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ETHYLENE- AND DIETHYLENE GLYCOL METABOLISM, TOXICITY AND TREATMENT

The Ohio State University  PH.D.  1986

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ETHELENE- AND DIETHYLENE GLYCOL METABOLISM, 
TOXICITY AND TREATMENT

DISSERTATION

Presented in Partial Fulfillment of the Requirements 
for the Degree Doctor of Philosophy in the Graduate 
School of The Ohio State University

By

Harvey Lewis Wiener, B. S.

* * * * *

The Ohio State University
1986

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SOCITIES
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American Chemical Society
American Institute of Chemists

PRESENTATIONS
Competition between guests for sites in the host
tetrakis(4-methylpyridine)nickel(II) isothiocyanate, H. L.
Wiener and N. O. Smith, at the American Chemical Society
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Undergraduate Research Symposium, 1981.

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FIELDS OF STUDY
Major Field: Physiological Chemistry
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<tr>
<td>A</td>
<td>Absorbance</td>
</tr>
<tr>
<td>ADH</td>
<td>Alcohol dehydrogenase</td>
</tr>
<tr>
<td>ALDH</td>
<td>Aldehyde dehydrogenase</td>
</tr>
<tr>
<td>alpha-KGDH</td>
<td>alpha-Ketoglutarate dehydrogenase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>B6</td>
<td>Vitamin B6</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Ci</td>
<td>Curie</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>CPM</td>
<td>Counts per minute</td>
</tr>
<tr>
<td>DAO</td>
<td>D-Amino acid oxidase</td>
</tr>
<tr>
<td>DCIP</td>
<td>2,6-Dichlorophenol-indophenol</td>
</tr>
<tr>
<td>DEAE</td>
<td>Diethylaminoethyl</td>
</tr>
<tr>
<td>DEG</td>
<td>Diethylene glycol</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPM</td>
<td>Disintegrations per minute</td>
</tr>
<tr>
<td>EC</td>
<td>Enzyme Commission</td>
</tr>
<tr>
<td>EG</td>
<td>Ethylene glycol</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ETO</td>
<td>Ethylene oxide</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FMN</td>
<td>Flavin mononucleotide</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-Aminobutyric acid</td>
</tr>
<tr>
<td>GAD</td>
<td>Glycolic acid dehydrogenase</td>
</tr>
<tr>
<td>GAO</td>
<td>Glycolic acid oxidase</td>
</tr>
<tr>
<td>GDH</td>
<td>D-Glycerate dehydrogenase</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal system</td>
</tr>
<tr>
<td>GR</td>
<td>Glyoxylate reductase</td>
</tr>
<tr>
<td>HEAA</td>
<td>(2-Hydroxyethoxy)acetic acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>Km</td>
<td>Michaelis constant</td>
</tr>
<tr>
<td>LD(_{50})</td>
<td>Median lethal dose</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>ME</td>
<td>Malic enzyme</td>
</tr>
<tr>
<td>MEOS</td>
<td>Microsomal ethanol oxidizing system</td>
</tr>
<tr>
<td>MO</td>
<td>Measured osmolality</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide (oxidized)</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide (reduced)</td>
</tr>
<tr>
<td>NADP</td>
<td>Nicotinamide adenine dinucleotide phosphate (oxidized)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate (reduced)</td>
</tr>
<tr>
<td>OG</td>
<td>Serum osmolal gap</td>
</tr>
<tr>
<td>PKU</td>
<td>Phenylketonuria</td>
</tr>
<tr>
<td>RPHPLC</td>
<td>Reversed phase high performance liquid chromatography</td>
</tr>
<tr>
<td>TA</td>
<td>Transaminase</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TPP</td>
<td>Thiamine pyrophosphate</td>
</tr>
<tr>
<td>Tri-EG</td>
<td>Triethylene glycol</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)-aminomethane</td>
</tr>
<tr>
<td>U</td>
<td>Unit of enzyme activity</td>
</tr>
<tr>
<td>x g</td>
<td>Times gravity</td>
</tr>
<tr>
<td>XO</td>
<td>Xanthine oxidase</td>
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INTRODUCTION

Each year numerous men, women, children, and domestic animals suffer from ethylene glycol (EG) poisoning. The metabolism and toxicity of EG has been well described in the scientific and medical literature as have numerous methods of treatment. Current methods of treating EG poisoning evolved from a knowledge of the metabolic pathway of EG and the clinical signs and symptoms observed. The present approach to treating EG poisoning by administering ethanol is aimed at preventing the oxidation of EG to glycolic acid, the toxic mediator. When treatment is delayed or when the amount of EG consumed is large, successful treatment is rarely obtained since the concentration of glycolic acid becomes excessive. This suggests that a method to enhance the removal of glycolic acid may be effective in treating EG poisoning when treatment is delayed or when the amount of EG consumed is excessive.

In an effort to develop a better approach to treating EG poisoning, studies were conducted to determine the feasibility of using highly purified glycolic acid oxidase
(GAO) as a means of enzyme therapy since it is well accepted that GAO is one of two rate limiting enzymes in the metabolism of EG. Enzyme therapy with GAO was aimed at reducing the concentration of glycolic acid, the toxic mediator in EG poisoning.

Diethylene glycol (DEG) when ingested also results in toxicity. Presently, the metabolism and mechanism of action by which DEG exerts its toxic effects are unknown as is an acceptable means of therapy. To establish the metabolic pathway, the mechanism of action, and a program of treatment for DEG toxicity, studies were undertaken in vivo using male Wistar rats and radiolabeled DEG.
CHAPTER I
STUDIES ON ETHYLENE GLYCOL METABOLISM, TOXICITY, AND TREATMENT

LITERATURE REVIEW:

Ethylene glycol (1,2-ethanediol; EG) is prepared on the industrial scale by the hydration of ethylene oxide (Windholz et al., 1983). In 1984, 4.84 billion pounds of EG were produced in the United States, ranking EG as the number 28 chemical manufactured (Webber, 1985a). Additionally, in 1984, the United States consumed 40% while Western Europe and Asia each consumed 14% of the EG produced (Weber, 1985b).

Ethylene glycol has the following chemical structure:

\[
\begin{align*}
\text{CH}_2\text{OH} \\
\text{CH}_2\text{OH}
\end{align*}
\]

Its molecular weight is 62.07 grams mole\(^{-1}\). Ethylene glycol is a colorless, odorless, slightly viscous liquid having a warm (Hanzlik et al., 1931; Berman et al., 1957), sweet taste (Hanzlik et al., 1931; Pons and Custer, 1946), and a low vapor pressure at room temperature. It is
extremely hygroscopic, absorbing up to twice its weight of water at 100% relative humidity. Ethylene glycol is miscible with water, lower aliphatic alcohols, glycerol, acetic acid, acetone, aldehydes, and pyridine. It is insoluble in benzene, chlorinated hydrocarbons, petroleum ether, and oils (Hanzlik et al., 1931; Windholz et al., 1983).

The uses of EG are many and include: antifreeze and deicers, polishes, cosmetics, hydraulic fluids, heat exchangers, solvents, chemical intermediates, and detergents (Windholz et al., 1983; Winek et al., 1978). The ready availability of antifreeze mixtures makes EG intoxication a significant medical and veterinary problem.

The first reported case of human EG intoxication, in the United States, appeared in 1930 (Anonymous, 1930). Since then numerous cases have been reported in both the medical and veterinary literature. Annually, in the United States, approximately 40 - 60 deaths due to either intentional or accidental ingestion of EG are reported in the medical literature (Haggerty, 1959). Reports in the British medical literature are less frequent (Gorden and Hunter, 1982), perhaps due to a smaller number of motor vehicles.

Despite the publication of the first reported case of EG intoxication and the problems given below, von Oettingen
and Jirouch (1931) stated that "EG may be of interest for therapeutic use, as solvent and as vehicle". Furthermore, they go on to state the following problems: 1. large doses orally produce severe gastroenteritis and systemic symptoms; 2. for intramuscular injections only small doses should be used; 3. subcutaneous injections should be avoided; 4. intravenous administration would appear to be dangerous. Hanzlik et al. (1931) stated that EG was "comparatively innocuous as a solvent for medicinals", ignoring their own data as did von Oettingen and Jirouch.

The incidence of EG intoxication in veterinary medicine is quite significant, for countless numbers of poisonings in dogs and cats occur annually. Many cases are not reported and many cases are not even diagnosed. Mueller (1982) stated that "EG intoxication is a problem of greater magnitude than generally supposed".

There is a seasonal distribution of cases of EG intoxication. This distribution is bimodal with the primary peak occurring during the month of November and the secondary peak occurring during the month of May. The November peak most likely corresponds to the changing of automotive coolant while the cause of the secondary peak appears to be due to the opening of summer homes whose plumbing had been winterized with antifreeze solutions.
The reasons for EG intoxication include the warm, sweet taste and ready availability. Ethylene glycol is a popular agent for suicide and a "poor man's" substitute for alcohol. Pons and Custer (1946) remarked that "the ingenuity displayed by some in finding substitutes for alcoholic beverages is remarkable". Many accidental ingestions occur since EG solutions are frequently stored in old liquor bottles. The warm, sweet taste also contributes to the pediatric risk.

METABOLISM OF ETHYLENE GLYCOL, GLYCOLALDEHYDE, GLYCOLATE, GLYOXYLATE AND OXALATE:

Figure 1 shows the pathway for the metabolism of EG as presented by Chou and Richardson (1978). In the first reaction, that catalyzed by alcohol dehydrogenase (Alcohol:NAD⁺ oxidoreductase, EC 1.1.1.1), EG is oxidized to glycolaldehyde as NAD⁺ is reduced to NADH (von Wartburg et al., 1964; Blair and Vallee, 1966; Coen and Weiss, 1966) at a rate one tenth that of ethanol (von Wartburg et al., 1964). Alcohol dehydrogenase (ADH) is one of two rate limiting enzymes in the metabolism of EG (Chou and Richardson, 1978). Alcohol dehydrogenase is a cytosolic enzyme containing zinc as a structural and functional component participating in the mechanism of enzymatic
Figure 1: The pathway for the metabolism of EG (after Chou and Richardson, 1978).
action (Vallee and Hoch, 1955; von Wartburg et al., 1964). The Michaelis constant (Km) for human liver ADH was reported as 1 mM and 30 mM for ethanol and EG respectively (Blair and Vallee, 1966).

As the metabolic flux through the ADH reaction is increased, the cytoplasmic NADH/NAD⁺ ratio increases resulting in decreased Krebs' Cycle activity since the malate dehydrogenase (L-Malate:NAD⁺ oxidoreductase, EC 1.1.1.37) reaction is shifted to favor the production of malate from oxaloacetate (Rawat, 1968).

The increased NADH/NAD⁺ ratio also results in an increased lactate/pyruvate ratio (Rawat, 1968) through lactate dehydrogenase (L-Lactate:NAD⁺ oxidoreductase, EC 1.1.1.27; LDH). This elevated lactate/pyruvate ratio allows one to explain the metabolic acidosis that accompanies EG toxicity (Rajagopal et al., 1981). Similar effects are also observed with excessive ethanol ingestion. Aldehydes formed by the ADH reaction are capable of inhibiting both glycolysis and the Krebs’ cycle (Ruffo et al., 1962a,b). The glycolytic pathway is also decreased as a result of the inhibitory effect of the elevated NADH/NAD⁺ ratio on glyceraldehyde-phosphate dehydrogenase (D-Glyceraldehyde-3-phosphate:NAD⁺ oxidoreductase; EC 1.2.1.12) (Mezey, 1985). Decreased glycolytic activity decreases ATP production resulting in increased ADP levels
which stimulates mitochondrial respiration resulting in increased reoxidation of NADH to NAD$^+$ for use by ADH (Mezey, 1985). The effects of ethanol on the activities of gluconeogenic and glycolytic enzymes have been reported by Duruibe and Tejwani (1981) with similar results being observed.

When ethanol is metabolized by the ADH reaction acetaldehyde is formed. The cleavage of threonine to acetaldehyde and glycine by threonine aldolase (L-Threonine acetaldehyde-lyase, EC 4.1.2.5) is another source (Dixon and Webb, 1979).

The oxidation of EG to glycolaldehyde can also be catalyzed by catalase (Hydrogen-peroxide:hydrogen-peroxide oxidoreductase; EC 1.11.1.6) with hydrogen peroxide being reduced to water (Keilin and Hartree, 1945). As with ADH, liver and kidney contain high enough concentrations of catalase for this reaction to occur (Coen and Weiss, 1966). Although catalase is listed as one of three enzyme systems capable of oxidizing ethanol it is not considered to be important in vivo because administration of the potent catalase inhibitor, 3-amino-1,2,4-triazole, has no effect on ethanol metabolism (Mezey, 1985).

The conversion of the aldehyde moiety of glycolaldehyde to the corresponding carboxylic acid,
glycolate, is catalyzed by the mitochondrial enzyme aldehyde dehydrogenase (Aldehyde:NAD$^+$ oxidoreductase, EC 1.2.1.3; ALDH) and the cytosolic enzyme aldehyde oxidase (Aldehyde:oxygen oxidoreductase, EC 1.2.3.1). ALDH requires NAD as its cofactor (Racker, 1949), while aldehyde oxidase requires FAD as its cofactor (Palmer, 1962); both are present in mammalian liver. Acetaldehyde formed during ethanol metabolism is converted to acetic acid by both ALDH and aldehyde oxidase.

Glycolate is present in the diet in significant levels and is readily absorbed from the digestive tract (Harris and Richardson, 1980).

The alcohol group of glycolate may be oxidized to the corresponding aldehyde, glyoxylate, by two possible enzymes, LDH (Nakada and Weinhouse, 1953) and glycolic acid oxidase (Glycolate:oxygen oxidoreductase, EC 1.1.3.1) (Richardson and Tolbert, 1961). This is the second rate limiting step in the metabolism of EG (Chou and Richardson, 1978; Marshall, 1982). Glyoxylate is normally formed from glycine by the action of glycine oxidase (D-Aminoacid:oxygen oxidoreductase, EC 1.4.3.3) (Ratner, 1955).

Glycolic acid oxidase (GAO) is found in both animal and plant tissues. Liver GAO is peroxisomal in nature (Ushijima, 1973), requires FMN as a co-factor (Tolbert,
1981), and has broad substrate specificity for alpha-hydroxy acids in the L-configuration (Fry and Richardson, 1979a). The cellular distribution of glycolate metabolism is shown in Figure 2 (Nath et al., 1984).

The isolation and characterization of pig liver GAO was reported by Schuman and Massey (1971a,b). Glycolic acid oxidase isolated from pig liver contains two flavin chromophores, FMN and 6-hydroxy FMN (Jorns, 1978), thought to be located near one or more positively charged groups and a hydrophobic region of the protein as evident from characteristic changes in the visible spectrum upon the binding of a variety of anions including, sulfate, sulfite, chloride, heptanoate, oxalate and phosphate (Schuman and Massey, 1971a). Schuman and Massey (1971b) proposed that monocarboxylic acids bind to pig liver GAO by both electrostatic interaction between the negative carboxylate group and a positively charged group at the active site, and hydrophobic forces. They found straight chain monocarboxylic acids to act as noncompetitive inhibitors while the dicarboxylic acids act as competitive inhibitors when glycolate is the substrate. The Km for glycolate is 0.32 mM, the molecular weight by molecular sieve chromatography is 100,000 (Jorns, 1978), and the amino acid composition has been reported (Schuman and Massey, 1971a).
Figure 2: The cellular distribution of glycolate metabolism (Nath et al., 1984). Enzymes: DAO = D-Amino acid oxidase; TA = Transaminase; GAO = Glycolic acid oxidase; LDH = Lactate dehydrogenase; GAD = Glycolic acid dehydrogenase; GR = Glyoxylate reductase; ME = Malic enzyme; alpha-KGDH = alpha-Keto glutarate dehydrogenase.
Fry and Richardson (1979a) were the first to isolate and characterize human liver GAO. Their five-step procedure resulted in an over 3000-fold purification to a specific activity of 123 U/mg protein. Human liver GAO has a broad pH optimum (pH 8.2 - 8.8) depending on the substrate. The Km for glycolate and glyoxylate are 0.33 and 3.54 mM respectively. The molecular weight by disc electrophoresis was 105,000 Daltons. One can see that the properties of human liver GAO closely resemble those reported for pig liver GAO.

Schwam et al. (1979) reported their isolation and characterization of human liver GAO during the same year as Fry and Richardson. Schwam et al. employed a three-step procedure resulting in 1000-fold purification and a specific activity of 6.2 U/mg protein.

Glycolic acid oxidase partially purified from rat liver peroxisomes is identical to aliphatic L-alpha-hydroxy acid oxidase (L-2-Hydroxyacid:oxygen oxidoreductase, EC 1.1.3.15) based on a comparison of substrate specificities, kinetic parameters, activation energy, inhibition patterns, and pH optimum (Ushijima, 1973). The Km for glycolate reported by Ushijima is one-tenth that reported by others for pig liver GAO (Schuman and Massey, 1971a; Jorns, 1978), for human liver GAO (Fry and Richardson, 1979a; Schwam et al., 1979), and even for
purified rat liver GAO (Asker and Davies, 1983).

Richardson (1964) studied the effect of testosterone on the GAO levels in male and female rat liver. The adult male rat was shown to have an approximately 30% higher level of GAO than the adult female rat; furthermore, this difference was not observed until after puberty. Liver GAO levels in adult male rats dropped following castration but could be maintained by the subcutaneous administration of testosterone. When male rats were castrated before puberty, induction of GAO was prevented and this could not be reversed by testosterone administration. This difference between the two sexes explains why female rats have a greater resistance to the nephrotoxic effects of both EG and glycolic acid than male rats. Roberts and Seibold (1969) suggest that the same effect may also occur in primates.

As indicated above, the oxidation of glycolate to glyoxylate can also be catalyzed by LDH. However, when the NADH/NAD⁺ ratio is high this reaction favors the formation of glycolate from glyoxylate in a manner analogous to the conversion of pyruvate to lactate. The reduction of glyoxylate to glycolate by LDH was demonstrated by Meister (1952). In the liver, the major role of LDH is the oxidation of lactate to pyruvate for utilization in
gluconeogenesis (Richardson and Farinelli, 1981). Under such conditions it is safe to assume that the formation of glyoxylate from glycolate is catalyzed by GAO.

Glyoxylate is capable of being converted to a large number of compounds as indicated by Williams and Smith (1983) and shown in Figure 3. As shown in Figure 1, glyoxylate is broken down to carbon dioxide by the action of 2-oxoglutarate:glyoxylate carboligase (2-Hydroxy-3-oxoadipate glyoxylate-lyase(carboxylating), EC 4.1.3.15), an enzyme requiring thiamine pyrophosphate as its cofactor (Williams and Smith, 1983), in the cytosol. Deficiency of this enzyme results in Primary Hyperoxaluria Type I (Williams and Smith, 1983).

The metabolism of hydroxyproline, an intermediate in collagen metabolism, is a normal source of glyoxylate. Hydroxyproline is metabolized to glyoxylate and pyruvate with 4-hydroxy-2-ketoglutarate as the intermediate (Richardson and Fry, 1976). Williams and Smith (1983) indicate that oxalate biosynthesis resulting from hydroxyproline appears to be negligible.

