and isoelectric focusing. Although other methods such as ion exchange chromatography, hydrophobic chromatography, calcium phosphate gel absorption, and affinity chromatography with apparently appropriate ligands were attempted, they did not give satisfactory results. Use of ion exchange chromatography under a variety of conditions did meet with marginal success. DEAE-cellulose, phosphocellulose and CM cellulose were studied; only the DEAE-cellulose was effective in enzyme purification (4-6 fold). With the use of this ion exchange medium, however, the recovery was only 20-25%.

Table I illustrates a typical purification scheme for MG reductase. One of the better purification steps was the Blue Dextran column as illustrated in Figure 3. It should be pointed out that a similar purification scheme involving Blue Dextran Affinity chromatography as a final step yielded apparently pure enzyme as evidenced by a single band on SDS-PAGE. However, when isoelectric

TABLE I

PURIFICATION OF MG REDUCTASE FROM SHEEP LIVER

Step	Volume (ml)	Units per ml	Total Units	Protein (mg/ml)	Specific Activity	Percent Recovery	Purification Step	Factor Overall
Crude preparation	29	7.63	221	25.5	0.30	--	1.0	1.0
Ammonium sulfate fractionation	5.2	41.4	215	80	0.52	97	1.7	1.7
G-100 Sephadex	9	18	162	11.5	1.56	75	3	5.2
Blue Dextran Sepharose	24	4.36	106	0.85	5.3	65	3.4	17.6
IEF column	24	2.43	58	0.2	12.13	55	2.3	40


Protein (mg/ml) (●—●)
3.0
2.0
1.0
40
35
30
25
20
10
Fraction no.
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
Units per ml (○—○)

focusing was done on this enzyme preparation at least two bands were seen. This scheme involved gel filtration with Fractogel TSK which gave about a two fold purification. A major problem with this gel was the very slow flow rate. Possibly better purification could be achieved if higher flow rates were possible. Overall purification for the scheme illustrated in Table I was slightly higher than that of the scheme involving Blue Dextran as a final step. A major difficulty encountered in all steps of the purification was the enzyme's tendency to lose activity even when stored at -20°C. The overall purification factor following isoelectric focusing was only 40 which seems rather low compared to other enzymes of MG metabolism such as glyoxalase I. However, Dunkerton and James (31) obtained an apparently homogeneous preparation of MG dehydrogenase from sheep liver with only an 89 fold purification. The purified MG reductase from the first isoelectric focusing column exhibited a single band when subjected to SDS-PAGE and polyacrylamide gel isoelectric focusing. A single band at pH 9.0 was seen following isoelectric focusing of the purified preparation from subsequent isoelectric focusing columns although with a pH interval of 8-10 the band was very diffuse and could possibly be interpreted as two bands. This may indicate that there are two isozymes present which have slightly

differing pI's although there was only a difference of about 0.2 pH units from top to bottom of the diffuse band. Another possible explanation would be the presence of subunits of the enzyme with one band a monomer and the other perhaps a dimer.

Estimation of the molecular weight of MG reductase was done using molecular weight standards on SDS polyacrylamide gels. Figure 4 shows a plot of log molecular weight versus distance traveled for these standards and for MG reductase. Linear regression analysis was done on these values and the molecular weight of MG reductase was calculated to be approximately 44,000 daltons. However, when molecular weight studies were done using Sephadex G-150 gel filtration, a molecular weight of 87,000 daltons was obtained. This along with the data from isoelectric focusing may indicate that MG reductase is a dimer.

Figure 5 illustrates that the pH optimum of the purified enzyme is 7.0 and that there is almost no activity below pH 5.8 or above pH 9.5. This is an indication that the physiological substrate of the enzyme is probably MG rather than D-lactaldehyde; the latter serving as a substrate for the reverse reaction only at very high pH values and at very high concentrations (14). The enzyme reaction of crude sheep liver homogenate was apparently reversible, but the purified enzyme showed no activity

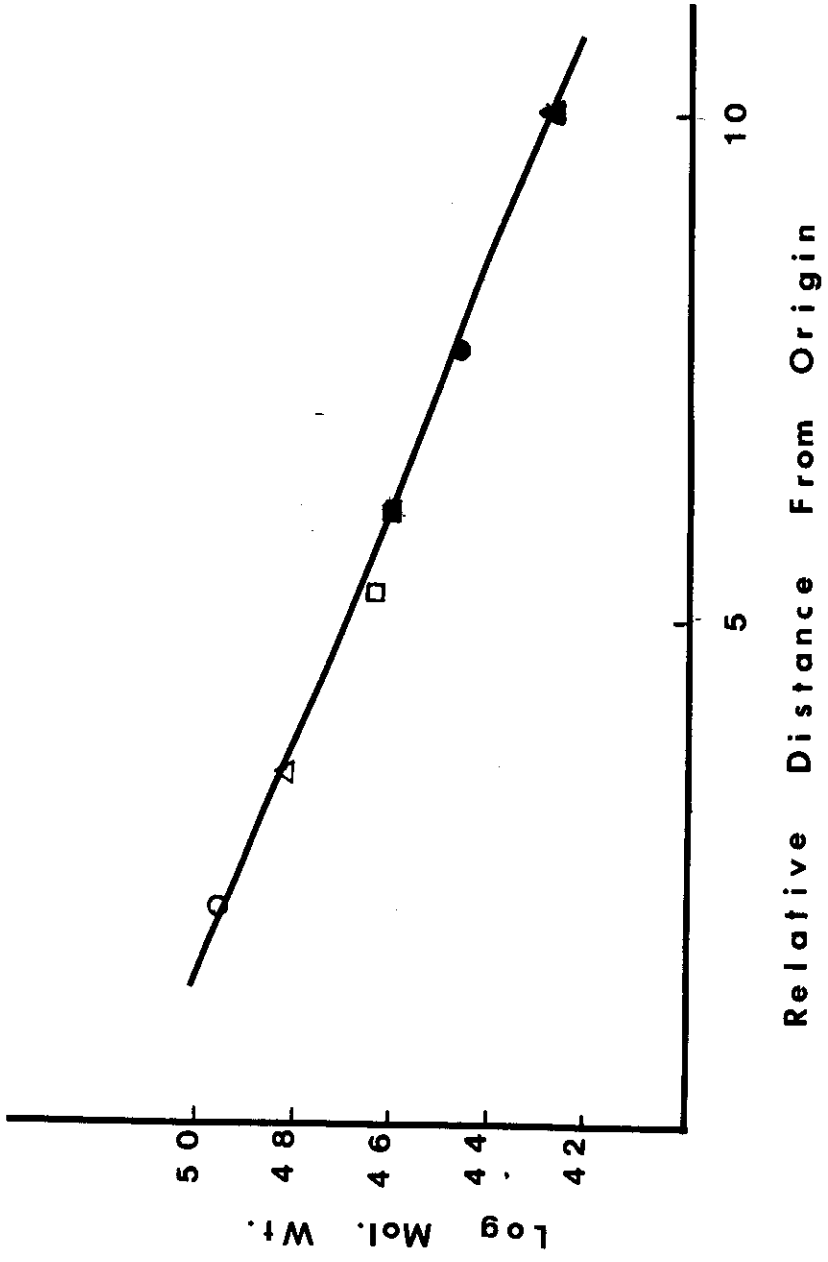