It is interesting to note that glyoxylate is an inhibitor of three Krebs' cycle enzymes, namely isocitrate dehydrogenase (NAD\(^+\)) (EC 1.1.1.41), alpha-ketoglutarate dehydrogenase (EC 1.2.4.2) (Bachman and Goldberg, 1971), and aconitate hydratase (EC 4.2.1.3) (Ruffo et al.,
Glyoxylate + [O] \rightarrow Oxalate

Glyoxylate + L-Glutamate $\leftrightarrow$ Glycine + alpha-Ketoglutarate

Glyoxylate + L-Ornithine $\leftrightarrow$ Glycine + Glutamic-gamma-semialdehyde

Glyoxylate + Other Amino Acids $\leftrightarrow$ Glycine + Keto acids

Glyoxylate $\leftrightarrow$ Glycolate

Glyoxylate + alpha-Ketoglutarate $\rightarrow$ alpha-Hydroxy-beta-keto adipate + CO$_2$

Glyoxylate + pyruvate $\leftrightarrow$ 2-Keto-4-hydroxyglutarate

Glyoxylate + CoASH $\rightarrow$ Formyl-S-CoA + CO$_2$

Figure 3: The enzymatic reactions in glyoxylate metabolism occurring in mammalian systems (Williams and Smith, 1983).
Bachman and Goldberg (1971) also demonstrated the inhibition of substrate level phosphorylation; furthermore they reported that respiration, oxidative phosphorylation, and Krebs' cycle activities were unaffected by EG, although substrate level phosphorylation was inhibited at concentrations greater than 10 mM. Oxalomalate (alpha-hydroxy-beta-oxalosuccinic acid), a nonenzymatic condensation product of glyoxylate and oxaloacetate, was shown by Ruffo et al. (1962a,b) to be a strong competitive inhibitor of aconitate hydratase resulting in the accumulation of citrate.

Conversion of the aldehyde moiety of glyoxylate to the corresponding carboxylic acid to form oxalate, an inert metabolic end product (Weinhouse and Freidman, 1951), is catalyzed by three possible enzymes as indicated in Figure 1, Reaction 5. These are GAO (Richardson and Tolbert, 1961), LDH (Sawaki et al., 1967) and xanthine oxidase (XO) (Gibbs and Watts, 1966). In the hydrated form, glyoxylate is structurally similar to glycolate (Tolbert, 1981).

Xanthine oxidase (Xanthine:oxygen oxidoreductase, EC 1.2.3.2) catalyzes the conversion of hypoxanthine to xanthine and xanthine to uric acid. Xanthine oxidase is a flavoprotein (FAD) containing iron and molybdenum. Inhibitors of XO, such as allopurinol (an uncompetitive inhibitor), at levels which completely inhibit purine
oxidation, do not significantly alter oxalate excretion and it is for this reason that the contribution of XO in the formation of oxalate is considered to be minor (Gibbs and Watts, 1966, 1967; King and Wainer, 1968). Gibbs and Watts (1966, 1967) go on to conclude that normal XO activity in vivo is not essential for oxalate production in man based on their inhibition studies and on observations in patients with xanthinuria, resulting from the congenital absence of XO, who excreted normal urinary levels of oxalate.

The main enzyme catalyzing the formation of oxalate from glyoxylate is GAO. Studies by Liao and Richardson (1973) on isolated perfused rat livers showed that the formation of oxalate from glycolate or glyoxylate can be completely inhibited by DL-phenyllactate, a competitive inhibitor, and n-heptanoate, a non-competitive inhibitor of GAO. DL-Phenyllactate was shown to have no inhibitory effect on XO or LDH. DL-Phenyllactate was also effective in vivo in the intact male rat, although it was shown to be highly toxic to the hepatectomized male rat (Farinelli and Richardson, 1983). Their studies indicated that, in rat liver, oxalate synthesis from glyoxylate is catalyzed exclusively by GAO. In previous studies, Liao and Richardson (1972) could not demonstrate the production of oxalate by isolated perfused rat kidneys indicating that
the major source of endogenous oxalate in the rat is the liver (Richardson, 1973). Liver contains all the necessary enzymes to oxidize EG, glycolaldehyde, glycolate, glyoxylate, and glycine to oxalate (Richardson, 1973). In studies using organs obtained from male Wistar rats, Coen and Weiss (1966) demonstrated that the kidney was the only organ besides the liver capable of oxidizing EG to glycolaldehyde at an appreciable rate, which was found to be approximately 25% that of liver.

As indicated above, liver GAO is a peroxisomal enzyme. Peroxisomal oxidases have pH optima around 8.5 (Tolbert, 1981). The compartmentalization of GAO and catalase in the peroxisomes may direct the metabolism of glycolate to oxalate due to the negative Gibbs free energy of the coupled reaction (Tolbert, 1981). Tolbert (1981) also offers a possible explanation for the compartmentalization of glycolate metabolism which is that "any glyoxylate in the cytoplasm would be instantaneously converted to glycolate and oxalate by excess LDH or oxidized nonenzymatically by a trace of hydrogen peroxide to carbon dioxide and formate". Asker and Davies (1983) state that peroxisomal glyoxylate may leak into or be transported into the cytosol, thus becoming a substrate for the LDH dismutation reaction leading one to believe that a glycolate-glyoxylate shuttle is occurring. Although no
evidence has been found in vivo for the existence of such a shuttle, Harris et al. (1982) present evidence for the existence of such a shuttle in isolated rat hepatocytes.

Richardson (1973) studied the effect of partial hepatectomy on the toxicity of EG, glycolate, glyoxylate and glycine in the rat. He observed that partial hepatectomy decreased the toxicity of EG and glycolate, but increased the toxicity of glyoxylate. Furthermore, Richardson observed that partial hepatectomy increased the urinary oxalate concentration of rats fed glyoxylate, but not that of rats fed EG, glycolate or glycine.

Richardson and coworkers (Richardson and Farinelli, 1981; Varalakshmi and Richardson, 1983; Farinelli and Richardson, 1983) studied the effect of total hepatectomy on the synthesis of oxalate from EG, glycolate, and glyoxylate in the male rat. Their results, which confirm the hypothesis that the liver plays a significant role in oxalate biosynthesis, are as follows: total hepatectomy reduced carbon dioxide production from glycolate by 85% and from glyoxylate by 67% (Richardson and Farinelli, 1981). Urinary oxalate from glycolate was decreased by 89% while that from glyoxylate was decreased by 50% following total hepatectomy (Richardson and Farinelli, 1981; Farinelli and Richardson, 1983). Furthermore, Richardson and Farinelli
(1981) reported that total oxalate synthesis from glycolate was reduced by 83% in the total hepatectomized rat while glycolate synthesis from glyoxylate was increased four fold. Oxalate synthesis in the hepatectomized rat is presumed to be catalyzed by extrahepatic LDH (Varalakshmi and Richardson, 1983; Farinelli and Richardson, 1983).

Chernoff and Richardson (1978) studied oxalate synthesis in patients with phenylketonuria (PKU). Phenylketonurics produce abnormally large amounts of L-phenyllactate, a compound that inhibits the biosynthesis of oxalate from glycolate and glyoxylate. In their study, patients with PKU were found to have elevated urinary levels of glycolate, glyoxylate, and oxalate. The elevated oxalate is due to the presence of excessive amounts of aromatic alpha-ketoacids, known oxalate precursors.

Another route in the metabolism of glyoxylate is the formation of glycine as indicated in Figure 1, Reaction 6. There are four aminotransferases that specifically use glyoxylate as the alpha-keto acid. These include: alanine-glyoxylate aminotransferase (L-Alanine:glyoxylate aminotransferase, EC 2.6.1.44) (Thompson and Richardson, 1967), glutamic-glyoxylate aminotransferase (Glycine:2-oxoglutarate aminotransferase, EC 2.6.1.4) (Thompson and Richardson, 1966), ornithine-glyoxylate aminotransferase (L-Ornithine:2-oxoacid aminotransferase, EC 2.6.1.13)
(Meister, 1954) and aromatic-amino acid-glyoxylate aminotransferase (Aromatic-aminoacid:glyoxylate aminotransferase, EC 2.6.1.60) (Harada et al., 1978).

Alanine-glyoxylate aminotransferase and glutamic-glyoxylate aminotransferase isolated from human liver were reported by Thompson and Richardson (1967, 1966). They reported both the irreversible nature of these reactions and the requirement of pyridoxal phosphate as a tightly bound coenzyme of the glutamate-glycine aminotransferase apoenzyme.

As indicated above, glyoxylate may be converted to oxalate by aromatic-amino acid-glyoxylate aminotransferase. This mitochondrial aminotransferase is hepatic in origin. Phenylalanine, kynurenine, tyrosine, and histidine can act as amino donors; glyoxylate, pyruvate, and hydroxypyruvate can act as amino acceptors (Harada et al., 1978).

Under conditions of a vitamin B₆ deficiency, the glyoxylate concentration increases, resulting in increased oxalate biosynthesis (Varalakshmi and Richardson, 1983) and increased urinary oxalate excretion (Richardson, 1967). It had been previously reported that vitamin B₆ deficient rats had a 10 - 18 fold increase in urinary oxalate excretion from glycolate, ethanolamine, and EG (Runyan and Gershoff, 1965). Although urinary oxalate levels are not decreased
when vitamin B₆ deficient rats are given diets high in magnesium, there is a protective effect afforded by magnesium against the deposition of calcium oxalate in the kidney by altering the solvent characteristic of urine (Gershoff and Andrus, 1962). Richardson (1967) demonstrated that vitamin B₆ deficiency did not affect the GAO level in either male or female rats although Murthy et al. (1982) recently reported that liver GAO levels are increased by pyridoxine deficiency. Sharma et al. (1984) reported that pyridoxine deficient animals also had increased liver glycolic acid dehydrogenase (Glycolate:(acceptor) oxidoreductase, EC 1.1.99.14, GAD) activity. Gershoff and coworkers (Sabo et al., 1971; Ribaya and Gershoff, 1979) reported that hepatic glucose-6-phosphate dehydrogenase (D-Glucose-6-phosphate:NADP⁺ 1-oxidoreductase, EC 1.1.1.49; G6PDH), malic enzyme (L-Malate:NAD⁺ oxidoreductase, EC 1.1.1.38, ME), ATP citrate lyase (ATP:citrate oxaloacetate-lyase, EC 4.1.3.8), and LDH levels are decreased in vitamin B₆ deficient rats. Ribaya and Gershoff (1979) reported the effects of oxalate, glyoxylylate, and glycolate to be as follows: oxalic acid inhibited the activities of LDH, G6PDH, and ME; glyoxylic acid inhibited LDH and ME; glycolic acid inhibited G6PDH and ME. They further reported that ATP citrate lyase was not effected by either glycolic acid, glyoxylic acid, or
oxalic acid and that glycine was without effect on the above enzymes. Gershoff and Faragalla (1959) reported that pyridoxine deficiency decreases the activities of most of the aminotransferases, including glycine:glyoxylate aminotransferase. Total hepatectomy reduced oxalate excretion and eliminated the vitamin B₆ effect (Varalakshmi and Richardson, 1983). Vitamin B₆ deficiency also enhances the reduction of respiratory carbon dioxide in the totally hepatectomized rat (Varalakshmi and Richardson, 1983). The results reported by Varalakshmi and Richardson (1983) on the totally hepatectomized male rat suggest a difference in oxalate metabolism between normal and vitamin B₆ deficient kidneys and possibly other tissues as well.

The interconversion of glycine and serine is shown in Figure 1, Reaction 7. This reversible reaction is catalyzed by the enzyme serine hydroxymethyltransferase (5,10-Methylenetetrahydrofolate:glycine hydroxymethyltransferase, EC 2.1.2.1). This pyridoxal-phosphate-protein also catalyzes the reaction of glycine with acetaldehyde to form L-threonine (Dixon and Webb, 1979).

Figure 4 shows the pathways for oxalate biosynthesis, including the direct conversion of glycolate to oxalate by GAD. In this reaction, the alcohol moiety of glycolate is
Figure 4: Pathways of Oxalate Biosynthesis (Richardson and Farinelli, 1981).
converted directly to the corresponding carboxylic acid. Glycolic acid dehydrogenase from the soluble fraction of human liver was first isolated and characterized by Fry and Richardson (1979b). It is interesting to note that glyoxylate is not formed as a free intermediate. Glyoxylate and DL-phenyllactate inhibit GAD, an enzyme which is specific for glycolate and exhibits no activity towards glycine and glyoxylate. The authors did not demonstrate requirements for cofactors. They reported that oxygen does not appear to be the electron acceptor. FMN, ascorbate, and cytochrome c were shown to stimulate activity. Human liver GAD has a sharp pH optimum of 6.1 and the Km for glycolate is $6.3 \times 10^{-5}$ M. The vitamin B$_6$ deficiency studies discussed above suggest that GAD is favored in producing oxalate from glycolate in the vitamin B$_6$ deficient rat.

Gambarrellia and Richardson (1977) investigated the pathways of oxalate formation from phenylalanine, tyrosine, tryptophan, and ascorbic acid in the rat. They concluded, based on their studies with radiolabeled compounds, that: phenylalanine and tyrosine are converted to oxalate via GAD with glycolate as an intermediate; tryptophan is converted to oxalate via GAO with glyoxyxlate as the intermediate; and that neither glycolate, glyoxyxlate, or the enzymes GAO and
GAD are involved in the formation of oxalate from ascorbic acid. A minor pathway in the catabolism of ascorbic acid is the formation of oxalate and a four carbon intermediate when ascorbic acid is cleaved between carbon 2 and carbon 3 by a reaction occurring in all animals and representing approximately 4-10% of ascorbate catabolism in man (Tolbert et al., 1975).

Hydroxypyruvate, the alpha-keto acid of serine, is metabolized to oxalate by the same pathway as EG (Liao and Richardson, 1978; Gambardella and Richardson, 1978). The major pathway for the conversion of hydroxypyruvate to oxalate involves the decarboxylation of hydroxypyruvate to glyceraldehyde (Liao and Richardson, 1978) and the subsequent oxidation of glyceraldehyde by the pathway discussed above for EG. Liao and Richardson (1978) indicate that the oxidation of serine to oxalate apparently proceeds via hydroxypyruvate rather than glycine or ethanolamine. Richardson's studies on hydroxypyruvate clearly identify it as a precursor of oxalate.

Hydroxypyruvate is effective in competing with glyoxylate for the active sites on LDH, thus inhibiting the synthesis of oxalate and glycolate from glyoxylate (Raghavan and Richardson, 1983a). Hydroxypyruvate also inhibits the synthesis of oxalate from glyoxylate catalyzed by GAO (Raghavan and Richardson, 1983b).
D-Glycerate dehydrogenase (D-Glycerate:NAD\textsuperscript{+} oxidoreductase, EC 1.1.1.29) catalyzes the reduction of hydroxypyruvate to D-glycerate, and it is this enzyme that has been reported to be deficient in the inherited metabolic disease Primary Hyperoxaluria Type II (L-Glyceric aciduria) (Williams and Smith, 1983). The increasing concentration of hydroxypyruvate in Primary Hyperoxaluria Type II plus the normal concentration of lactate and pyruvate saturate LDH, minimizing the interaction of LDH with glyoxylate (Raghavan and Richardson, 1983b). Raghavan and Richardson (1983b) further suggest a possible nonenzymatic mechanism that may be occurring in Primary Hyperoxaluria Type II. They unequivocally demonstrated, by high performance liquid chromatography and radioactive isotope dilution studies, that aged solutions of hydroxypyruvate spontaneously autooxidize forming oxalate at pH 7.4.

**TOXIC MEDIATOR:**

Numerous authors have stated that the toxicity of EG is not due to EG itself, but rather its metabolic products (Hunt, 1932; Milles, 1946; Bachman and Goldberg, 1971; Richardson, 1973; Clay and Murphy, 1977; Marshall, 1982), although the direct effect has been suggested by others
Ethylene glycol by itself is about as toxic as ethanol on the central nervous system (Beasley and Buck, 1980; Brown et al., 1983).

Toxicity is determined by the extent of oxidation to oxalate (Milles, 1946; Gessner et al., 1961), although data stating that oxalate is a minor factor has been given (Wiley et al., 1938; Roberts and Seibold, 1969; McChesney et al., 1971, 1972). The rat which produces large amounts of oxalate is less sensitive to EG toxicity than the monkey which forms minimal amounts of oxalate and is thus more sensitive to EG (McChesney et al., 1971). Certainly in chronic EG poisoning, calcium oxalate is a major factor; although in acute EG poisoning oxalic acid seems less detrimental. For the rat, the relative toxicity is EG < glycolate < glycolaldehyde < glyoxylate (Richardson, 1973). The serum and urine concentration of glycolaldehyde and glyoxylate in rats is extremely low following EG intoxication (Clay and Murphy, 1977; Chou and Richardson, 1978). There is a direct correlation between mortality and the urinary glycolate concentration in rats leading one to believe that the toxic mediator in EG poisoning is glycolic acid (Chou and Richardson, 1978).

Other evidence supporting the role of glycolic acid as the toxic mediator is seen by studying the metabolism of a structurally related glycol, namely propylene glycol, which
is metabolized via a different pathway and is non-toxic (Hanzlik et al., 1931; Pons and Custer, 1946; Milles, 1946; Berman et al., 1957). Further data supporting the toxic mediator hypothesis are seen in inhibition studies where either ethanol or pyrazole derivatives were used as ADH inhibitors and the mortality reduced due to decreased production of glycolic acid (Clay and Murphy, 1977; Chou and Richardson, 1978; Van Stee et al., 1975; Mundy et al., 1974). The source of the metabolic acidosis in monkeys has been suggested to be glycolic acid (McChesney et al., 1971; Clay and Murphy, 1977). Richardson’s (1973) partial hepatectomy data, discussed earlier, lends further support. Finally, one must remember that the reaction catalyzed by GAO is one of two rate limiting steps in the metabolic oxidative pathway of EG (Chou and Richardson, 1978).

PATHOBIOCHEMICAL SEQUENCE OF EVENTS IN ACUTE ETHYLENE GLYCOL INGESTION:

Figure 5 shows the pathobiochemical sequence of events in acute EG toxicity as given by Rajagopal et al. (1981). As one can see, once ingested, EG is rapidly absorbed and distributed evenly throughout the body tissues (McChesney et al., 1971) Peak levels of EG are reached between one and four hours following ingestion (Winek et al., 1978). After approximately 24 - 48 hours, no EG is detected in
Figure 5: The pathobiochemical sequence of events in acute ethylene glycol toxicity (Rajagopal et al., 1981).
either the urine or the tissues (Winek et al., 1978). Studies of McChesney et al. (1971) in the monkey revealed a plasma half-life of approximately 3 hours. These workers, assuming that the tissue half-life is the same as the plasma half-life, state that by twenty hours post ingestion the total body content of EG should not exceed 1% of the dose. Furthermore, since the excretory rate was even greater in the rat, McChesney et al. (1971) believe the tissue half-life of EG to be 2 - 2.5 hours.

The mechanism by which EG is excreted into the urine was studied in dogs by Swanson and Thompson (1969). Their studies suggest that glomerular filtration and passive reabsorption are the main mechanisms involved in the excretion of EG. Furthermore, they showed that the distal nephron, especially the collecting duct, has an extraordinarily high permeability to EG in contrast to the proximal nephron.

As indicated above, the metabolism of EG is an oxidative process, occurring primarily in the liver, in which EG is converted to glycolaldehyde, glycolate, and glyoxylate (Gessner et al., 1961), which are potent inhibitors of respiration and glucose metabolism in slices of rat brain, liver, and myocardium (Ruffo et al., 1962a). Only glycolaldehyde was effective in vivo (Ruffo et al., 1962a).
The mechanisms by which glucose metabolism is impaired are as follows: the activity of both hexokinase and glycogen synthetase are decreased, while the activity of phosphorylase is increased (Rajagopal and Ramakrishnan, 1978; Mezey, 1985) resulting in a rapid decrease in liver glycogen. There is an increased flux through the pentose phosphate pathway resulting in an increased NADPH/NADP⁺ ratio. Under conditions of acute EG toxicity, lipid biosynthesis appears normal (Rajagopal and Ramakrishnan, 1978) and the NADPH produced by the increased flux through the pentose phosphate pathway is utilized by the microsomal ethanol oxidizing system (MEOS) (Rajagopal and Ramakrishnan, 1978) which catalyzes the following reaction (using ethanol as the substrate):

\[
\text{ethanol} + \text{NADPH} + \text{H}^+ + \text{O}_2 \quad \xrightarrow{\text{MEOS}} \quad \text{acetaldehyde} + \text{NADP}^+ + 2\text{H}_2\text{O}
\]

The contribution of the MEOS to ethanol metabolism in vivo is estimated as 10-20% and increases at even higher ethanol concentrations (Mezey, 1985).

In addition to impaired glucose metabolism, metabolic acidosis is a serious consequence. Glycolic acid is the
cause of the metabolic acidosis observed in monkeys (McChesney et al., 1971). Inhibition of the Krebs' cycle, resulting in increased lactic acid production, is another cause. As a result of acidosis the plasma bicarbonate ion level decreases (Rajagopal et al., 1981).

A direct result of metabolic acidosis is augmented ammonia synthesis by the kidney in which the distal tubular cells produce more ammonia (Rajagopal et al., 1981). Augmented ammonia synthesis together with oxalate crystal deposits and oliguria result in the diffusion of ammonia into the systemic circulation giving the characteristic hyperammonemia frequently observed (Rajagopal et al., 1981).

Hyperammonemia has toxic effects on the brain. To alleviate the toxic effects of ammonia, the brain compensates by detoxifying the ammonia by increasing the synthesis of glutamine (Walser, 1983 and references therein) at the expense of glutamate, alpha-ketoglutarate, and the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) (Rajagopal et al., 1981). GABA enters the Krebs' cycle at the succinate level, thus providing the ATP needed for glutamine synthesis (Bessman and Bessman, 1955; Manning et al., 1964).
THREE CLINICAL STAGES OF ETHYLENE GLYCOL TOXICITY:

The three clinical stages of EG toxicity as defined by Berman et al. (1957) and Brown et al. (1983) are shown in Table 1.

Stage I is characterized by central nervous system (CNS), gastrointestinal (GI) system, and metabolic manifestations. This stage occurs within the first 12 hours post ingestion and patients observed during this stage exhibit the following signs and symptoms: Although the patient appears drunk, there is an absence of the odor of alcohol on the breath. Nausea, vomiting, convulsions, metabolic acidosis, and coma are also seen during this first stage (Berger and Ayyar, 1981). Gordon and Hunter (1982) attribute the cause of cerebral irritation to the aldehydes produced when EG is oxidized and also to oxalate production. In animals, EG is depressing and results in incoordination and ataxia within approximately 60 minutes following ingestion (Beasley and Buck, 1980).

It is during Stage II that the cardiorespiratory manifestations occur. Stage II occurs 12 - 24 hours post ingestion of EG and it is characterized by tachycardia, mild hypertension, tachypnea, congestive heart failure, and acute pulmonary edema as a result of prolonged CNS depression (Winek et al., 1978).
Table 1: Three clinical stages of ethylene glycol toxicity (Berman et al., 1957; Brown et al., 1983).

Stage I: CNS, GI, and Metabolic Manifestations
- 30 Minutes - 12 hours post ingestion
- Patient appears drunk
- Nausea
- Vomiting
- Convulsions
- Coma
- Metabolic acidosis

Stage II: Cardiorespiratory Manifestations
- 12-24 Hours post ingestion
- Tachycardia
- Mild hypertension
- Tachypnea
- Congestive heart failure, pulmonary edema

Stage III: Renal Manifestations
- 24-72 Hours post ingestion
- Tenderness, pain
- Acute tubular necrosis
Stage III manifestates itself in the renal system and develops one to three days post ingestion of EG. This stage is characterized by flank pain and tenderness (Berman et al., 1957), and acute tubular necrosis.

Other clinical signs indicative of EG toxicity include hyperkalemia (Beasley and Buck, 1980), hypocalcemia, an increased anion gap (Jacobsen et al., 1982a), an increased osmolal gap (Jacobsen et al., 1982a), calcium oxalate crystal deposition in the kidneys, urinary tract, and the engorged vessels of the brain and the meninges (Pons and Custer, 1946), and proteinuria.

The anion gap in serum as defined by Jacobsen et al. (1982a) is calculated as follows:

\[
\text{Anion Gap} = [\text{Na}^+ + \text{K}^+] - [\text{Cl}^- + \text{HCO}_3^-]
\]  

(1)

The osmolal gap in serum (OG) as defined by Jacobsen et al. (1982a) is the difference between the measured (MO) and calculated osmolality and is given by the following equation:

\[
\text{OG} = \text{MO} - \frac{(1.86)[\text{Na}^+] + [\text{Glucose}] + [\text{Urea}]}{0.93}
\]  

(2)

The normal anion gap is about 16 mM and the normal osmolal gap is -0.9 milliosmole kg\(^{-1}\) H\(_2\)O. In diabetic coma
both the anion gap and the osmolal gap increase due to the accumulation of ketoacids. In EG and methanol intoxication, both the anion gap and the osmolal gap also increase as it does in a fourth condition, namely, alcoholic acidosis. Thus, the determination of these values may lead the physician to suspect either EG or methanol toxicity in the absence of specific methods of chemical determination (Jacobsen et al., 1982a).

Agreement as to the type of crystals reported in EG intoxication is lacking due to difficulties in identification and similarities between calcium oxalate monohydrate and hippuric acid when viewed microscopically. Godolphin et al. (1980) definitively confirmed the presence of calcium oxalate monohydrate crystals by X-ray diffraction. In contrast, Kramer et al. (1984) report the presence of hippuric acid crystals by microscopy. There are two basic problems to be resolved here: 1. microscopic evaluation is not a valid means of determination, for only X-ray diffraction can give definitive structural elucidation; and 2. for hippuric acid to be produced an exogenous source of benzoic acid must be supplied to conjugate with glycine (Gessner et al., 1961).
CURRENT METHODS USED IN TREATING ETHYLENE GLYCOL TOXICITY:

Currently, the treatment of EG toxicity is largely supportive and symptomatic (Winek et al., 1978) for the physician strives to correct the metabolic abnormalities observed. One of the major problems faced by the physician trying to effectively treat a case of EG toxicity is proper diagnosis; another is the delay between consumption and the initiation of treatment.

Ethanol, a competitive substrate for ADH, is the currently accepted treatment (Borden and Bidwell, 1968; Beckett and Shields, 1971) although it is only beneficial if started within the first four to six hours post ingestion (Parry and Wallach, 1974; Peterson et al., 1981). Indeed, Borden and Bidwell (1968) achieved 90% survival in rats when treatment with ethanol was initiated within 15 minutes following ingestion. Ethanol therapy was first used experimentally in 1963 in both rats and monkeys (Peterson et al., 1963) with great success leading Wacker et al. (1965) to use ethanol in treating two cases of EG intoxication in humans. von Wartburg et al. (1964), based on their kinetic studies of human liver ADH, proposed using ethanol to treat both EG and methanol toxicity.

Other ADH inhibitors used in man and animals in treating EG toxicity include pyrazole and its derivatives
(Mundy et al., 1974; Van Stee et al., 1975; Chou and Richardson, 1978; Blomstrand et al., 1980; McMartin et al., 1980) and alkyl diols (Holman et al., 1979; Hewlett et al., 1983; Murphy et al., 1984). Although a potent and long lasting ADH inhibitor, pyrazole is toxic (Goldstein and Pal, 1971) and therefore poses unacceptable risks. 4-Methylpyrazole offers promise in treating EG intoxication since was shown to be non-toxic in rats and mice at doses sufficient to inhibit ADH (Blomstrand et al., 1980). Of the alkyl diols, 1,3-butanediol has been shown to be an effective means of therapy in mice and dogs suffering from EG intoxication. 1,3-Butanediol offers great promise, perhaps as the new drug of choice, since it is safer and more efficacious than ethanol (Holman et al., 1979).

Gastric lavage, a method commonly used to empty the stomach contents of EG, is effective only if the stomach still contains EG. Activated charcoal has also been used in treating EG intoxication in dogs (Adaudi and Oehme, 1981). Their studies indicated that hemoperfusion onto activated charcoal canisters lowered the blood concentration of EG but not to an extent significant to increase survival. Their failure to observe a protective effect may be the result of the activated charcoal becoming saturated with EG. The main problem with these therapeutic modalities is their limited value when treatment is
delayed.

As indicated above, therapy is largely supportive. Acidosis is corrected by the administration of sodium bicarbonate (Borden and Bidwell, 1968; Beckett and Shields, 1971). Clay and Murphy (1977) state that animals respond to therapy when their blood glycolate decreases with a corresponding increase in blood bicarbonate levels. Increased fluid intake or intravenous mannitol (to produce an osmotic diuresis) are methods used to increase the urinary excretion of both EG and its metabolites. Hemodialysis (Collins et al., 1970; Peterson et al., 1981; Jacobsen et al., 1982b) is frequently used to decrease the serum levels of both EG and its metabolic intermediates. Jacobsen et al. (1982b) proposed that EG intoxication is an absolute indication of hemodialysis. Central nervous system depression and convulsions are also treated by supportive means as are the cardiorespiratory manifestations. Thiamine and pyridoxine, precursors for the cofactors needed to degrade EG to products less toxic than oxalate, are frequently administered also.

Hypocalcemia is treated by either of two methods, namely, calcium chloride or calcium gluconate administration. It is interesting to note that calcium excretion is decreased when sodium bicarbonate is
administered (Borden and Bidwell, 1968). The main problem in administering calcium salts are that they may increase calcium oxalate deposits. Winek et al. (1978) question the use of calcium salts in treating EG intoxication, although they indicate that the implied rationale for calcium gluconate is for treating hypocalcemic tetany.

Treatment is not very successful in cases where the dose of EG is excessive or where treatment is delayed for an extended period so that the concentration of glycolate is built up above acceptable levels. Under these conditions ethanol therapy is certainly ineffective and death usually results due to the high concentration of glycolate, the toxic mediator. Effective treatment after an extended time period would require the detoxification or removal of glycolate. Enhanced oxidation of glycolate may allow for effective treatment of both cases of excessive dose and delayed onset of treatment.

ENZYME THERAPY:

Enzymes have been called unique therapeutic agents for small amounts of these biological catalysts can rapidly produce large, very specific effects at physiological pH and temperature (Holcenberg, 1982). Numerous examples are given in the literature demonstrating the use of enzymes as therapeutic agents (Cooney and Rosenbluth, 1975; Holcenberg
and Roberts, 1977; Holcenberg, 1982). Enzymes have been used for treating cancer, genetic diseases, clotting disorders, and toxic reactions.

Holcenberg (1982) made the following statement concerning the use of enzymes in treating toxicological conditions:

"Enzymes should be ideal agents for removal of toxins since they usually have high affinity to compete with plasma proteins and tissue sites for the toxin. This approach depends on the discovery of an appropriate enzyme, the elimination of any toxic products of the enzymatic reaction, and the avoidance of allergic reactions to the enzymes."

Examples of the use of enzyme therapy in treating toxic conditions include the use of carboxypeptidase G (Cooney and Rosenbluth, 1975) to reverse the toxic effects of methotrexate in man, and the use of uricase, in Europe, to treat high levels of uric acid in patients with leukemia. It is worth noting that the main problem in uricase therapy is removal of allantoin, a product of the enzymatic reaction (Holcenberg, 1982).

Holcenberg and Roberts (1977) list the characteristics of therapeutically useful enzymes. Their list is given in Table 2. As one can see, for use in enzyme therapy, the enzyme under consideration must meet a rigorous list of demands to be of therapeutic value. Before an enzyme can
Table 2: Characteristics of therapeutically useful enzymes (Holcenberg and Roberts, 1977).

1. High activity and stability at physiological pH.

2. Retention of activity and stability in animal serum and whole blood.

3. High affinity for substrate.

4. Slow clearance from circulation when injected into animals.

5. No inhibition by products or other constituents normally found in body fluids.

6. No requirements for exogenous cofactors.

7. Effective irreversibility of the enzymatic reaction under physiological conditions.

8. Availability from a nonpathogenic organism that contains little endotoxin.
be used therapeutically, one must first determine its effectiveness in vitro in whole blood. Next, one must determine the biological half-life and if the enzyme is taken up by the tissues. Determination of activity in vivo is crucial. One may also consider using enzyme therapy in addition to the current method of treatment. Finally one must determine the maximum dose of toxin treatable by enzyme therapy and the maximum time delay permitted between ingestion and initiation of treatment.

Holcenberg and Roberts (1977) also point out the disadvantages of using enzymes as drugs, which include: the need for exhaustive purification, their high cost to prepare, their rapid inactivation or degradation in the body giving rise to the need for frequent administration, their limited distribution within the host, and the antigenicity of foreign enzymes.

Once injected intravenously, enzymatic activity in plasma decreases exponentially over time (Holcenberg, 1982). The reasons for this include distribution of the enzyme to other fluids and tissues, proteolysis, and excretion (Holcenberg, 1982). Although very little is known about the mechanisms by which endogenous or exogenous proteins and enzymes are cleared (Posen, 1970), the rate of degradation of some enzymes appears to be related to either the accessibility of free amino groups or the surface
charge (Holcenberg, 1982). For exogenous enzymes to be effective, they must exhibit prolonged activity in the circulation and elicit minimal immunological responses (Holcenberg, 1982).

What are some of the solutions to the problems encountered in enzyme therapy? To deal with the limited availability of enzymes an abundant source must be identified. Microorganisms generally constitute the most practical source (Holcenberg and Roberts, 1977). The recent advances in recombinant DNA technology, as evident in the production of both human insulin and growth hormone, offer much promise to the scientist studying enzyme therapy since this offers a method by which large amounts of human enzymes can be produced. Increased use of human enzymes and proteins would also eliminate the immunological response characteristic of foreign proteins.

Other methods have been developed by which foreign enzymes can be improved upon. The chemical, kinetic, immunological, or biological properties can be changed by either covalent modification or encapsulation. Enzymes may be immobilized by a variety of methods, including: absorption, ionic interaction or binding, cross linking, entrapment, and covalent attachment. It should be noted that these methods frequently increase the Km and decrease
the maximal velocity of the enzyme, although they may improve upon the immunological properties, shift the pH optimum in a more favorable direction, or even increase the biological half-life.

MATERIALS AND METHODS

DCIP ASSAY OF GLYCOLIC ACID OXIDASE:

Glycolic acid oxidase may be assayed by the method of Zelitch and Ochoa (1953) as modified by Asker and Davies (1983). This method is based on the reduction of 2,6-dichlorophenol-indophenol (DCIP) and was as follows:

A stock solution containing the following was prepared: sodium glycolate (2 mM; BDH Chemicals Limited, Poole, England), DCIP (35 mg l⁻¹; Sigma Chemical Company, St. Louis, Missouri), and bicine buffer (N,N-bis(2-hydroxyethyl)glycine, 80 mM, pH 8.5; Calbiochem, Los Angeles, California).

For the assay, 2.85 ml of stock solution was added to a 1 cm plastic cuvette and deoxygenated by bubbling with N₂(g). To this cuvette, 0.15 ml of enzyme solution were added and the decrease in absorbance at 620 nm was monitored with a Gilford recording spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, Ohio). All assays were
performed at 25 °C.

One unit of enzymic activity was defined as the amount of enzyme catalyzing the formation of one micromole of product from substrate per minute. For this assay, Asker and Davies (1983) state that one unit is equivalent to an absorbance change of 7.93 minute\(^{-1}\) at 620 nm.

GLYOXYLATE PHENYLHYDRAZONE ASSAY OF GLYCOLIC ACID OXIDASE:

Glycolic acid oxidase may also be assayed by the method of Baker and Tolbert (1966). This method, based on the formation of glyoxylate phenylhydrazone, was as follows:

The following reagent solutions were prepared:

- Potassium phosphate buffer, 0.1 M, pH 8.3
- Sodium glycolate, 0.04 M, adjusted to pH 8
- Phenylhydrazine-HCl (Eastman Organic Chemicals, Rochester, New York), 0.1 M, adjusted to pH 6
- L-Cysteine-HCl (Fisher Scientific Company, Fairlawn, New Jersey), 0.1 M, prepared fresh, adjusted to pH 6

The following reagents were added to a 1 cm cuvette:

- 2.0 ml Potassium phosphate buffer
- 0.1 ml Sodium glycolate
- 0.1 ml Phenylhydrazine-HCl
- 0.1 ml Cysteine-HCl
- 0.6 ml Water
To initiate the reaction, 0.1 ml of enzyme solution was added. The progress of the reaction was monitored spectrophotometrically by following the increase in absorbancy at 324 nm with a Gilford recording spectrophotometer.

One unit of enzymic activity was defined as the amount of enzyme catalyzing the formation of one micromole of glyoxylate from glycolate per minute. For this assay, Baker and Tolbert (1966) state that one unit is equivalent to an absorbance change of 5.67 minute$^{-1}$ at 324 nm.

**PROTEIN DETERMINATION:**

Protein was determined by the binding of Coomassie Brilliant Blue G-250 to proteins by the Bradford (1976) method using the Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories, Richmond, California) with bovine serum albumin (BSA; Sigma) serving as standard. The standard curve (Figure 6) was linear from 20 to 70 micrograms of protein. These data were fitted to a straight line by least squares analysis and Equation 3 was used to calculate the protein concentration in micrograms:

\[
\text{Protein} = \frac{A_{595} + 0.0321}{0.0093}
\]  

(3)
Figure 6: Bradford protein determination standard curve.
Where the slope was 0.0093 and the y-axis intercept was -0.0321 by least squares analysis.

ELECTROPHORESIS:

Disc electrophoresis using polyacrylamide gels was performed using the method of Davis (1964). The method of Chrambach et al. (1967) was used for rapid fixing and staining using 12.5% trichloroacetic acid (TCA) and Coomassie Brilliant Blue R-250 (Sigma).