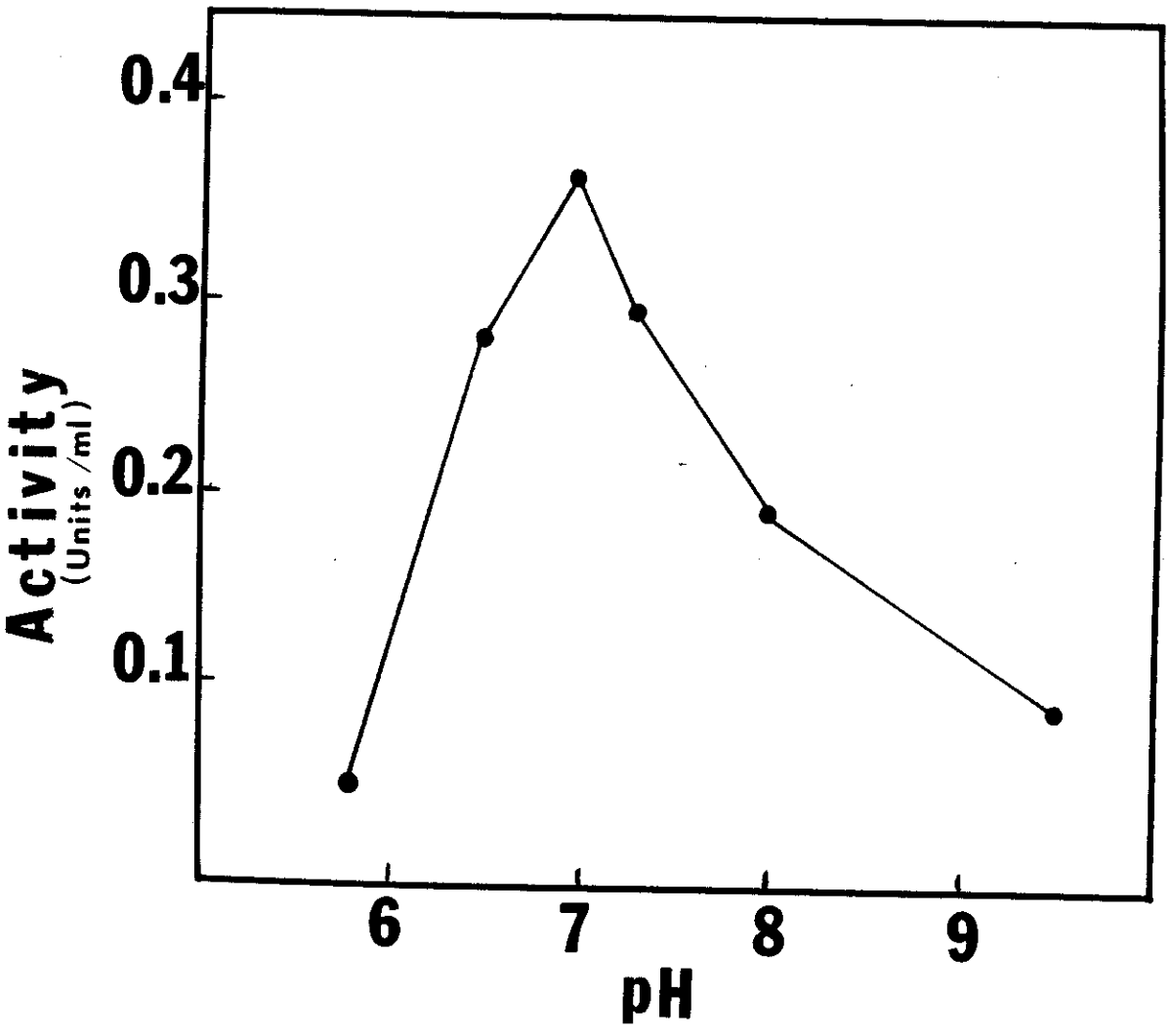

Log Mol. Wt.
5.0
4.8
4.6
4.4
4.2
5
10
Relative Distance From Origin

Figure 5

Effect of pH on Methylglyoxal Reductase

The standard assay system was used. For pH 5.8, 50 mM sodium citrate buffer was used. For pH 6.5, 7.0, 7.3 and 8.0, 50 mM potassium phosphate buffer was used and for pH 9.5, 50 mM sodium pyrophosphate buffer was used.


0.4
0.3
0.2
0.1
Activity
(Units /ml)
6
7
8
9
pH

with D-lactaldehyde as substrate under the same conditions. The D-lactaldehyde oxidizing activity in the crude preparation could be due to the presence of another enzyme capable of using D-lactaldehyde as substrate.

The coenzyme requirements for MG reductase were investigated and it was found that NADPH shows approximately 16% of the activity seen with NADH. In contrast to the enzyme in Bacillus subtilis (16), ATP was found to have no effect on activity. In an effort to determine if a metal ion was required for activity, a partially purified enzyme preparation was incubated with EDTA at a concentration of 1 mM. The enzyme activity showed no significant change.

Michaelis constants were determined at 25°C in 100 mM potassium phosphate buffer pH 7.0. Values for MG with constant levels of NADH ranged from 1.42 mM at 0.1 mM NADH (Figure 6) to 1.49 mM at 0.35 mM NADH. It was impossible to measure reaction rates at saturating levels of NADH since at higher levels enzyme activity was significantly depressed. This could be the result of inhibitors present in the NADH solution (as per Sigma Chemical Co. product bulletin) or to actual substrate inhibition. The K_m value for NADH was determined using 10 mM MG and a value of 0.04 mM was obtained as seen in Figure 7. The K_m value for NADPH was determined to be 1.59 mM using the same level of MG (Figure 8).

Figure 6

Determination of Apparent Michaelis Constant for Methylglyoxal

Each assay mixture contained 0.1 mM NADH and a constant amount of MG reductase. Concentrations of MG were 0.5, 1.0, 2.0, and 3.0 mM. Velocity is expressed as units/ml.

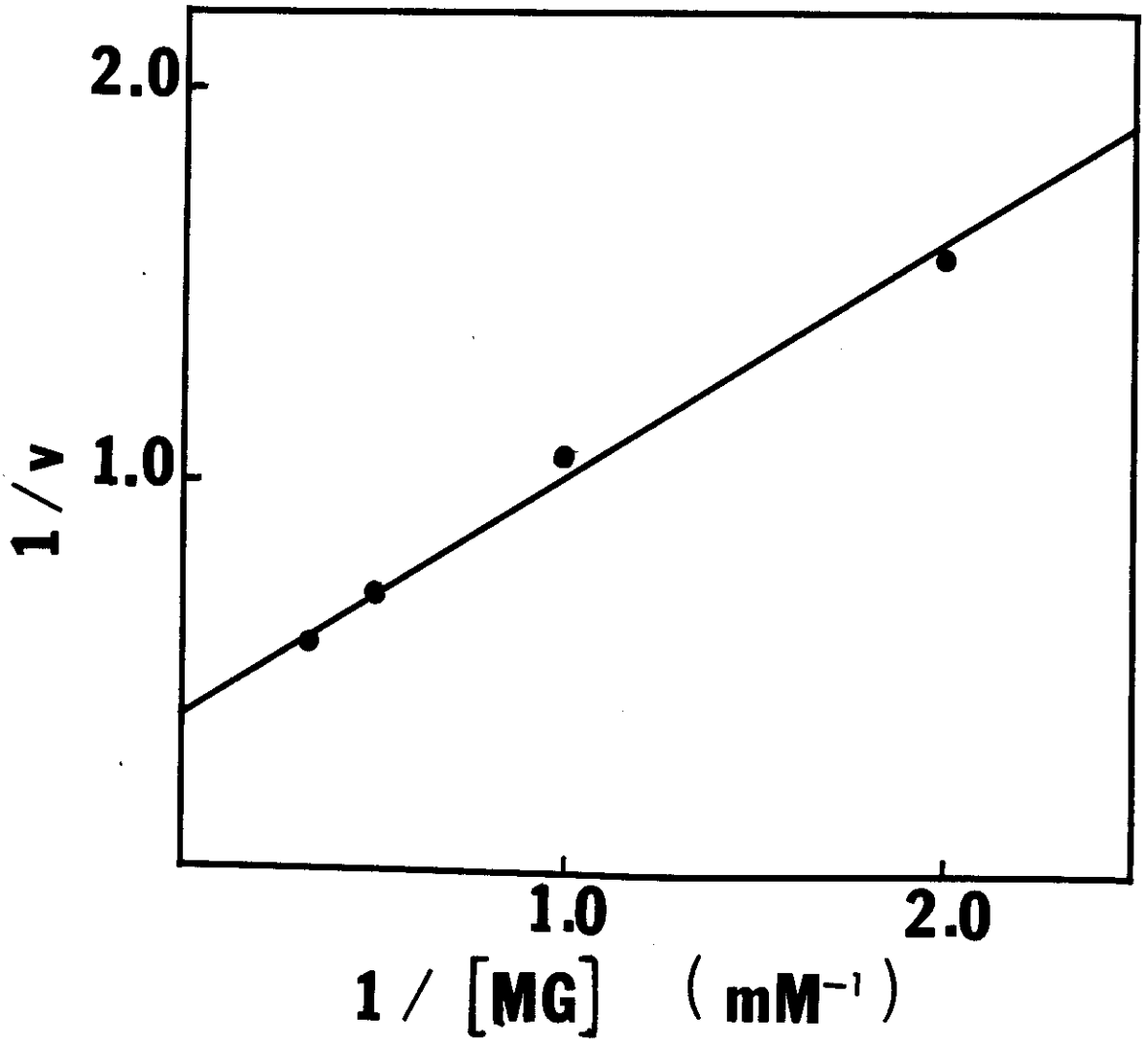

2.0
1.0
1/v
1.0
2.0
1 / [MG] (mM⁻¹)