VISIBLE SPECTROSCOPY:

The visible spectrum of pig liver glycolic acid oxidase at pH 7.0 in 0.1 M sodium phosphate buffer was determined at room temperature using a Cary Model 17-DX spectrophotometer coupled to an On-Line Instruments Systems (Jefferson, Georgia) Model 3820 Data Collection System.

DEAE-CELLULOSE COLUMN PREPARATION:

DEAE-cellulose chromatography was made possible using DE-32 (Whatman, Kent, England), a product that must be "pre-cycled" prior to using. To prepare this media for use the following procedure was employed:

Pre-cycling:

An appropriate amount of the DE-32 anion
exchanger was weighed assuming an approximate bed volume of 5 ml/dry gram. This powder was slowly stirred into 15 volumes of 0.5 M HCl. Care was taken not to use a magnetic stirrer during the preparation of DE-32.

After at least 0.5 h but not longer than 2 h the exchanger was filtered on a sintered polyethylene Buchner funnel. The exchanger was then washed with distilled water until the effluent was pH 4.

The DE-32 was then stirred into 15 volumes of 0.5 M NaOH and was filtered after 0.5 h. This step was then repeated.

Following the second NaOH treatment, the exchanger was filtered and washed until the pH reaches neutrality.

Equilibration:

The anion exchanger was equilibrated by stirring it into the appropriate buffer that was ten times as concentrated as the desired starting buffer and allowing a ten minute equilibration. The volume of buffer used should be 15 - 30 ml/dry gram. After the equilibration period, the supernatant was decanted and additional buffer was added. This was repeated until the buffer and the supernatant solution have the same pH.
Removal of Fines:

Before packing the column the fines must be removed to insure good flow. The anion exchanger was dispersed into buffer (30 ml/dry gram) and the slurry was allowed to settle into a cylinder for a time period calculated by the following equation:

\[ T = nH \]  

(4)

where \( T \) is the time allowed for settling, \( n \) is a factor supplied by the manufacturer (1.3 for DE-32), and \( H \) is the total height of the slurry in the cylinder (in centimeters). After time \( T \), the supernatant was decanted and the wet settled volume was noted.

Buffer was then added to 150% of the wet settled volume and the column was packed.

Packing the DE-32 Column:

The column was filled half way with buffer and the slurry was added allowing free drainage. At the appropriate time, the top flow adapter was inserted and starting buffer was pumped through to allow for final equilibration. At least a ten fold excess of starting buffer should be used.
Regeneration After Use:

DE-32 may be used repeatedly if it was first rinsed with starting buffer containing 1.0 M KCl. One must remember to equilibrate the DE-32 with starting buffer before future use.

HYDROXYLAPATITE COLUMN PREPARATION:

Hydroxylapatite column chromatography was performed using BioGel-HTP (Bio-Rad). BioGel-HTP for column chromatography was prepared as follows:

An appropriate amount of BioGel-HTP was weighed assuming an approximate bed volume of 3 ml/dry gram. This was added to six times the starting buffer by swirling. Care must be taken to use neither a magnetic stirrer nor a glass rod since these devices will greatly increase the number of fines and damage the chromatography media.

After ten minutes, the supernatant was decanted and the buffer was replaced. This was then repeated and the column was then ready to be packed.

The column was first filled half way with starting buffer and the BioGel-HTP was added and allowed to settle by gravity. When the bed was stable, the column was allowed to flow and two times the bed volume of starting buffer was allowed to pass.
The top flow adapter was then inserted and the pump connected.

SEPHADEX G-25 M COLUMN PREPARATION:

Sephadex G-25 M (Pharmacia Fine Chemicals, Piscataway, New Jersey) was prepared for use as a desalting column by the following procedure:

An appropriate amount of Sephadex G-25 M was weighed assuming an approximate bed volume of 5 ml/dry gram. This was slowly added to the starting buffer with stirring. Care must be taken not to use a magnetic stirrer when working with Sephadex. The Sephadex was allowed to swell for 3 h at room temperature.

Following swelling, the fines were removed. The column was then packed by the same procedure described for DEAE-cellulose.

The homogeneity of the packed bed and the void volume were determined by monitoring the elution profile of Blue Dextran 2000 (2 mg/ml, Pharmacia) as suggested by Andrews (1965). Blue Dextran 2000, with an average molecular weight of 2,000,000, is completely excluded from Sephadex G-25 M and elutes in the void volume. Any heterogeneity of the packed bed
was readily revealed by visual inspection during the elution of Blue Dextran 2000.

PURIFICATION OF PIG LIVER GLYCOLIC ACID OXIDASE:

Glycolic acid oxidase was purified by modifying the procedures of Schuman and Massey (1971a) and Fry and Richardson (1979a).

The procedure used was as follows:

Pig liver was purchased from the Ohio Packing Co. (Columbus, Ohio), divided into 1 kg portions, and frozen until needed.

Each 1 kg of liver was cut into small pieces and washed 3 times with 2 l of 0.15 M KCl. The washed liver was then homogenized in 2 l of 0.15 M KCl for 90 seconds in a Waring Blender at medium speed. Any foam formed during the homogenization step was removed by aspiration.

The crude homogenate was acidified to pH 4.8 using 2 M acetic acid with constant stirring. This acidic homogenate was then centrifuged at 9,000 x g for 0.5 h in a refrigerated Sorvall centrifuge. The precipitate was discarded.

To the supernatant, 1 M NaOH was added with constant stirring and the pH was adjusted to pH 8.0. The alkalinized homogenate was allowed to settle
overnight.

Following overnight settling, the alkaninized homogenate was centrifuged at 12,000 x g for 0.5 h. Again the precipitate was discarded.

The supernatant was fractionated between 45 and 60% (NH₄)₂SO₄. Solid (NH₄)₂SO₄ (Special Enzyme Grade, Schwarz/Mann, Cambridge, Massachusetts) was used and added in accordance with the table of Green and Hughs (1955).

The 60% (NH₄)₂SO₄ precipitate was resuspended in 100 ml of 5 mM Tris-HCl (Sigma) buffer (pH 8.0) and dialyzed against 4 l of the same for 24 h with 3 changes of the dialysis buffer.

After dialysis, the resuspended solution was applied to a DEAE-cellulose column (Whatman DE-32) that was previously equilibrated with the same buffer. The dimensions of the column were 2.5 x 25 cm and the flow rate was 104 ml h⁻¹. Ten minute fractions were collected. The column was eluted with a 500 ml linear gradient of 0 - 0.5 M KCl in 5 mM Tris-HCl buffer (pH 8.0). Those tubes exhibiting the greatest amount of GAO activity using the DCIP assay were pooled and dialyzed overnight against 4 l of 5 mM sodium phosphate buffer (pH 6.1). If desired, the preparation, at this point, may be stored as an 80%
(NH₄)₂SO₄ suspension.

At the end of this dialysis period, the pooled fraction was centrifuged for 0.5 h at 12,000 x g. The slight precipitate was discarded and the supernatant was applied to a hydroxylapatite (Bio-Rad BioGel-HTP) column that was 2.6 x 25 cm having a flow rate of 114 ml h⁻¹ and 10 minute fractions were collected. This column was eluted with a 1 liter linear gradient of 0.4 - 1.4 M (NH₄)₂SO₄ in 0.1 M sodium phosphate (pH 6.1). Those fractions having the greatest GAO activity were pooled and may be stored as a 100% (4.1 M) (NH₄)₂SO₄ suspension.

The pooled fractions from the hydroxylapatite column were concentrated by 100% (NH₄)₂SO₄ fractionation. Solid (NH₄)₂SO₄ was slowly added to 4.1 M (100% saturation) and mixed for 1 h, after which time the fraction was centrifuged for 0.5 h at 35,000 x g. The supernatant was decanted and the yellow enzyme pellet was resuspended in a minimal amount of 5 mM sodium phosphate buffer (pH 6.1).

Following concentration, the enzyme was desalted using a Sephadex G-25 M (1 x 25 cm; Pharmacia) gel permeation column that was previously equilibrated with 5 mM sodium phosphate buffer (pH 6.1). The flow
rate of this column was 60 ml h\(^{-1}\). The yellow enzyme solution was collected, assayed, and further purified with a second hydroxylapatite column.

The second hydroxylapatite column was 1.5 x 30 cm and had a flow rate of 104 ml h\(^{-1}\). This column was eluted with the same gradient used earlier. Ten minute fractions were collected. Those fractions having the greatest GAO activity were pooled. This too may be stored as a 100% \((\text{NH}_4)_2\text{SO}_4\) suspension.

All procedures were conducted at 4 °C.

DETERMINATION OF GLYOXYLATE:

Glyoxylate in rat blood may be determined by modification of the procedure of Baker and Tolbert (1966). This method, based on the formation of glyoxylate phenylhydrazone, was as follows:

The following solutions were prepared:

- Potassium phosphate buffer, 0.1 M, pH 8.3
- Phenylhydrazine-HCl, 0.1 M, adjusted to pH 6
- L-Cysteine-HCl, 0.1 M, prepared fresh, adjusted to pH 6
- Trichloroacetic acid, 10% (weight/volume)

To perform the determination a 0.4 ml aliquot of blood was withdrawn and centrifuged for 10 minutes. A 0.1 ml aliquot of the supernatant serum was added to 0.1 ml
trichloroacetic acid. This was then centrifuged and a 0.1 ml aliquot of supernatant was withdrawn and added to a 1 cm cuvette containing 0.2 ml phenylhydrazine-HCl and allowed to incubate for 15 minutes. Following the incubation period, 2 ml of phosphate buffer, 0.1 ml of cysteine-HCl, and 0.6 ml of water were added and the absorbance at 324 nm was determined using a Gilford recording spectrophotometer. The concentration of glyoxyxlate was then determined using a standard curve of absorbance versus glyoxyxlate concentration (Figure 7) which was linear from 50 to 500 nanomoles of glyoxyxlate. These data were fit to a straight line having the following equation by linear regression:

$$\text{Glyoxyxlate (nanomoles)} = \frac{A_{324} + 0.087}{0.00398}$$

where the slope was 0.00398 and the y-axis intercept was -0.087.

**OXIDATION OF GLYCOLIC ACID TO GLYOXYLIC ACID IN VITRO IN WHOLE RAT BLOOD BY PIG LIVER GLYCOLIC ACID OXIDASE:**

To determine if pig liver GAO was active in vitro in heparinized whole rat blood the following experiment was performed:

Glycolic acid oxidase stored frozen as an 100%
Figure 7: Glyoxylate determination standard curve.
(NH₄)₂SO₄ suspension was thawed and centrifuged for 0.5 h at 35,000 x g in a refrigerated Sorvall centrifuge. The supernatant solution was decanted. To each enzyme pellet (1.24 U, 2.48 U, and 3.72 U), 4.0 ml of heparinized whole rat blood was added. (The blood was generously provided by Dr. G. P. Brierley.) A male Sprague-Dawley rat was heparinized by intraperitoneal injection of 1300 U of heparin one hour prior to sacrifice. Upon decapitation, blood was collected in a heparinized beaker.

Upon complete mixing, two 0.4 ml aliquots were removed and centrifuged for 10 minutes in an Eppendorf centrifuge. One 0.1 ml aliquot of supernatant serum was immediately added to 0.1 ml of 10% TCA, this was to become the blank used in the determination of glyoxylate. The second 0.1 ml aliquot of supernatant serum was assayed for GAO activity by the method of Baker and Tolbert (1966) as described previously.

After verifying enzymic activity, 120 umol of glycolate (240 ul of 0.5 M glycolic acid adjusted to pH 8.0) were added to each sample of blood and an 0.4 ml aliquot was immediately removed for determination of glyoxylate by the modified procedure of Baker and Tolbert (1966) as described previously except that the sample was brought to the TCA stage; this was the 10 minute sample. Samples were then withdrawn for determinations at 30, 60,
90, and 120 minutes. Incubations were carried out in a Dubnoff incubator at 26 °C. At 120 minutes a second 0.4 ml aliquot was removed and assayed for enzymic activity.

At the conclusion of the incubation period, the TCA samples were centrifuged and the glyoxylate produced was determined.

**OXIDATION OF \[^{14}C\]-GLYCOLIC ACID TO \[^{14}C\]-GLYOXYLIC ACID in vitro IN WHOLE RAT BLOOD BY PIG LIVER GLYCOLIC ACID OXIDASE:**

To determine if pig liver GAO was active in vitro in heparinized whole rat blood the following experiment was performed:

Both a 4 and 16 U aliquot of GAO stored frozen as an 100% (NH\(_4\))\(_2\)SO\(_4\) suspension were thawed and centrifuged for 0.5 h at 35,000 x g in a refrigerated Sorvall centrifuge. The supernatant solution was decanted. To each enzyme pellet, 5.0 ml of heparinized whole rat blood were added.

Upon complete mixing, 1 mmol of sodium glycolate containing 5 uCi of \[^{14}C\]\(_1\)-sodium glycolate (5.5 mCi mmol\(^{-1}\), Amersham International, Arlington Heights, Illinois) was added to each blood sample and incubated for 4 h in a Dubnoff incubator at 24 °C.

At both 1.75 and 3.75 h, a 2 ml aliquot was removed from each blood sample and centrifuged for 0.25 h in an
Eppendorf centrifuge to separate the cells from the plasma. Each plasma sample was then deproteinized by centrifugation in a desk top clinical centrifuge using an Amicon (Danvers, Massachusetts) YMT membrane filter set.

For each incubation, a 100 ul aliquot of deproteinized plasma was then analyzed in duplicate by the anion exclusion high performance liquid chromatography (HPLC) method described below and the percentage of $[^{14}C]$-glyoxylate determined.

CARE OF LABORATORY ANIMALS:

Male Wistar Furth (WF/Hsd BR) rats were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, Indiana) and were cared for in the Department of Animal Labs facilities, under the supervision of a veterinarian, which included a 12 hour light - 12 hour dark cycle. The regulated temperature was 72±2°F and the humidity was between 40 and 60%. The animals were housed in groups of four to a cage and were fed a diet of Purina Rodent Laboratory Chow #5001 (Ralston Purina Company, St. Louis, Missouri) and water \textit{ad libitum}.

DETERMINATION OF COUNTING EFFICIENCY:

The counting efficiency for $^{14}C$ with the Beckman LS 6800 liquid scintillation counter was determined by a
standard quench series plot of percentage efficiency versus H number (Figure 8). A standard quench series of 51,000 DPM (New England Nuclear Research Products, Boston, Massachusetts) was counted in channel one using a lower discriminator setting of 0 and an upper discriminator setting of 670. A two sigma preset counting error of two and a preset counting time of ten minutes were employed. These data were fitted to a straight line having the following equation by linear regression:

\[
\text{Percentage Efficiency} = -0.103(H\#) + 104.9 \quad (6)
\]

where the slope was -0.103 and the y-axis intercept was 104.9.

DETERMINATION OF RESPIRATORY \(^{14}\text{C}\)-CARBON DIOXIDE:

Respiratory \(^{14}\text{CO}_2\) was analyzed by modification of the procedure of Farinelli and Richardson (1983). In this procedure, respired \text{CO}_2 was trapped as sodium carbonate (Weinhouse and Friedman, 1951) in 40% (weight/volume) NaOH containing an added 10% isoamyl alcohol (3-methyl-1-butanol, Baker Chemical Company, Phillipsburg, New Jersey) as an antifoaming agent. A 0.2 ml aliquot was removed from the aqueous NaOH phase lying below the isoamyl alcohol. To this aliquot, 1 ml of water and 10 ml of Complete
Figure 8: Quench curve for the Beckman LS 6800 liquid scintillation counter.
Scintillation Solution Neutralizer (Research Products International, Elk Grove Village, Illinois) were added. The radioactivity was then determined, following overnight adaptation, with the Beckman LS 6800 liquid scintillation counter as described earlier using the H# method to correct for quench.

DETERMINATION OF URINARY RADIOLABELED METABOLITES:

Radiolabeled urinary metabolites were determined by high performance liquid chromatography (HPLC) by modification of the procedure of Marshall (1982). Urine was first centrifuged for 15 minutes in an Eppendorf centrifuge and then filtered using an Acrodisc-CR, 0.45 micrometer, filter (Gelman, Ann Arbor, Michigan).

A 100 ul aliquot of filtered urine was then chromatographed using an Aminex HPX-87 (Bio-Rad) anion exclusion HPLC column (7.8 x 300 mm) at 40°C. The column was eluted using an isocratic mobile phase of 0.0065 M H₂SO₄ at a flow rate of 0.6 ml minute⁻¹. This HPLC procedure was also used to determine the radiochemical purity of the various radiolabeled compounds employed in these studies.

Radioactivity was monitored with the aid of a Flow-One Beta, Model IC, Flow Through Detector (Radiometric Instruments and Chemical Company, Tampa, Florida) using
Flow-Scint III (Radiometric Instruments) as the scintillation cocktail.

To determine the total radioactivity in urine, a 20 ul aliquot was removed and Thrift Solve (Kew Scientific, Columbus, Ohio) was added. Total radioactivity was then determined using the Beckman LS 6800 liquid scintillation counter as described earlier.

**In vivo STUDIES USING PIG LIVER GLYCOLIC ACID OXIDASE:**

The biological half-life of injected pig liver GAO in male rats was determined by injecting either 1.1 U, 2 U, or 5 U of pig liver GAO (isolated as described previously) into male Sprague-Dawley rats (350-530 g, generously provided by Dr. G. P. Brierley) via either the femoral or caudal vein. Blood was collected by transecting the caudal vein at 1, 5, 10, 15, 25, 35, 45, and 60 minutes following the injection of GAO. The blood collected was then centrifuged for 15 minutes in an Eppendorf centrifuge. Duplicate 0.1 ml aliquots of serum were assayed for enzymic activity by the method of Baker and Tolbert (1966) as described earlier.

To determine if pig liver GAO was active in vivo the following experiments were performed. Male Wistar rats (300-600 g, Harlan) were injected with 2 ml kg⁻¹ EG
(Eastman) containing 5 uCi of \([U-^{14}C]\)-EG (4.7 mCi mmol\(^{-1}\), New England Nuclear) via a 24 gauge, 0.75 inch Angiocath Intravenous Catheter Placement Unit (The Desert Company, Sandy, Utah) inserted into the caudal vein under ether anesthesia.

The rat was placed in a metabolism chamber (Farinelli, 1981) for 6 h. The \(^{14}\)CO\(_2\) evolved was collected into 40% NaOH. Additionally, urine was collected. This apparatus is depicted in Figure 9.

A continuous infusion, via the caudal vein cannula, of either 0.15 M NaCl, 4 U, 8 U, or 16 U of GAO in 0.15 M NaCl over a four hour period was performed with the rat in the metabolism chamber. For the remaining 2 h, 0.15 M NaCl was infused.

The GAO used in these experiments was prepared as described previously and stored frozen as a 100% (NH\(_4\))\(_2\)SO\(_4\) suspension. The appropriate amount was thawed and centrifuged at 35,000 x g for 0.5 h and the supernatant was discarded. The yellow GAO pellet was resuspended in 10 ml of 0.15 M NaCl and centrifuged for 0.25 h in an Eppendorf centrifuge immediately prior to injection.

At the end of the six hour period, the rat was exsanguinated by cardiac puncture. The blood collected was centrifuged for 0.25 h in an Eppendorf centrifuge and the serum was frozen for later analysis if needed.
Figure 9: Metabolism chamber allowing for continuous infusion and the collection of urine and CO$_2$. Symbols: A - Metabolic chamber; B - Restraining cage; C - Urine receptacle; D - Caudal vein catheter; E - NaOH trap for atmospheric CO$_2$; F - NaOH trap for respiratory CO$_2$. From Farinelli, 1981.
contents of the bladder were collected and pooled with the urine collected during the six hour period of the experiment. Both the $^{14}\text{CO}_2$ and urine were analyzed by the methods described previously.

Additional studies of this type were conducted using male Wistar rats (420 - 450 g) in an analogous manner except that either 0.15 M NaCl, 16 U or 32 U of pig liver GAO were used as the treatment and sodium glycolate (5 mmol/kg) containing 5 uCi $[^{14}\text{C}]$-sodium glycolate (5.5 mCi mmol$^{-1}$, Amersham) was used as the radiolabeled substrate.

RESULTS AND DISCUSSION

Figures 10 - 12 show the elution profiles of a typical pig liver GAO preparation obtained by chromatography on DEAE-cellulose and hydroxylapatite assayed using DCIP. The DCIP assay could not be used with crude tissue homogenates for DCIP was rapidly reduced in the absence of glycolate. In Figure 10 those fractions having the greatest GAO activity were pooled, they eluted between 0.19 and 0.33 M KCl. Figure 11 is the elution profile for the first hydroxylapatite column. Two peaks were observed having GAO activity; the major peak was collected. It eluted between 0.83 and 1.03 M (NH$_4$)$_2$SO$_4$. Figure 12 is the elution profile obtained by rechromatography on a second
Figure 10: DEAE-cellulose chromatography elution profile for a typical preparation of pig liver glycolic acid oxidase.
Figure 11: First hydroxylapatite column elution profile for a typical preparation of pig liver glycolic acid oxidase.
Figure 12: Second hydroxylapatite column elution profile for a typical preparation of pig liver glycolic acid oxidase.
hydroxylapatite column. The pooled fractions eluted between 0.66 and 0.92 M (NH₄)₂SO₄ and were judged to be homogeneous by disc electrophoresis in polyacrylamide gels.

Table 3 summarizes the overall purification scheme for pig liver GAO which was purified 210 fold, with a 70% recovery, and a specific activity of 0.84 U mg⁻¹ protein. Although frozen liver was used, no significant loss of activity was observed upon storage at -20 °C. Since crude homogenates were able to reduce DCIP in the absence of sodium glycolate the recovery for stages prior to (NH₄)₂SO₄ fractionation was not calculated. Furthermore, recovery was reported for the pooled DEAE-cellulose fractions after dialysis since KCl was found to decrease the activity of pig liver GAO (Figure 13).

The visible spectrum of pig liver GAO obtained at room temperature in 0.1 M sodium phosphate buffer (pH 7.0) is shown in Figure 14. Absorbance maxima occurred at 406 and 447.5 nm with a shoulder occurring at 475 nm. The spectrum obtained was found to be similar to that reported by Schuman and Massey (1971a).

Figures 15 and 16 illustrate the results of the in vivo studies on glyoxylate production from glycolate in heparinized whole rat blood as catalyzed by pig liver GAO. In Figure 15 the slope of each line by linear regression is 48.65, 88.43, and 159.66 for 1.24, 2.48, and 3.72 units of
Table 3: Purification of pig liver glycolic acid oxidase.

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>Total Activity (Units)</th>
<th>Specific Activity (U/mg)</th>
<th>Recovery (%)</th>
<th>Fold Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>45-60% (NH₄)₂SO₄ after dialysis</td>
<td>175</td>
<td>18,200</td>
<td>79</td>
<td>0.004</td>
<td>100</td>
<td>----</td>
</tr>
<tr>
<td>DE-32 eluate after dialysis</td>
<td>145</td>
<td>13,574</td>
<td>83</td>
<td>0.006</td>
<td>105</td>
<td>1.5</td>
</tr>
<tr>
<td>First BioGel-HTP eluate</td>
<td>260</td>
<td>170</td>
<td>91</td>
<td>0.54</td>
<td>115</td>
<td>135</td>
</tr>
<tr>
<td>Second BioGel-HTP eluate</td>
<td>250</td>
<td>65</td>
<td>55</td>
<td>0.84</td>
<td>70</td>
<td>210</td>
</tr>
</tbody>
</table>
Figure 13: The effect of potassium chloride on pig liver glycolic acid oxidase activity.
Figure 14: Visible spectrum of pig liver glycolic acid oxidase.
Figure 15: Glyoxylate production from glycolate by pig liver glycolic acid oxidase in vitro in rat blood. Explanation of symbols: Squares = 1.24 units; Triangles = 2.48 units; Diamonds = 3.72 units.
Figure 16: $^{14}$C-Glyoxylate production from $^{14}$C-glycolate by pig liver glycolic acid oxidase in vitro in rat blood. Explanation of symbols: Squares = 4 units; Triangles = 16 units.
GAO respectively. As the amount of GAO employed doubled from 1.24 to 2.48 units, the slope increased by a factor of 1.8. As the amount of GAO employed tripled from 1.24 to 3.72 units, the slope of the line increased by a factor of 3.3. Thus, glyoxylate production in whole blood in vitro as catalyzed by pig liver GAO was directly proportional to the amount of GAO employed. Additionally, the glyoxylate production increased steadily with time despite increasing red blood cell hemolysis during the course of the experiment. Enzymic activity as measured by the method of Baker and Tolbert (1966) was elevated at the conclusion of the incubation period due to glyoxylate production during this time and its subsequent reaction with the phenylhydrazine reagent.

A similar experiment was performed using [\(^{14}\text{C}_1\)]-glycolate and the results are summarized in Figure 16. As one can see, [\(^{14}\text{C}\)]-glyoxylate production increased with time. However, as the amount of GAO employed was increased from four to sixteen units the glyoxylate production did not increase by the same factor of four. The higher levels of glyoxylate produced in the experiments with radiolabeled glycolate reflect the higher substrate concentration (1 mmole) in contrast to the experiments with unlabeled glycolate where the substrate concentration was 120 umoles. In conclusion Figures 15 and 16 show that glyoxylate
production from glycolate in vitro in whole rat blood as catalyzed by pig liver GAO increases with incubation time and the amount of enzyme employed.

To determine the biological half-life of pig liver GAO in male Wistar rats, a 1.1, 2, or 5 unit bolus of pig liver GAO was administered intravenously and blood withdrawn for the determination of GAO activity over time by the method of Baker and Tolbert (1966). The results of these experiments are shown in Figure 17 where the percentage maximal activity of GAO in blood versus time are graphed. GAO activity reached a maximum within five minutes and decreased to approximately 25% maximum within one hour. When these data were averaged assuming that the different dosages of exogenous pig liver GAO were eliminated by the same mechanism, the biological half-life was found to be approximately twenty to twenty five minutes (Figure 18).

The glyoxylate phenylhydrazone assay described by Baker and Tolbert depends on the production of the reaction product, glyoxylate. This assay did not exhibit endogenous activity in the absence of substrate as was observed with the DCIP assay of Asker and Davies (1983). The reduction of DCIP occurs rapidly in the presence of ascorbic acid (Tolbert et al., 1975) and most likely accounts for the endogenous activity observed when the DCIP assay was used.
Figure 17: In vivo half-life determination for pig liver GAO. Explanation of symbols: Squares = 1.12 units. Triangles = 2 units. Diamonds = 5 units.
Figure 18: Average in vivo half-life determination.
with rat serum since the rat synthesizes ascorbic acid. The glyoxylate phenylhydrazone assay of Baker and Tolbert was found to be an effective means of assaying GAO activity in rat serum as well as tissue homogenates.

Figure 19 shows the removal rate observed for the five unit GAO bolus. This plot displays the logarithm of percentage maximal activity versus time beginning at 100% maximum activity with all data fitted to a straight line by linear regression. From this graph, one disappearance rate was recognizable and the observed biological half-life was approximately thirty five minutes. Enzymes, like most other circulating substances, are removed at a constant fractional rate with characteristic biological half-lives depending on the enzyme and the species involved (Posen, 1970). The concentration of any plasma constituent at any given time depends on many variables, including the rate of delivery into the intravascular compartment, the volume of this compartment, the rate of entry from the intravascular to the extravascular compartment(s) and vice versa, and the metabolic breakdown and excretion of the substance under investigation (Posen, 1970).

Posen (1970) further states that in most injection experiments more than one disappearance rate is recognizable by a biphasic plot. The early, rapid phase attributed to the transfer of enzyme into extravascular
Figure 19: Log (percent maximal activity) versus time for 5 units pig liver GAO.
compartments described by Posen (1970) is not evident in Figure 19 since these data were fitted by linear regression. The disappearance rate shown in Figure 19 is believed to result from enzyme breakdown. Posen (1970) states that biphasic disappearance rates are observed in situations where mixing is assumed to be complete. The mechanism of enzyme removal from the circulatory system is not well understood; although, the likely site for enzyme degradation is the reticulo-endothelial system (Posen, 1970).

Having established an approximate biological half-life of twenty five minutes, studies were conducted to determine if this exogenous pig liver GAO was active in vivo. In these studies 2 ml kg⁻¹ EG containing 5 uCi [U-¹⁴C]-EG was injected intravenously and GAO was infused at a constant rate of 1, 2, or 4 units per hour for four hours. During this time respiratory ¹⁴CO₂ was trapped and urine collected for further analysis by anion exclusion HPLC. When the radioactivity of respiratory ¹⁴CO₂ trapped in sodium hydroxide was determined it was necessary to allow for overnight adaptation before the samples were counted in the liquid scintillation counter. Figure 20 shows the results of repetitive counting over time for sodium hydroxide samples taken from the atmospheric trap and the trap for respiratory carbon dioxide. As one can see, overnight
Figure 20: Counts per minute versus time for respiratory carbon dioxide samples. Explanation of symbols: Squares = samples derived from atmospheric trap, unlabeled carbon dioxide. Triangles = samples derived from respiratory trap containing $^{14}$C-labeled carbon dioxide.
adaptation was necessary to achieve stable counting.

Urine collected during these studies was analyzed by anion exclusion HPLC by modification of the procedure of Marshall (1982). A radioactive flow detector was employed. Table 4 lists the average retention times for EG, glycolate, glyoxylate, and oxalate. The most oxidized compound, oxalate, eluted first and the least oxidized compound, EG, eluted last. Glyoxylate was not detected in the urine of rats receiving [U-\textsuperscript{14}C]-EG. Previous investigators (Gessner et al., 1962; Chou and Richardson, 1978; Marshall, 1982) have reported that the urinary concentration of glyoxylate and glycolaldehyde are extremely low following EG administration. Marshall (1982) reported that glycolaldehyde and glycolate co-elute from the anion exclusion HPLC column employed for these determinations. Any radioactivity eluting with an average retention time of 15.6 minutes was assumed to be glycolate. The lower limit of detection for the HPLC procedure utilized for radiochemical analysis was 0.25 nCi, which would equal 0.025% of a 1 uCi dose, or 0.005% of a 5 uCi dose.

The metabolism of EG in male Wistar rats serving as controls is summarized in Tables 5 and 6. Rats in the 450 - 500 gram range excreted more glycolate than rats weighing between 325 and 360 grams (22.88±0.67% compared to
Table 4: Average retention times of radiolabeled ethylene glycol metabolites.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Average Retention Time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxalic Acid</td>
<td>10.6</td>
</tr>
<tr>
<td>Glyoxylic Acid</td>
<td>12.5</td>
</tr>
<tr>
<td>Glycolic Acid</td>
<td>15.6</td>
</tr>
<tr>
<td>Ethylene Glycol</td>
<td>19.4</td>
</tr>
</tbody>
</table>

[U-\textsuperscript{14}C]-ethylene glycol was purchased from New England Nuclear. \textsuperscript{14}C\textsubscript{1}-glyoxylate (sodium salt), \textsuperscript{14}C\textsubscript{1}-glycolate (sodium salt), and \textsuperscript{14}C-oxalic acid were from Amersham.
Table 5: Metabolism of ethylene glycol (2 ml kg\(^{-1}\)) in male Wistar rats: \(^{14}\)CO\(_2\) Production.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group 1*</th>
<th>Group 2*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight range (g)</td>
<td>325-360</td>
<td>450-500</td>
</tr>
<tr>
<td>Number</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>uCi (^{14})CO(_2) Produced</td>
<td>0.10±0.05</td>
<td>0.12±0.04</td>
</tr>
<tr>
<td>uCi (^{14})CO(_2) kg(^{-1})</td>
<td>0.31±0.16</td>
<td>0.25±0.08</td>
</tr>
<tr>
<td>%(^{14})CO(_2) dose(^{-1})</td>
<td>2.1±1.0</td>
<td>2.5±0.9</td>
</tr>
</tbody>
</table>

*Mean±Standard Deviation
Table 6: Metabolism of ethylene glycol (2 ml kg\(^{-1}\)) in male Wistar rats: Urine analysis.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group 1*</th>
<th>Group 2*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight range (g)</td>
<td>325-360</td>
<td>450-500</td>
</tr>
<tr>
<td>Number</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>uCi Recovered</td>
<td>0.87±0.47</td>
<td>1.23±0.63</td>
</tr>
<tr>
<td>% Dose</td>
<td>17.3±9.3</td>
<td>24.7±12.6</td>
</tr>
<tr>
<td>uCi kg(^{-1})</td>
<td>2.60±1.49</td>
<td>2.52±1.21</td>
</tr>
<tr>
<td>% EG</td>
<td>88.25±0.48</td>
<td>75.20±1.98</td>
</tr>
<tr>
<td>% Glycolate</td>
<td>9.33±1.00</td>
<td>22.88±0.67</td>
</tr>
<tr>
<td>% Oxalate</td>
<td>2.44±0.52</td>
<td>1.93±1.36</td>
</tr>
<tr>
<td>uCi EG</td>
<td>0.77±0.42</td>
<td>0.94±0.50</td>
</tr>
<tr>
<td>uCi Glycolate</td>
<td>0.08±0.04</td>
<td>0.28±0.13</td>
</tr>
<tr>
<td>uCi Oxalate</td>
<td>0.03±0.02</td>
<td>0.02±0.01</td>
</tr>
<tr>
<td>uCi kg(^{-1}) EG</td>
<td>2.30±1.33</td>
<td>1.92±0.97</td>
</tr>
<tr>
<td>uCi kg(^{-1}) Glycolate</td>
<td>0.23±0.12</td>
<td>0.57±0.26</td>
</tr>
<tr>
<td>uCi kg(^{-1}) Oxalate</td>
<td>0.08±0.05</td>
<td>0.03±0.02</td>
</tr>
<tr>
<td>% Dose EG</td>
<td>15.3±8.3</td>
<td>18.8±10.1</td>
</tr>
<tr>
<td>% Dose Glycolate</td>
<td>1.5±0.7</td>
<td>5.6±2.7</td>
</tr>
<tr>
<td>% Dose Oxalate</td>
<td>0.5±0.3</td>
<td>0.3±0.2</td>
</tr>
</tbody>
</table>

*Mean±Standard Deviation
This increase in glycolate production is most likely due to an increased ADH level in the larger rats. Alcohol dehydrogenase is one of two rate limiting enzymes in the metabolic pathway of EG (Chou and Richardson, 1978). One would expect EG to be more toxic to those animals with a higher ADH level since they would produce more glycolate, the toxic mediator.

Tables 7 and 8 report the results of studies in which 2 ml kg⁻¹ EG containing 5 uCi [U⁻¹⁴C]-EG were injected intravenously into male Wistar rats and GAO administered at a rate of 1, 2, or 4 units per hour for four hours. All rats receiving 2 ml kg⁻¹ EG displayed a non-persistent hemoglobinuria immediately following EG administration; Marshall (1982) also reported this same phenomenon. No statistically significant difference in metabolic profile was observed between control rats and those receiving GAO therapy. It was expected that those rats receiving GAO therapy would produce higher levels of oxalate and ¹⁴CO₂ than control rats receiving saline. The Michaelis constant for glycolate is 0.32 mM (Jorns, 1978). One possible explanation for GAO therapy being without effect is that the concentration of glycolate in the blood was not sufficiently adequate for the exogenous pig liver GAO to have an effect. Other possible explanations are that larger amounts of GAO should be employed or that the GAO
Table 7: Metabolism of ethylene glycol (2 ml kg\(^{-1}\)) in male Wistar rats: \(^{14}\)CO\(_2\) Production for GAO treated animals.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control*</th>
<th>4 U GAO*</th>
<th>8 U GAO*</th>
<th>16 U GAO*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight range (g)</td>
<td>450-500</td>
<td>400-440</td>
<td>425-500</td>
<td>467-493</td>
</tr>
<tr>
<td>Number</td>
<td>3</td>
<td>6</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>(\text{uCi} , ^{14}\text{CO}_2)</td>
<td>0.12±0.04</td>
<td>0.15±0.03</td>
<td>0.12±0.01</td>
<td>0.15±0.02</td>
</tr>
<tr>
<td>(\text{uCi} , ^{14}\text{CO}_2 , \text{kg}^{-1})</td>
<td>0.25±0.08</td>
<td>0.34±0.07</td>
<td>0.26±0.01</td>
<td>0.28±0.06</td>
</tr>
<tr>
<td>%(^{14}\text{CO}_2) dose(^{-1})</td>
<td>2.5±0.9</td>
<td>3.0±0.7</td>
<td>2.4±0.2</td>
<td>3.1±0.3</td>
</tr>
</tbody>
</table>

*Mean ± Standard Deviation
Table 8: Metabolism of ethylene glycol (2 ml kg\(^{-1}\)) in male Wistar rats receiving GAO: Urine analysis.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control*</th>
<th>4 U GAO*</th>
<th>8 U GAO*</th>
<th>16 U GAO*</th>
</tr>
</thead>
<tbody>
<tr>
<td>uCi Recovered</td>
<td>1.23±0.63</td>
<td>1.59±0.52</td>
<td>2.07±0.67</td>
<td>0.99±0.41</td>
</tr>
<tr>
<td>% Dose</td>
<td>24.7±12.6</td>
<td>31.7±9.56</td>
<td>41.4±13.4</td>
<td>19.7±8.3</td>
</tr>
<tr>
<td>uCi kg(^{-1})</td>
<td>2.52±1.21</td>
<td>3.76±1.22</td>
<td>4.39±1.10</td>
<td>1.85±0.81</td>
</tr>
<tr>
<td>% EG</td>
<td>75.2±2.0</td>
<td>73.2±4.3</td>
<td>75.6±5.1</td>
<td>75.8±1.1</td>
</tr>
<tr>
<td>% Glycolate</td>
<td>22.9±0.7</td>
<td>25.3±4.0</td>
<td>22.7±5.5</td>
<td>22.6±0.4</td>
</tr>
<tr>
<td>% Oxalate</td>
<td>1.9±1.4</td>
<td>1.6±0.9</td>
<td>1.7±0.4</td>
<td>1.5±1.1</td>
</tr>
<tr>
<td>uCi EG</td>
<td>0.94±0.50</td>
<td>1.17±0.39</td>
<td>1.53±0.40</td>
<td>0.74±0.31</td>
</tr>
<tr>
<td>uCi Glycolate</td>
<td>0.28±0.13</td>
<td>0.39±0.11</td>
<td>0.51±0.27</td>
<td>0.22±0.09</td>
</tr>
<tr>
<td>uCi Oxalate</td>
<td>0.02±0.01</td>
<td>0.02±0.01</td>
<td>0.04±0.01</td>
<td>0.02±0.02</td>
</tr>
<tr>
<td>uCi kg(^{-1}) EG</td>
<td>1.92±0.97</td>
<td>2.78±0.99</td>
<td>3.26±0.60</td>
<td>1.39±0.60</td>
</tr>
<tr>
<td>uCi kg(^{-1}) Glycolate</td>
<td>0.57±0.26</td>
<td>0.92±0.29</td>
<td>1.05±0.49</td>
<td>0.42±0.18</td>
</tr>
<tr>
<td>uCi kg(^{-1}) Oxalate</td>
<td>0.03±0.02</td>
<td>0.05±0.03</td>
<td>0.08±0.01</td>
<td>0.04±0.03</td>
</tr>
<tr>
<td>% Dose EG</td>
<td>18.8±10.1</td>
<td>23.4±7.8</td>
<td>30.6±8.0</td>
<td>14.8±6.1</td>
</tr>
<tr>
<td>% Dose Glycolate</td>
<td>5.6±2.7</td>
<td>7.8±2.3</td>
<td>10.1±5.3</td>
<td>4.5±1.8</td>
</tr>
<tr>
<td>% Dose Oxalate</td>
<td>0.3±0.2</td>
<td>0.5±0.3</td>
<td>0.7±0.1</td>
<td>0.4±0.3</td>
</tr>
</tbody>
</table>

*Mean±Standard Deviation
was inactive or displayed less than optimal activity in vivo, although pig liver GAO was active in vitro in rat blood as discussed earlier.