Figure 7

Determination of Apparent Michaelis Constant for NADH

Each assay mixture contained 10 mM methylglyoxal and a constant amount of MG reductase. Concentrations of NADH were 4, 5, 10, 20, and 50 µM. Velocity is expressed as units/ml.

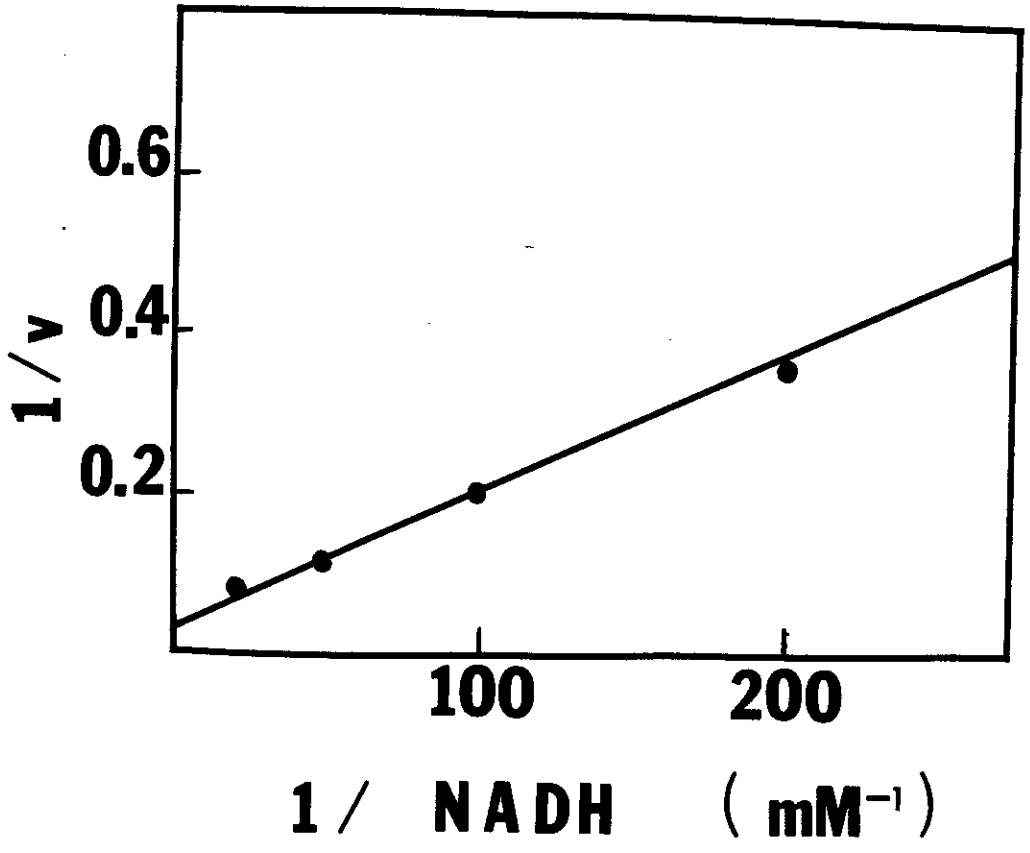
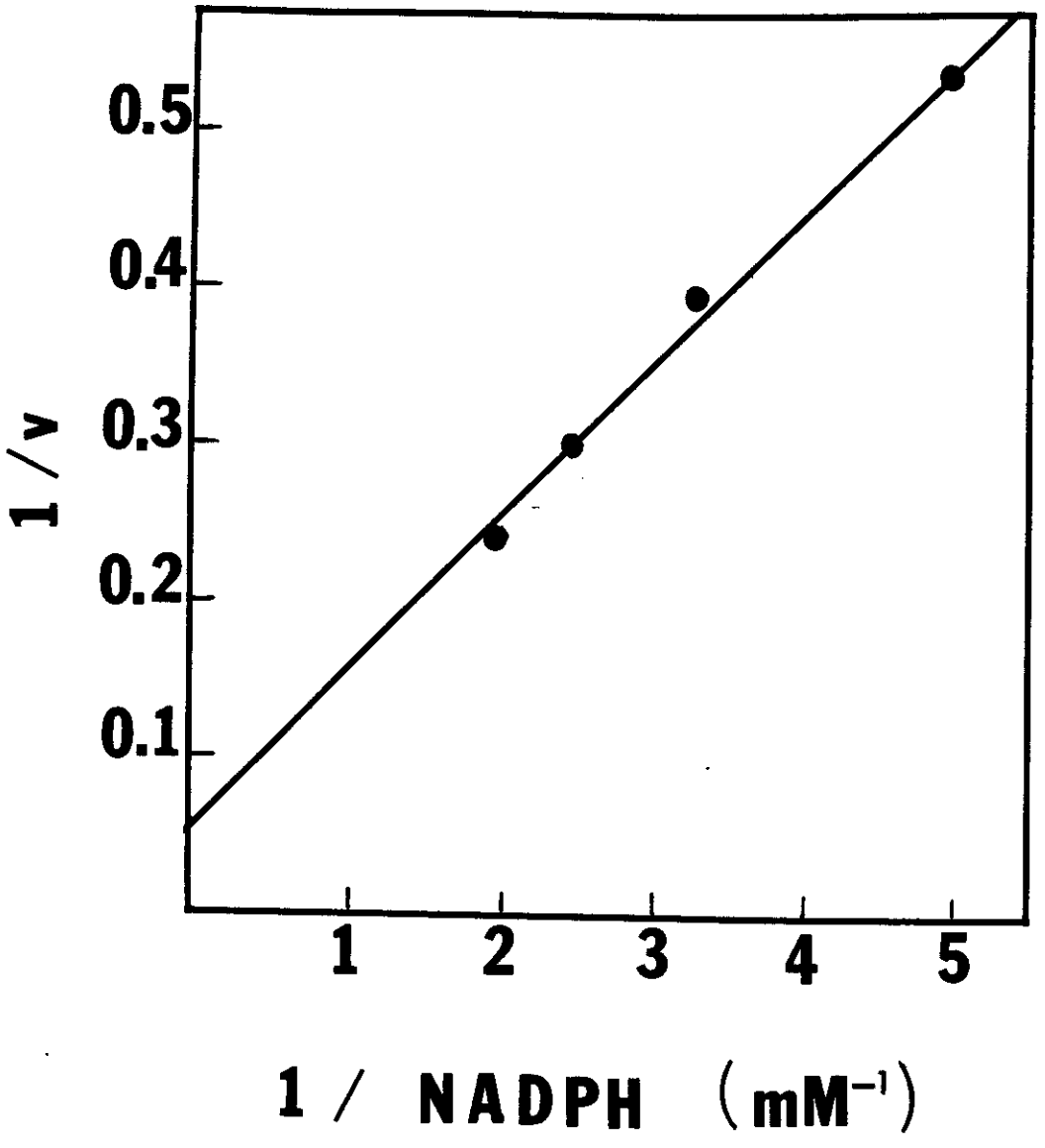