In an effort to further determine if pig liver GAO was active in vivo similar studies were conducted in which 5 mmole kg\(^{-1}\) glycolate containing 5 uCi \([^{14}C_1]\)-glycolate and either four or eight units of GAO per hour for four hours were administered. The results of these studies are given in Tables 9 and 10. No statistically significant difference was observed between the GAO treated rats and control even with glycolate being used as substrate.

For enzyme therapy with exogenous pig liver GAO to have been effective it is likely that more GAO would have been needed. Enzyme therapy at this level was ineffective despite the fact that the enzyme was active in vitro and had an approximate biological half-life of twenty five minutes. Although these experiments demonstrated that the approach employed was not feasible, enzyme therapy with GAO for EG poisoning should not be completely ruled out at this time. Future work should consider utilizing GAO from other sources or larger dosages of GAO, although this may not be practical. The half-life of GAO may be increased by covalently attaching it to serum albumin. Immobilization onto polyethylene glycol to lower the immunological response (Abuchowski et al., 1977) may allow for a larger
Table 9: Metabolism of glycolate (5 mmol kg$^{-1}$) in male Wistar rats treated with GAO: $^{14}$CO$_2$ Production.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>16 U GAO</th>
<th>32 U GAO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (grams)</td>
<td>424</td>
<td>439</td>
<td>444</td>
</tr>
<tr>
<td>uCi $^{14}$CO$_2$</td>
<td>0.43</td>
<td>0.61</td>
<td>0.42</td>
</tr>
<tr>
<td>uCi $^{14}$CO$_2$ kg$^{-1}$</td>
<td>1.0</td>
<td>1.4</td>
<td>0.94</td>
</tr>
<tr>
<td>$^{14}$CO$_2$ dose$^{-1}$</td>
<td>8.6</td>
<td>12.2</td>
<td>8.4</td>
</tr>
</tbody>
</table>
Table 10: Metabolism of glycolate (5 mmol kg\(^{-1}\)) in male Wistar rats treated with GAO: Urine analysis.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>16 U GAO</th>
<th>32 U GAO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (grams)</td>
<td>424</td>
<td>439</td>
<td>444</td>
</tr>
<tr>
<td>uCi Recovered</td>
<td>2.45</td>
<td>2.51</td>
<td>2.25</td>
</tr>
<tr>
<td>% Dose</td>
<td>49.0</td>
<td>50.2</td>
<td>45.0</td>
</tr>
<tr>
<td>uCi kg(^{-1})</td>
<td>5.78</td>
<td>5.72</td>
<td>5.07</td>
</tr>
<tr>
<td>% Glycolate</td>
<td>93.87</td>
<td>91.96</td>
<td>95.68</td>
</tr>
<tr>
<td>% Oxalate</td>
<td>6.13</td>
<td>8.04</td>
<td>4.32</td>
</tr>
<tr>
<td>uCi Glycolate</td>
<td>2.30</td>
<td>2.31</td>
<td>2.15</td>
</tr>
<tr>
<td>uCi Oxalate</td>
<td>0.15</td>
<td>0.20</td>
<td>0.10</td>
</tr>
<tr>
<td>uCi kg(^{-1}) Glycolate</td>
<td>5.42</td>
<td>5.26</td>
<td>4.84</td>
</tr>
<tr>
<td>uCi kg(^{-1}) Oxalate</td>
<td>0.35</td>
<td>0.46</td>
<td>0.23</td>
</tr>
<tr>
<td>% Dose Glycolate</td>
<td>46.0</td>
<td>46.2</td>
<td>43.0</td>
</tr>
<tr>
<td>% Dose Oxalate</td>
<td>3.0</td>
<td>4.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>
dose of GAO to be infused. Immobilization to shift the pH optimum to that pH of blood may be beneficial. An additional avenue that may be explored in the future could be the use of GAO as an extracorporeal shunt. Lavin et al (1985) demonstrated that the bilirubin concentration in rat blood was lowered when passed through an extracorporeal shunt packed with immobilized bilirubin oxidase. The use of immobilized GAO as an extracorporeal shunt together with ADH inhibition may prove to be an effective means of therapy for EG intoxication.

Although treatment with exogenous pig liver GAO as reported here was without effect, these studies were necessary in studying the feasibility of such an enzyme therapy and will serve to direct future research into the use of enzyme therapy for EG poisoning.

Enzyme therapy with GAO, one of two rate limiting enzymes in the oxidation of EG, was aimed at reducing the concentration of glycolic acid, the toxic mediator. Pig liver GAO was active in vitro in rat blood, oxidizing glycolic acid to glyoxylic acid. When injected intravenously into male Wistar Furth rats, GAO had an approximate half-life of twenty five minutes and its elimination followed first order kinetics. Despite activity in vitro, native pig liver GAO did not display detectable activity in vivo.
LITERATURE REVIEW:

Throughout the history of therapeutics, drug toxicity has been a continuing problem for the pharmaceutical industry, the physician, and most importantly, the patient. The first massive drug toxicity incident involved Elixir of Sulfanilamide. This came to be known as the "Massengil disaster". Elixir of Sulfanilamide employed a toxic vehicle, which composed 72% of the preparation. This toxic vehicle was diethylene glycol (DEG).

Diethylene glycol (2,2'-oxybisethanol) is a colorless, hygroscopic, practically odorless liquid with a sharply sweetish taste (Windholz et al., 1983). Its molecular weight is 106.12 grams mole⁻¹, its boiling point is 245°C, and its density is 1.118 grams milliliter⁻¹.

Diethylene glycol is prepared on the industrial scale by the reaction of ethylene oxide with ethylene glycol. The structural formula of DEG is as follows:
Diethylene glycol is miscible with water, ethanol, ether, acetone, and EG. It is insoluble in benzene and carbon tetrachloride (Windholz et al., 1983). Smyth et al (1941) reported the oral LD$_{50}$ to be 20.76 and 13.21 grams kilogram$^{-1}$ for rats and guinea pigs, respectively.

The uses of DEG are many and include antifreeze solutions for sprinkler systems; water seals for gas tanks; lubricating and finishing agents for wool, worsted, cotton, rayon, and silk; a solvent for vat dyes; in composition corks, glue, gelatin, casein, and pastes to prevent drying out (Windholz et al., 1983).

An additional use of DEG was described recently in a series of articles appearing in The New York Times (Prial, 1985; Tagliabue, 1985; Molotsky, 1985). Wine producers in Austria took advantage of the sweetish taste of DEG to produce a sweeter wine which would demand a higher price at the market place. As much as 48 grams liter$^{-1}$ have reportedly been found (Prial, 1985). Fortunately, no incidents of toxicity from this practice have been reported.
to date (Tagliabue, 1985) despite the fact that the practice of adding DEG to wine in Austria may date back to 1979 (Prial, 1985). The impact of this practice remains to be seen.

Diethylene glycol poisoning was first reported in 1937 as a result of the "Massengil tragedy". As indicated above, Elixir of Sulfanilamide contained approximately 70% DEG, a compound with a cumulative toxicity. During the months of September and October 1937, over 100 deaths occurred in the United States (Geiling and Cannon, 1938). Apparently, the Massengil Company was unaware of their products toxic effects, for Elixir of Sulfanilamide was distributed without adequate animal testing (Geiling and Cannon, 1938). As a direct result of the "Massengil disaster", the Federal Food, Drug, and Cosmetic Act of 1938 was passed requiring toxicological testing of drugs in experimental animals before their use in humans (Balazs et al., 1982).

Two other incidents of DEG poisoning have been reported in the medical literature. Wordley (1947) reported on two cases, and Bowie and McKenzie (1972) reported the deaths of seven children during a three month period in 1969. A check of the literature failed to reveal any veterinary cases.
A review of the literature reveals that little is known about the metabolism and mechanism of action of DEG. It has been suggested (Durand et al., 1976; Hebert et al., 1978) that DEG follows the same metabolic pathway as EG, although this has not been demonstrated experimentally.

Durand et al (1976) reported that acute intoxication with DEG in male rats is associated with considerable urinary oxalate excretion. Furthermore, they report that the excretion of oxalate is significantly decreased by alkalinization and/or intraperitoneal injection of ethanol. Further work by Hebert et al (1978) reported the presence of calcium oxalate crystals in renal tubules following acute intoxication by DEG in male rats. Morris et al (1942) reported the presence of urinary calculi composed primarily of calcium oxalate in male rats following long term ingestion of DEG. Morris et al (1942) go on to state that the formation of calcium oxalate following DEG ingestion is almost conclusive proof that in the rat the ether linkage of DEG is broken and the end products so formed are converted to oxalic acid. The observance of calcium oxalate in male rats following DEG ingestion was also reported by Fitzhugh and Nelson (1946), Hanzlik et al (1947), and Weil et al (1967).
Other workers have reported that calcium oxalate is not formed following DEG intoxication. Brown (1938) based on the chemical structure of DEG stated that the possibility of oxidizing DEG to oxalic acid does not exist. Wiley et al (1938) reported that DEG did not increase urinary oxalic acid, suggesting that this seems to be evidence supporting the hypothesis that the ether linkage is not broken, for if it was broken by hydrolysis in the body EG and, subsequently, oxalic acid should have been formed. Similar findings have also been reported recently. Winek et al (1978) showed that the oxalate concentration in rat blood and kidneys after ingestion of EG were much higher than those following ingestion of DEG. Balazs et al (1982) reported that calcium oxalate crystal deposition is not a feature of DEG nephrotoxicity in either humans or animals.

The utilization of DEG has been studied in the perfused cat liver (Newman et al., 1940). They reported that DEG was not utilized by the cat liver, although it caused a decrease in both oxygen consumption and carbon dioxide production by the liver. They also reported a reduction of liver glycogen and an increase in lactic acid formation.
METABOLISM OF DIETHYLENE GLYCOL BY BACTERIA:

Alcaligenes MC11 (Harada and Sawada, 1977), Pseudomonas P400 (Thelu et al., 1980), and Acinetobacter S8 (Pearce and Heydeman, 1980) have been shown to grow utilizing DEG as the sole carbon source. Harada and Sawada (1977) reported on the occurrence of an NAD-dependent dehydrogenase with high specificity towards ether-alcohol compounds. This enzyme was able to act on DEG, triethylene glycol, and ethylene glycol monomethylether but not on EG or ethanol.

Thelu et al (1980) reported on the occurrence of an inducible polyethylene glycol dehydrogenase from Pseudomonas P400. This enzyme required both FAD and potassium cyanide for activity and actively oxidized DEG. The authors proposed that polyethylene glycol dehydrogenase works through the following mechanism where DCIP is an electron acceptor:

\[
R\text{-O-CH}_2\text{-CH}_2\text{OH} + \text{DCIP}_{\text{oxidized}}
\]

\[
\downarrow
\]

\[
[R\text{-O-CH=CHOH}] + \text{DCIP}_{\text{reduced}}
\]
Pearce and Heydeman (1980) working on Acinetobacter S8, a Gram-negative bacterium, reported the presence of DEG lyase, a novel type, membrane-bound oxygen sensitive enzyme catalyzing the non-oxidative removal of ethylene oxide units as acetaldehyde leaving EG. They proposed the following mechanism by which DEG lyase cleaves DEG leaving EG and acetaldehyde:

\[
[R-O-CH=CHOH] + H_2O \\
\text{\rightarrow} \\
R-O-CHOH-CH_2OH
\]
Diethyl ether is metabolized in man and experimental animals. Van Dyke and Chenoweth (1965) proposed that diethyl ether is metabolized to acetaldehyde and ethanol by the enzymatic addition of a hydroxyl group at the ether linkage. Further support for the formation of an aldehyde group when the ether linkage is cleaved was gained during investigations of methoxyflurane (2,2-dichloro-1,1-difluoroethylmethyl ether) metabolism (Van Dyke, 1966). Methoxyflurane undergoes O-demethylation resulting in the formation of formaldehyde (Van Dyke, 1966).

The metabolism of ethers occur in liver microsomes (Van Dyke and Chenoweth, 1965). The ether metabolizing enzymes require NADPH and oxygen for activity and are induced by methoxyflurane and phenobarbital (Van Dyke, 1966).

Green and Cohen (1971) studied the metabolism of $^{14}$C-diethyl ether in the mouse. They reported that the non-volatile metabolites of diethyl ether metabolism were cholesterol, fatty acids (palmitic acid, stearic acid, and oleic acid), and mono-, di- and triglycerides. This lends further support to the hypothesis of Van Dyke and Chenoweth discussed above.
Aune et al (1978) and Morland et al (1980) studied the metabolism of diethyl ether in man. They reported on the formation of acetaldehyde following diethyl ether administration lending further support to the Van Dyke and Chenoweth hypothesis.

It appears that diethyl ether is cleaved to yield ethanol and acetaldehyde as first proposed by Van Dyke and Chenoweth (1965). This is followed by the oxidation of ethanol to acetaldehyde by ADH as discussed previously. Acetaldehyde is further oxidized to acetate by ALDH and the acetate formed can enter the bodies carbon pool for subsequent conversion to fatty acids, cholesterol, and/or carbon dioxide.

METABOLISM OF STRUCTURALLY RELATED COMPOUNDS; 2-ETHOXYETHANOL:

2-Ethoxyethanol metabolism in male Sprague-Dawley rats has recently been studied by Cheever et al (1984). Using $^{14}$C radiolabeled 2-ethoxyethanol they showed that the main pathway for the oxidation of 2-ethoxyethanol is to the corresponding carboxylic acid, 2-ethoxyacetic acid. They also reported some subsequent conjugation with glycine forming N-ethoxyacetylglycine.

Furthermore, Cheever et al (1984) studied the metabolism of 2-ethoxyethanol with the radiolabel
incorporated at different positions. When 2-ethoxyethanol [ethanol-1,2-\(^{14}\)C] was administered orally to male Sprague-Dawley rats 4.6% of the administered dose was eliminated as \(^{14}\)CO\(_2\). When 2-ethoxyethanol [ethoxy-1-\(^{14}\)C] was administered 11.7% was eliminated as \(^{14}\)CO\(_2\). Their work shows that the ether linkage in 2-ethoxyethanol is cleaved to at least 11.7% in the rat. If the ether cleavage occurred by the mechanism proposed by Van Dyke and Chenoweth (1965) then the products formed as a result of this cleavage would be acetaldehyde from the ethoxy portion of the molecule and EG from the ethanol portion of 2-ethoxyethanol. Based on this hypothesis, it is not surprising to see greater \(^{14}\)CO\(_2\) production from the ethoxy portion of the molecule since it would be subsequently metabolized as acetaldehyde whereas the ethanol portion of 2-hydroxyethanol would be further metabolized as EG.

METABOLISM OF STRUCTURALLY RELATED COMPOUNDS; 1,4-DIOXANE:

1,4-Dioxane is an organic solvent used in both laboratories and industries for a variety of applications (Braun and Young, 1977). The metabolism of 1,4-dioxane in rats (Braun and Young, 1977) and humans (Young et al., 1976) has been reported. In rats \([U-^{14}\text{C}]\)-1,4-dioxane is metabolized to form (2-hydroxyethoxy)acetic acid as the major urinary metabolite (Braun and Young, 1977).
Additionally, two other compounds were reported to be found in the urine, namely, unmetabolized 1,4-dioxane and DEG. Braun and Young (1977) also reported that their experiments did not confirm the presence of either diglycolic acid or oxalic acid in the urine of rats receiving [U-\textsuperscript{14}C]-1,4-dioxane, thus, eliminating the possibility of further oxidation of (2-hydroxyethoxy)acetic acid. Since respiratory carbon dioxide was not collected by Braun and Young no statements can be made about the integrity of the ether linkage in 1,4-dioxane or (2-hydroxyethoxy)acetic acid. Humans exposed to 1,4-dioxane vapors excreted both 1,4-dioxane and (2-hydroxyethoxy)acetic acid in their urine (Young et al., 1976).

CLINICAL SIGNS AND SYMPTOMS OF DIETHYLENE GLYCOL POISONING:

The clinical signs and symptoms reported in DEG poisoning are similar to those of EG poisoning except that they occur somewhat slower (Balazs et al., 1982). The "Massengil disaster" led to the report of the signs and symptoms displayed by man when poisoned with DEG. Geiling and Cannon (1938) reported the following signs and symptoms: heart burn, nausea, abdominal cramps, dizziness, malaise, vomiting, and flank pain all occurring approximately 24 hours after ingesting DEG. These were
followed by oliguria and anuria, pulmonary edema, progressive loss of consciousness from drowsy to comatose, and death from uremic coma occurring from two to seven days after the onset of anuria.

The signs and symptoms of DEG poisoning in children were reported by Bowie and McKenzie (1972). They reported the following: dehydration, metabolic acidosis, vomiting, anuria, diarrhea, hepatomegaly, depression of consciousness, irritability, palpable kidneys, elevated blood urea levels, and elevated blood alanine transaminase levels.

The signs and symptoms observed in laboratory animals are well documented, especially for rats. Laug et al (1939) and Durand et al (1976) reported the following: stupor, polyuria, dehydration due to osmotic diuresis, and acute renal failure. Metabolic acidosis and renal tubular necrosis were reported by Hebert et al (1978). The pathology of the renal lesions observed due to DEG intoxication were well characterized by Kesten et al (1937) and by Oliver et al (1951). Gyrd-Hansen (1974) reported on the diminution of alkaline phosphatase activity in the microvilli of proximal renal tubular cells in rats ingesting DEG.
TREATMENT OF DIETHYLENE GLYCOL POISONING:

A case documenting the successful treatment of DEG poisoning in humans has yet to be reported. At the time of the "Massengil disaster", Geiling and Cannon (1938) reported that there is no known cure for DEG poisoning. Bowie and McKenzie (1972) treated seven cases of DEG poisoning in children by rehydration, bicarbonate to correct metabolic acidosis, peritoneal dialysis, and other supportive measures. Despite their action all seven children succumbed.

The treatment of DEG poisoning in rats has been reported by Durand et al (1976) and Hebert et al (1978). They used hydration together with pyridoxine administration to lower the mortality rate to 20%. Ethanol was also administered, together with bicarbonate, to completely eliminate mortality and the observed metabolic acidosis.

MATERIALS AND METHODS

This section on materials and methods describes procedures unique to studying the metabolism and toxicity of DEG. The interested reader is referred to the EG section for the following four methods:

1. Care of Laboratory Animals
2. Determination of Counting Efficiency
3. Determination of Respiratory $[^{14}\text{C}]$-Carbon Dioxide

4. Determination of Urinary Radiolabeled Metabolites

SYNTHESIS OF $[^{14}\text{C}]-\text{DIETHYLENE GLYCOL}$:

$[^{14}\text{C}]-\text{Ethylene glycol (4.7 mCi mmol}^{-1}; 250 \text{ uCi})$ in methanol was purchased from New England Nuclear Research Products and transferred to a 16 x 150 mm test tube. The original ampoule was rinsed twice with methanol and the rinsings added to the original test tube. The methanol was then blown off with $\text{N}_2(g)$. To the test tube were added 5 ml of n-pentane (Mallinckrodt, Inc., Paris, Kentucky) and 10 ul $\text{H}_2\text{SO}_4$ (Fisher Scientific Co., Fairlawn, New Jersey). Ethylene oxide (ETO; generously provided by Dr. H. W. Sprecher) was then slowly bubbled through for 20 minutes with n-pentane being replenished periodically as necessary to maintain an approximate volume of 5 ml. At the conclusion of the ETO administration, 10 ul of 40% NaOH (weight/volume) were added and the n-pentane blown off using $\text{N}_2(g)$. Following the removal of the n-pentane, 300 ul of water were added and the solution filtered through a "pre-wetted" Acrodisc-CR filter. The filter was rinsed with 500 ul of water and the preparation was purified by reversed phase high performance liquid chromatography (RPHPLC). All procedures except RPHPLC purification were conducted in a fume hood.
PURIFICATION OF [14C]-DIETHYLENE GLYCOL BY REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY:

[14C]-Diethylene glycol synthesized as described above was purified by RPHPLC using an Ultrasphere-ODS column (10 x 250 mm) generously provided by Dr. C. Ryzewski of the Bioanalytical Systems Group, Beckman Instruments, Arlington Heights, Illinois. The column was eluted with a premixed isocratic mobile phase consisting of 50 mM potassium phosphate (HPLC grade, MCB) buffer (pH 6.5) containing 5% methanol (HPLC grade, Fisher) at a flow rate of 4.7 ml minute⁻¹.

The elution profile for a 100 ul injection was monitored using a Beckman Model 156 Differential Refractive Index Detector also generously provided by Dr. Ryzewski. The chart recorder was operated at a chart speed of 10 mm minute⁻¹ and an attenuation of 2⁷ was employed. Multiple injections were made and those fractions corresponding to unreacted EG were pooled. Similarly those fractions corresponding to DEG were also pooled. The methanol was blown off from the pooled DEG fraction and the percentage yield calculated using the following equation:

\[
\text{Percentage Yield} = \frac{\text{uCi}[^{14}\text{C}]-\text{DEG recovered}}{250 \text{ uCi}} \times 100 \quad (7)
\]
GAS-LIQUID CHROMATOGRAPHIC SEPARATION AND DETERMINATION OF ETHYLENE- AND DIETHYLENE GLYCOL:

Ethylene glycol, 1,2-propylene glycol, 1,2-butane diol, and glycerol were separated on Carbowax 20M by Wachowiak and Connors (1979). This liquid phase was utilized for the gas-liquid chromatographic separation and determination of EG and DEG.

Chromatography was made possible using a Varian 6000 Vista Series gas chromatograph with a flame ionization detector. An eight foot long, 2 mm internal diameter glass column custom packed by Supelco, Inc. (Bellefonte, Pennsylvania) containing 5% Carbowax 20M on 40/60 Chromosorb T was employed. Dry helium at 30 ml minute$^{-1}$ was the carrier gas. The injection port and the detector were set at 250°C, and the column was operated isothermally at 170°C. One microliter samples (100 ng total glycol) were injected directly onto the column.

ASSAY OF ALCOHOL DEHYDROGENASE:

Horse liver ADH was purchased from Sigma and assayed as follows:

The following solutions were prepared:

Glycine (Sigma) buffer, 50 mM, pH 8.8
NAD$^+$ (Boehringer-Mannheim, Grade I, Free Acid), 0.01 grams dissolved in 15 ml glycine buffer

Ethanol (Aaper Alcohol and Chemical Co., Louisville, Kentucky), 9 ul dissolved in 15 ml glycine buffer

Alcohol dehydrogenase (Sigma, 1.5 U mg$^{-1}$), 0.001 grams dissolved in 15 ml glycine buffer

The following reagents were added to a 4 ml, 1 cm pathlength cuvette:

- 0.1 ml Alcohol dehydrogenase
- 1.0 ml Ethanol solution
- 0.9 ml Glycine buffer

To initiate the reaction, 1.0 ml of NAD$^+$ was added. The progress of the reaction was monitored spectrophotometrically by following the increase in absorbancy at 340 nm with a Gilford recording spectrophotometer at 25°C.

ASSAY OF ALDEHYDE DEHYDROGENASE:

Potassium activated Baker's yeast ALDH was purchased from Sigma and assayed as follows:

The following solutions were prepared:

- Tris-HCl (Sigma) buffer, 1 M, pH 8.0
- Tris-HCl buffer, 0.1 M, pH 8.0, containing 0.002% (weight/volume) bovine serum albumin (BSA, Sigma)
- Acetaldehyde sodium bisulfite (Eastman), 0.1 M
- Potassium chloride (MCB), 3 M
2-Mer captoethanol (Sigma), 1 M

NAD⁺ (Boehringer-Mannheim, Grade I, free acid), 20 mM

Aldehyde dehydrogenase (Sigma, 8.7 U mg⁻¹), 0.5 U ml⁻¹ in 0.1 M Tris-HCl buffer containing BSA

The following reagents were added to a 4 ml, 1 cm pathlength cuvette:

0.3 ml Tris-HCl buffer
0.1 ml NAD⁺
0.1 ml KCl
0.03 ml 2-Mercaptoethanol
0.05 ml Acetaldehyde sodium bisulfite
2.32 ml Water (distilled, deionized)

To initiate the reaction, 0.1 ml of enzyme solution was added. The progress of the reaction was monitored spectrophotometrically by following the increase in absorbancy at 340 nm with a Gilford recording spectrophotometer at 25°C.

In vitro STUDIES WITH ALCOHOL DEHYDROGENASE, ALDEHYDE DEHYDROGENASE, AND RAT LIVER HOMOGENATES:

The oxidation of DEG by horse liver ADH was studied in the following experiments using DEG and [¹⁴C]-DEG:

Horse liver ADH was assayed as described above using either ethanol, EG, or DEG as substrate and the reduction of NAD⁺ monitored spectrophotometrically.
Horse liver ADH was assayed using DEG as substrate and the stoichiometric reduction of NAD\(^+\) was determined since 1 umole NADH in 3 ml, 1 cm light path, has an optical density of 2.07 at 340 nm.

The oxidation reaction of DEG and \([^{14}\text{C}]\)-DEG in the presence of horse liver ADH and NAD\(^+\) was allowed to run to completion, after which the ADH was denatured by heating. The reaction mixture was cooled and then centrifuged. The supernatant solution was then oxidized by ALDH and the reaction allowed to run to completion. The products of both the ADH incubation and the ALDH incubation were determined by anion exclusion HPLC.

The oxidation of \([^{14}\text{C}]\)-DEG by rat liver homogenates was also studied. Five grams of rat liver were minced and washed three times in 0.15 M KCl. The liver was then homogenized in 10 ml of 0.15 M KCL with a Duall tissue grinder (Kontes Glass Co.). The homogenate was centrifuged for 0.5 hours at 27,000 x g in a refrigerated Sorvall centrifuge. All procedures up to this point were conducted at 0 - 4°C. The pellet was discarded; 0.5 uCi \([^{14}\text{C}]\)-DEG added to the supernatant and incubated at room temperature in a Dubnoff shaker for five hours. Aliquots (1 ml) were
withdrawn hourly and added to 0.1 ml TCA (10% weight/volume). At the conclusion of the incubation period, the samples were centrifuged, filtered, and analyzed by anion exclusion HPLC.

STUDIES USING [$^{14}$C]-DIETHYLENE GLYCOL In vivo:

Six male Wistar Furth rats (255 - 535 grams) were divided into three groups. Group I received 1 ml kg$^{-1}$ DEG (Fisher) containing 1 uCi [$^{14}$C]-DEG via intragastric intubation. Group II received 3 mmole kg$^{-1}$ pyrazole (K & K Laboratories, Inc., Plainview, New York) in normal saline by intraperitoneal injection followed by 1 ml kg$^{-1}$ DEG containing 1 uCi [$^{14}$C]-DEG four hours later. Group III received 4 mmole kg$^{-1}$ diethylidithiocarbamate (Eastman) in water by intraperitoneal injection followed by 1 ml kg$^{-1}$ DEG containing 1 uCi [$^{14}$C]-DEG 16 hours later. All animals were fasted for 24 hours prior to receiving either DEG (Group I), pyrazole (Group II), or diethylidithiocarbamate (Group III), although water was allowed ad libitum. All intraperitoneal injections were made with the animals under light ether anesthesia. Each rat was placed in a glass metabolism chamber (Figure 21) for six hours allowing for the collection of urine and respiratory carbon dioxide. At the conclusion of the six hour period, each rat was euthanized with by ether. The
Figure 21: Metabolic chamber allowing for the collection of urine and respiratory carbon dioxide. Explanation of symbols: A - Metabolic chamber; B - Wire mesh; C - Urine receptacle; D - NaOH trap for atmospheric carbon dioxide; E - NaOH trap for respiratory carbon dioxide.
total radioactivity in the urine was determined by liquid scintillation counting and the urine was further analyzed by anion exclusion and reversed phase HPLC.

A similar study was also conducted using two male Wistar Furth rats (420 and 510 grams) receiving the same dose of DEG through a caudal vein catheter.

TOXICITY STUDIES:

Twenty four male Wistar Furth rats (115 - 150 grams) were fasted for twenty four hours and randomly divided into four groups. Group I received 20.76 g kg\(^{-1}\) DEG, the LD\(_{50}\) as reported by Smyth et al (1941). Group II received 25.95 g kg\(^{-1}\) DEG, a dose 25% above the LD\(_{50}\) value. Group III received 3 mmole kg\(^{-1}\) pyrazole four hours prior to the administration of 20.76 g kg\(^{-1}\) DEG. Group IV received 3 mmole kg\(^{-1}\) pyrazole four hours before the administration of 25.95 g kg\(^{-1}\) DEG. Diethylene glycol was administered by intragastric intubation. With the rat under light ether anesthesia, pyrazole (prepared in normal saline) was administered by intraperitoneal injection to those animals in Groups III and IV four hours before DEG administration. All animals were housed individually in metabolism cages allowing for the collection of urine and feces. Urine was collected into receptacles containing 3 ml of 6 N HCl and saved for later analysis if needed. Water was allowed ad
libitum, and food was provided 24 hours after each rat received DEG.

This study was also performed on twenty four male Wistar Furth rats weighing 330 - 480 grams.

RESULTS AND DISCUSSION

As indicated in the introduction and the literature review, a lack of knowledge of the metabolic pathway of DEG suggested this project. Durand et al (1976) and Hebert et al (1978) studied the metabolism of DEG in male rats and reported increased urinary oxalate excretion and the presence of calcium oxalate crystals in renal tubules following acute intoxication by DEG. Since these workers hypothesized that DEG is metabolized by the same pathway as EG and that DEG poisoning should be treated by the same methods as EG poisoning, consideration of their hypothesis is necessary to developing a better understanding of the metabolism, toxicity, and treatment of DEG.

Durand et al and Hebert et al administered unlabeled DEG and stated that this induced oxalate excretion. To determine if the urinary oxalate excreted is derived from DEG, the studies described in the previous section were undertaken utilizing $[^{14}\text{C}]$-DEG.

$[^{14}\text{C}]$-Diethylene glycol is not available commercially
in the United States and this necessitated its synthesis. DEG is prepared on the industrial scale by the reaction of EG with ETO. ETO is introduced into a large excess of EG in the presence of acid and heat. The DEG produced can then be purified by fractional distillation.

In the laboratory the synthesis and purification of \([^{14}\text{C}]-\text{DEG}\) from \([\text{U}^{14}\text{C}]-\text{EG}\) and ETO is not as simple. Due to the expense and limited volume of \([\text{U}^{14}\text{C}]-\text{EG}\), ETO can not be introduced directly into the EG. Instead the EG must be dissolved in a relatively unreactive solvent. A good solvent for the synthesis of \([^{14}\text{C}]-\text{DEG}\) should also be miscible with EG and low boiling. Initial studies on the synthesis of \([^{14}\text{C}]-\text{DEG}\) employed tetrahydrofuran (THF) as the solvent due to the structural similarity between THF and ETO, and the low boiling point of THF (66°C). THF was abandoned as a solvent since it was found to react under the conditions employed and produced peaks interfering in the reversed phase chromatographic separation of EG, DEG, and triethylene glycol.

The troublesome nature of THF led to the selection of n-pentane as the solvent used in synthesis of \([^{14}\text{C}]-\text{DEG}\). n-Pentane is miscible with alcohol, ether, and many organic solvents. Furthermore, it lacks functional groups that may react under the conditions employed, it is low boiling (36°C), and it does not interfere with the chromatographic
procedure employed.

Having selected a solvent in which to run the reaction it was necessary to select an appropriate acid catalyst and the duration of ETO administration. Organic acids were ruled out since the reaction of a primary alcohol (EG) and a carboxylic acid would result in the synthesis of an ester. For this reason sulfuric acid was chosen. The volume of sulfuric acid employed was to be minimized since any water present in the reaction mixture will react with ETO in the presence of acid and produce EG, DEG, and longer members of the ethylene glycol series, as well as lower the specific activity of the labeled EG and DEG. For the same reasons it was necessary to determine the duration for ETO administration.

A study was conducted in which 5 ul EG containing an added 5 uCi [U-\textsuperscript{14}C]-EG was incubated with either 10 or 25 ul \textsubscript{2}H\textsubscript{2}SO\textsubscript{4} and ETO was bubbled through for either 10, 20, or 30 minutes. Each preparation was subjected to reversed phase HPLC, the peaks collected and their radioactivity determined. The preparation containing 10 ul \textsubscript{2}H\textsubscript{2}SO\textsubscript{4} and receiving a 20 minute ETO administration displayed the greatest yield and these conditions were employed for the synthesis of [\textsuperscript{14}C]-DEG.

[\textsuperscript{14}C]-DEG was purified from the reaction mixture by
reversed phase HPLC on an Ultrasphere-ODS column. Initial studies on the separation of EG, DEG, and triethylene glycol utilized Sephadex G-15 (Pharmacia) to separate these compounds by gel permeation. Sephadex G-15 proved inconvenient to use since it required a series of at least three 100 cm columns packed in tandem and was quite time consuming. These problems led to the investigation of HPLC techniques to achieve separation of EG, DEG, and triethylene glycol.

A series of HPLC columns were provided by Dr. C. Ryzewski and the possibility of using each was explored. A 4.6 x 150 mm Ultrasphere-ODS column failed to give complete resolution of EG and DEG when 5% methanol was the mobile phase. A 4.6 x 250 mm Ultrasphere-ODS column gave complete resolution of EG, DEG, and triethylene glycol with 5% methanol as the mobile phase although the time between the EG and DEG peak was short. These two columns in series gave an increased separation time between the EG and DEG peaks and allowed for a higher concentration of EG and DEG to be separated, although the elution of DEG and triethylene glycol were close.

Adsorption chromatography using an Ultrasphere-Cyano column (4.6 x 150 mm) with 5% methanol as the mobile phase failed to completely resolve EG, DEG, triethylene glycol (MCB), and tetraethylene glycol (Aldrich). Reversed phase
chromatography on an Ultrasphere-Octyl column (4.6 x 150 mm) with 5% methanol as the mobile phase gave similar results. Ligand exchange chromatography using a uSpherogel Carbohydrate column (6.5 x 300 mm) at 90°C with water as the mobile phase gave complete resolution of only tetraethylene glycol; EG, DEG, and triethylene glycol co-eluted.

The column showing the most promise proved to be the Ultrasphere-ODS column. When 50 mM phosphate buffer (pH 6.5) containing an added 5% methanol was employed as the mobile phase complete resolution was achieved between EG, DEG, and triethylene glycol. The addition of phosphate buffer provided a salting out effect. Having achieved a useful separation, this procedure was then transferred to a semipreparative Ultrasphere-ODS column (10 x 250 mm). Table 11 gives the retention times for EG, DEG, and triethylene glycol on the 10 x 250 mm Ultrasphere-ODS column. Figure 22 shows the elution profile obtained from a typical injection of the $[^{14}C]$-DEG synthesis reaction mixture. The percentage yield for the synthesis of $[^{14}C]$-DEG calculated by Equation 7 was 6.7%. When one corrects for the [U-$^{14}$C]-EG recovered the percentage yield was 8%. Although the percentage yield was low this procedure resulted in the production of $[^{14}C]$-DEG that was
Table 11: Retention times for reversed phase HPLC on an Ultrasphere-ODS (10 x 250 mm) column.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention Times Average (Minutes)</th>
<th>Relative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethylene glycol</td>
<td>2.3</td>
<td>0.51</td>
</tr>
<tr>
<td>Diethylene glycol</td>
<td>4.5</td>
<td>1.00</td>
</tr>
<tr>
<td>Triethylene glycol</td>
<td>9.6</td>
<td>2.13</td>
</tr>
</tbody>
</table>

Ethylene glycol was purchased from Eastman Organic Chemicals. Diethylene glycol was purchased from Fisher Scientific, Co. Triethylene glycol was purchased from MCB.
Figure 22: The elution profile for a typical injection of the \([^{13}C]\)-DEG synthesis reaction mixture chromatographed on an Ultrasphere-ODS (10 x 250 mm) column.
chromatographically pure by both reversed phase and anion exclusion HPLC. Furthermore, the low yield was found acceptable since radiolabeled DEG is not commercially available and a preparation free from any contaminating radiolabeled EG was achieved.