0.6
0.4
0.2
1/v
100
200
1/ NADH (mM⁻¹)

Figure 8

Determination of Apparent Michaelis Constant for NADPH

Each assay mixture contained 10 mM methylglyoxal and a constant amount of MG reductase. Concentrations of NADPH were 0.2, 0.3, 0.4, and 0.5 mM. Velocity is expressed as units/ml.


0.5
0.4
0.3
0.2
0.1
1/v
1
2
3
4
5
1 / NADPH (mM⁻¹)

Results of the T_3 and PTU experiments in rats indicated that rat liver MG reductase was affected *in vivo* by these two compounds. Malic enzyme, which catalyzes the oxidative decarboxylation of malic acid to yield pyruvate, is known to be induced by injection of T_3. Goodridge (32) found that T_3 caused a 60 fold increase in relative synthesis of malic enzyme in liver cells in culture. One of the functions of this enzyme is to generate NADPH for fatty acid synthesis in the cytoplasm. Evidence indicates that thyroid hormones initiate their effects by stimulating transcription of DNA. Rat liver has been shown to contain nuclear T_3 binding proteins, cytosol T_3 binding proteins, and mitochondrial T_3 binding proteins (32). Thus it has been postulated that thyroid hormones have a dual action--(1) an action on nuclear transcription and RNA and protein synthesis and (2) activation of mitochondrial energy metabolism.