As indicated in the Materials and Methods section, a refractive index detector was used to monitor the elution profile of the various columns tested. EG, DEG, and triethylene glycol have similar refractive indices and are detected quite easily with a refractive index detector. When using a refractive index detector certain precautions should be observed. Since refractive index varies with temperature, the column and the detector should be at the same temperature. Furthermore, since refractive index varies with concentration, an isocratic mobile phase should be employed since it is difficult to simulate the same gradient composition in both the sample and reference cell.

All mobile phases of two or more components should be premixed by the user instead of being prepared in the mixing chamber of the HPLC for mobile phases which are mixed in a chamber will exhibit troublesome air bubbles if a pressure restricter is not utilized. These air bubbles result in spikes in the chromatogram when a refractive index detector is used. Pressure restricters must not be employed with a refractive index detector since the maximal
pressure tolerated by the detector flow cell is approximately 50 psi. Finally, if more than one detector is to be used in series, the refractive index detector should be last since a blockage at a point past this detector would result in severe damage.

The unlabeled DEG used for these studies was found to be free of any contaminating EG by gas-liquid chromatography on 5% Carbowax 20M. Table 12 gives the retention times for EG and DEG. These glycols were completely resolved isothermally at 170°C without derivatization. Wachowiak and Connors (1979) derivatized hydroxy compounds by acetylation with acetic anhydride as catalyzed by N-methylimidazole. N-Methylimidazole (Aldrich) was found to have a longer retention time than either EG or DEG and was not used since separation was achieved with underivatized aqueous samples.

Having prepared [14C]-DEG and having determined that the unlabeled DEG was free of any EG, studies were undertaken both in vivo in male Wistar Furth rats and in vitro with ADH, ALDH, and rat liver homogenates. When 1 ml kg⁻¹ DEG containing 1 uCi [14C]-DEG was injected into male Wistar Furth rats a single peak in addition to DEG was observed by anion exclusion HPLC with a radioactive flow through detector. Since the unidentified peak eluted
Table 12: Retention times for gas-liquid chromatography on a 5% Carbowax 20M column at 170°C.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention Time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethylene glycol</td>
<td>2.03</td>
</tr>
<tr>
<td>Diethylene glycol</td>
<td>6.93</td>
</tr>
</tbody>
</table>


before DEG it was thought to be an oxidation product of DEG. In an attempt to identify this peak in vitro studies were undertaken.

When ADH was assayed in the presence of equal concentrations of ethanol, EG, or DEG the initial velocity of the reaction with ethanol as substrate was 33 times faster than that with EG as substrate, and 67 times faster than that with DEG as substrate. A stoichiometric study of the oxidation of DEG by ADH showed that one equivalent of NAD$^+$ was reduced to NADH for each DEG oxidized. This leads one to believe that only one of the alcohol groups in DEG is oxidized by ADH.

When $[^{14}\text{C}]-\text{DEG}$ was incubated with ADH and the results assayed by anion exclusion HPLC a single peak corresponding to DEG was observed despite the fact that NAD$^+$ reduction was observed spectrophotometrically. This observation proved troublesome for some time and will be discussed further below.

When $[^{14}\text{C}]-\text{DEG}$ was incubated with ADH followed by ALDH and the products assayed by anion exclusion HPLC again a single peak corresponding to DEG was produced despite the observed reduction of NAD$^+$. Studies with rat liver homogenates also yielded similar results which are discussed below.

When $[^{14}\text{C}]-\text{DEG}$ was injected into male Wistar Furth
rats the following results were obtained. No $^{14}\text{CO}_2$ was detected. The limit of detection for $^{14}\text{CO}_2$ was approximately 10 nCi which is 1% of a 1 uCi dose or 0.5% of a 2 uCi dose. Furthermore, no radiolabeled EG, glycolate, or oxalate were detected by anion exclusion HPLC. This led to the conclusion that the ether linkage of DEG was not cleaved \textit{in vivo} in male Wistar Furth rats.

As indicated above a single radiolabeled peak in addition to unreacted DEG was observed in the urine of rats receiving $[^{14}\text{C}]$-DEG. This peak was later identified as (2-hydroxyethoxy)acetic acid (HEAA), the main urinary metabolite observed for p-dioxane by Braun and Young (1977). Diglycolic acid was not detected in the urine of rats receiving $[^{14}\text{C}]$-DEG further confirming the work of Braun and Young (1977). Table 13 summarizes the retention times of DEG, HEAA, and diglycolic acid by anion exclusion HPLC, while Table 14 summarizes the retention times of DEG and HEAA by reversed phase HPLC.

(2-Hydroxyethoxy)acetic acid generously provided by Dr. A. M. Schumann of the Mammalian and Environmental Toxicology Research Laboratory of Dow Chemical U. S. A., Midland, Michigan, was found to have the same retention time as the unidentified urinary peak by both anion exclusion and reversed phase HPLC and this previously
Table 13: Relative retention times for anion exclusion HPLC on a Bio-Rad HPX-87 column (7.8 x 300 mm).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Relative Retention Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diethylene glycol</td>
<td>1.00</td>
</tr>
<tr>
<td>(2-Hydroxyethoxy)acetic acid</td>
<td>0.84</td>
</tr>
<tr>
<td>Diglycolic acid</td>
<td>0.70</td>
</tr>
</tbody>
</table>

Diglycolic acid was purchased from Aldrich. (2-Hydroxyethoxy)acetic acid was generously provided by Dr. A. M. Schumann of the Mammalian and Environmental Research Laboratory, Dow Chemical U. S. A., Midland, Michigan.
Table 14: Relative retention times for reversed phase HPLC on an Ultrasphere-ODS column (10 x 250 mm).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Relative Retention Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diethylene glycol</td>
<td>1.00</td>
</tr>
<tr>
<td>(2-Hydroxyethoxy)acetic acid</td>
<td>0.64</td>
</tr>
</tbody>
</table>
unidentified peak has been tentatively defined as HEAA.

Tables 15, 16, and 17 present the results of the urine analysis of rats receiving DEG, DEG and pyrazole (an ADH inhibitor), and DEG and diethyldithiocarbamate (an ALDH inhibitor) respectively.

Male Wistar Furth rats receiving DEG by intragastric intubation as well as those receiving DEG by intravenous administration displayed similar metabolic profiles. After six hours the urine of rats receiving DEG by intragastric intubation contained approximately 20.5% HEAA. After six hours the urine of rats receiving DEG by intravenous administration contained approximately 18% HEAA, and this increased to approximately 32% HEAA at 12 hours following DEG administration. No difference in metabolism occurred based on a comparison of two different routes of administration.

The formation of HEAA in male Wistar Furth rats receiving 3 mmole kg\(^{-1}\) pyrazole four hours prior to the administration of DEG was inhibited by approximately 91%. Pyrazole inhibition of the formation of HEAA lends support to the hypothesis that the first step in the oxidation of DEG is catalyzed by ADH. ADH catalyzed oxidation of DEG should result in the formation of (2-hydroxyethoxy)acetaldehyde.

In an effort to detect (2-hydroxyethoxy)acetaldehyde
Table 15: Metabolism of diethylene glycol in male Wistar rats receiving diethylene glycol by intragastric intubation: Urine analysis.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Rat 1</th>
<th>Rat 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (grams)</td>
<td>515</td>
<td>534</td>
</tr>
<tr>
<td>uCi Recovered</td>
<td>0.39</td>
<td>0.46</td>
</tr>
<tr>
<td>% Dose</td>
<td>39</td>
<td>46</td>
</tr>
<tr>
<td>uCi kg⁻¹</td>
<td>0.76</td>
<td>0.87</td>
</tr>
<tr>
<td>% DEG</td>
<td>79.19</td>
<td>79.78</td>
</tr>
<tr>
<td>% HEAA</td>
<td>20.81</td>
<td>20.22</td>
</tr>
<tr>
<td>uCi DEG</td>
<td>0.31</td>
<td>0.37</td>
</tr>
<tr>
<td>uCi HEAA</td>
<td>0.08</td>
<td>0.09</td>
</tr>
<tr>
<td>uCi kg⁻¹ DEG</td>
<td>0.60</td>
<td>0.69</td>
</tr>
<tr>
<td>uCi kg⁻¹ HEAA</td>
<td>0.16</td>
<td>0.17</td>
</tr>
<tr>
<td>% Dose DEG</td>
<td>31</td>
<td>37</td>
</tr>
<tr>
<td>% Dose HEAA</td>
<td>8</td>
<td>9</td>
</tr>
</tbody>
</table>

Abbreviations: DEG - Diethylene glycol; HEAA - (2-Hydroxyethoxy)acetic acid.
Table 16: Metabolism of diethylene glycol in male Wistar rats receiving diethylene glycol by intragastric intubation and pyrazole by intraperitoneal injection: Urine analysis.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Rat 3</th>
<th>Rat 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (grams)</td>
<td>430</td>
<td>440</td>
</tr>
<tr>
<td>uCi Recovered</td>
<td>0.23</td>
<td>0.07</td>
</tr>
<tr>
<td>% Dose</td>
<td>23</td>
<td>7</td>
</tr>
<tr>
<td>uCi kg⁻¹</td>
<td>0.52</td>
<td>0.15</td>
</tr>
<tr>
<td>% DEG</td>
<td>98.83</td>
<td>97.29</td>
</tr>
<tr>
<td>% HEAA</td>
<td>1.17</td>
<td>2.71</td>
</tr>
<tr>
<td>uCi DEG</td>
<td>0.23</td>
<td>0.07</td>
</tr>
<tr>
<td>uCi HEAA</td>
<td>0.002</td>
<td>0.001</td>
</tr>
<tr>
<td>uCi kg⁻¹ DEG</td>
<td>0.53</td>
<td>0.16</td>
</tr>
<tr>
<td>uCi kg⁻¹ HEAA</td>
<td>0.005</td>
<td>0.002</td>
</tr>
<tr>
<td>% Dose DEG</td>
<td>23</td>
<td>7</td>
</tr>
<tr>
<td>% Dose HEAA</td>
<td>0.2</td>
<td>0.1</td>
</tr>
</tbody>
</table>
Table 17: Metabolism of diethylene glycol in male Wistar rats receiving diethylene glycol by intragastric intubation and diethyldithiocarbamate by intraperitoneal injection: Urine analysis.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Rat 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (grams)</td>
<td>255</td>
</tr>
<tr>
<td>uCi Recovered</td>
<td>0.07</td>
</tr>
<tr>
<td>% Dose</td>
<td>7</td>
</tr>
<tr>
<td>uCi kg⁻¹</td>
<td>0.29</td>
</tr>
<tr>
<td>% DEG</td>
<td>93.05</td>
</tr>
<tr>
<td>% HEAA</td>
<td>6.95</td>
</tr>
<tr>
<td>uCi DEG</td>
<td>0.07</td>
</tr>
<tr>
<td>uCi HEAA</td>
<td>0.005</td>
</tr>
<tr>
<td>uCi kg⁻¹ DEG</td>
<td>0.27</td>
</tr>
<tr>
<td>uCi kg⁻¹ HEAA</td>
<td>0.02</td>
</tr>
<tr>
<td>% Dose DEG</td>
<td>7</td>
</tr>
<tr>
<td>% Dose HEAA</td>
<td>0.5</td>
</tr>
</tbody>
</table>
in rat urine studies were undertaken in which rats were injected intraperitoneally with diethyldithiocarbamate, a potent \textit{in vivo} ALDH inhibitor (Dietrich and Erwin, 1971). Diethyldithiocarbamate is the reduction product of disulfiram \textit{in vivo} (Dietrich and Erwin, 1971). Rats receiving diethyldithiocarbamate exhibited an approximately 66% reduction in the formation of HEAA. Furthermore, it was interesting to note that the appearance of a peak corresponding to (2-hydroxyethoxy)acetaldehyde was not observed. This led one to believe that either (2-hydroxyethoxy)acetaldehyde co-eluted with DEG on the anion exclusion column or that diethyldithiocarbamate also inhibited ADH \textit{in vivo}. The inhibition of ADH by diethyldithiocarbamate has not been reported in the literature. One should remember that in EG metabolism, the ADH reaction is one of two rate limiting steps (Chou and Richardson, 1978) and that glycolaldehyde does not accumulate. Based on this, one would not expect (2-hydroxyethoxy)acetaldehyde to accumulate either.

To determine if ADH was inhibited by diethyldithiocarbamate studies were undertaken in which horse liver ADH was assayed spectrophotometrically in the presence of 1, 2, 3, 4, or 5 umoles of diethyldithiocarbamate with ethanol being used as substrate. Diethyldithiocarbamate at the indicated
concentrations had no effect on the activity of ADH. For this reason that it was believed that (2-hydroxyethoxy)acetaldehyde and DEG co-eluted on the anion exclusion column.

That DEG and (2-hydroxyethoxy)acetaldehyde co-elute would explain the in vitro studies with ADH in which only one radioactive anion exclusion HPLC peak was observed. However, this would fail to explain why only one peak was observed when this reaction was coupled to ALDH, or when a rat liver homogenate was employed. One peak in the ALDH studies and in the rat liver homogenate studies may be due to the fact that the concentration of (2-hydroxyethoxy)acetaldehyde produced was quite low and the activity of ALDH limited.

Based on the results presented here, Figure 23 outlines the oxidative pathway for DEG. DEG is first oxidized by ADH producing (2-hydroxyethoxy)acetaldehyde which is further oxidized by ADLH to HEAA. Both enzymatic reactions require NAD$^+$ as their cofactor. Further oxidation of HEAA to diglycolic acid was not observed. This is in accordance with the results observed by Braun and Young (1977) for HEAA is capable of forming a cyclic compound, as shown in Figure 24, that inhibits further oxidation of HEAA.
Figure 23: Proposed metabolic pathway for the oxidation of diethylene glycol in male Wistar Furth rats.
Figure 24: The cyclization of (2-hydroxyethoxy)acetic acid to 1,4-dioxanone under acid conditions. After Braun and Young (1977) and references therein.
The remaining issue to be discussed when considering the metabolism of DEG is the source of the increased oxalate excretion observed by Durand et al (1976) and by Hebert et al (1978). Since these workers administered unlabeled DEG, a definitive statement as to the source of the calcium oxalate cannot be made. DEG may be capable of augmenting oxalate synthesis from other sources, or the DEG administered by these workers may have been contaminated with EG. The studies reported here demonstrate that the increased oxalate excreted as reported by Durand et al and Hebert et al is not derived from DEG.

To determine if DEG toxicity could be treated using ADH inhibitors and if the products of DEG oxidation contribute to the toxicity of DEG, studies were conducted in which male Wistar Furth rats received either the LD$_{50}$ or 25% above the LD$_{50}$ by intragastric intubation. Pyrazole, an ADH inhibitor, was administered to one half of these animals and their mortality monitored for 48 hours. Tables 18 and 19 report the mortality rates for male Wistar Furth rats weighing between 115 and 150 grams, and between 330 and 480 grams respectively.

Pyrazole was found to protect against the LD$_{50}$ dose of DEG in rats between 115 and 150 grams, although it did not protect those rats receiving 25% above the LD$_{50}$ dosage. The oxidation of DEG produces HEAA which enhances the
Table 18: Mortality study of male Wistar Furth rats (115 - 150 grams) receiving diethylene glycol and pyrazole.

<table>
<thead>
<tr>
<th>Group</th>
<th>Average Weight</th>
<th>Mortality (Dead/Total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>24 Hours</td>
</tr>
<tr>
<td>I</td>
<td>120±6</td>
<td>0/6</td>
</tr>
<tr>
<td>II</td>
<td>132±8</td>
<td>1/6</td>
</tr>
<tr>
<td>III</td>
<td>126±4</td>
<td>0/6</td>
</tr>
<tr>
<td>IV</td>
<td>130±11</td>
<td>1/5</td>
</tr>
</tbody>
</table>

* Average Weight ± Standard Deviation

Groups I and III received the LD$_{50}$ by intragastric intubation. Groups II and IV received 25% above the LD$_{50}$. Groups III and IV received 3 mmol kg$^{-1}$ pyrazole by intraperitoneal injection four hours prior to receiving diethylene glycol.
Table 19: Mortality study of male Wistar Furth rats (330 - 480 grams) receiving diethylene glycol and pyrazole.

<table>
<thead>
<tr>
<th>Group</th>
<th>Average Weight*</th>
<th>Mortality (Dead/Total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>24 Hours</td>
</tr>
<tr>
<td>I</td>
<td>405±33</td>
<td>4/6</td>
</tr>
<tr>
<td>II</td>
<td>382±36</td>
<td>6/6</td>
</tr>
<tr>
<td>III</td>
<td>430±37</td>
<td>1/5</td>
</tr>
<tr>
<td>IV</td>
<td>414±39</td>
<td>4/6</td>
</tr>
</tbody>
</table>

* Average Weight ± Standard Deviation

Groups I and III received the LD$_{50}$ by intragastric intubation. Groups II and IV received 25% above the LD$_{50}$. Groups III and IV received 3 mmol kg$^{-1}$ pyrazole by intraperitoneal injection four hours prior to receiving diethylene glycol.
toxicity of DEG. However, these studies do not indicate if the toxicity is due strictly to HEAA.

Pyrazole did have a slight protective effect in rats weighing between 330 and 480 grams despite the fact that the LD$_{50}$ dosage of DEG was over estimated for this group. The LD$_{50}$ employed was that of Smyth et al (1941) which was reported for male Wistar rats weighing between 90 and 120 grams. Laug et al (1939) reported the LD$_{50}$ to be 14.8 grams kilogram$^{-1}$ for both male and female rats between 175 and 325 grams. It appears that the LD$_{50}$ decreases as the weight of the animal increases. These differences in weight range and sex may explain the differences reported by these two groups and the over estimation of the LD$_{50}$ value for the heavier animals.

Alcohol dehydrogenase inhibition as therapy for DEG toxicity appears to be justified since pyrazole was found to protect against the LD$_{50}$ dose. For pyrazole to be effective for dosages exceeding the LD$_{50}$, multiple treatments with pyrazole may be required. The half-life of pyrazole is 14 hours (Goldstein and Pal, 1971). Since pyrazole is a reversible inhibitor of ADH it may be necessary to give multiple doses to compensate for its elimination and the subsequent oxidation of DEG by ADH since the half-life of DEG is longer than that of EG (Winek et al., 1978). This hypothesis is supported by the
toxicity studies using rats between 330 and 480 grams since the mortality rate for rats receiving the LD$_{50}$ dosage of DEG was 66% at twenty four hours following DEG administration in contrast to those rats receiving pyrazole whose mortality rate was decreased to 20%.

The metabolism and toxicity of DEG was investigated in male Wistar Furth rats using [$^{14}$C]-DEG which was synthesized from [U-$^{14}$C]-EG and ETO and purified by RPHPLC. HEAA was identified as the major product of DEG oxidation. Further oxidation of HEAA to diglycolic acid was not observed. The ether linkage of DEG was apparently not cleaved since no radiolabeled EG, glycolate, oxalate, or carbon dioxide could be detected. The oxidation of DEG was inhibited by pyrazole, an alcohol dehydrogenase inhibitor, and diethyldithiocarbamate, an aldehyde dehydrogenase inhibitor. Pyrazole was found to protect against the toxicity of an LD$_{50}$ dose of DEG. These results suggest that the treatment of DEG poisoning should follow the same regimen as treatment for EG poisoning.