PTU provides partial inhibition of the biological effects of administered T_3. It may block the conversion of T_4 to T_3 or reduce the number of thyrotropin receptors in the thyroid gland (33). Evidence has also been presented that PTU exerts its effect by blocking thyroid peroxidase activity. At high concentrations, the drug blocks the first step in the iodination of tyrosyl (33).

In our present study, to determine the reliability of our procedure, we assayed rat liver homogenates for malic enzyme and found that it was increased over 7 fold with T_3 injections. As Table II indicates, MG reductase was also significantly affected by T_3 injection but in the opposite direction. MG reductase activity in T_3 treated rats was only 59% that of controls. Also of interest is the stimulation of MG reductase activity by PTU above the control levels. Glyoxalase I activity was apparently marginally affected by T_3, but the effect is not statistically significant in these initial studies. The data of two separate experiments, in which controls and T_3 treated and controls and PTU treated rats were compared, were subjected to statistical analysis. The results of a Student's t test (34) indicated a significant difference between controls and T_3 treated animals with a p less than 0.05. A significant difference between controls and PTU animals was also found with p less than 0.01. Since MG reductase activity is depressed following T_3 injection, it would seem that its function was not related to increased metabolic rate or increased protein synthesis.

The present studies do not give conclusive evidence of the nature of the repression of MG reductase due to T_3 injection. Further studies of the effect of T_3 and

PTU on other enzymes in the metabolic pathway should give a better explanation of this effect. One such enzyme, diacetyl reductase (L-glycol dehydrogenase), is capable of reduction of α-hydroxycarbonyls to L-glycols and might possibly convert D-lactaldehyde to 1,2-propanediol in the cell. If this enzyme was also affected, the results of the present study would be of more significance.

TABLE II

EFFECT OF T_3 AND PTU ON RAT LIVER ENZYMES

Group	MG Reductase			Malic Enzyme		
	Activity (units/ml)	S.D.	p	Activity (units/ml)	S.D.	p
Control	0.0125	0.005		0.047	0.01	
T_3	0.06	0.01		0.036	0.04	
			< 0.05			< 0.01
Control	0.07	0.021		0.06	0.014	
PTU	0.19	0.029		0.03	0.008	
			< 0.01			< 0.1

CHAPTER IV

CONCLUSION

The physiological role of MG reductase is difficult to assess at this point. The possibility that it is involved in regulation of cell growth by participating in control of intracellular concentrations of MG should not be dismissed. An alternative explanation would be that it performs a simple detoxifying role by removing MG. The possibility that enzymes concerned with MG metabolism could be inhibited as a means of controlling tumor growth has already been discussed.

Methods have been developed in the present studies for the purification of an enzyme which is capable of catalyzing the reduction of MG and concomitant oxidation of NADH at a physiologically relevant pH. Molecular weight estimations using SDS-PAGE indicate a molecular weight of approximately 44,000 daltons. On the other hand, gel filtration experiments indicate a molecular weight of 87,000 daltons, introducing the possibility that the enzyme is a dimer. The pure enzyme showed no activity with D-lactaldehyde as substrate; the product of MG reduction has not been analytically shown to be D-lactaldehyde. Studies of coenzyme requirements show

NADPH to be a poor substrate with only 16% of the activity of NADH. The K_m for NADH was determined to be 0.04 mM; significant depression of enzyme activity was seen at concentrations of 0.35 mM and higher. Whether this latter finding is due to the presence of inhibitors in the NADH solution or to substrate inhibition could not be determined. Results of experiments involving T_3 and PTU administration to rats were certainly interesting but no definite conclusions as to their significance can be made at this time.

The studies described herein have provided information of an enzyme activity which is capable of reducing methylglyoxal in the presence of NADH. The studies also point to new directions where additional investigations should provide a better understanding of ketoaldehyde metabolism.

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