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OXIDATION OF POLYETHYLENE GLYCOLS BY ALCOHOL DEHYDROGENASE

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Abstract—The present studies were undertaken to investigate the enzymology of a fatal toxic syndrome that resulted from the absorption and subsequent oxidation of polyethylene glycol (PEG). The presence of organic acids of PEG in the blood of poisoned patients and in an animal model suggested that the metabolism of PEG involved sequential oxidations by alcohol dehydrogenase (ADH) and aldehyde dehydrogenase. A key question concerned the ability of ADH to initiate this pathway for oxidation of PEG. In the present studies the oxidation of PEG homologues by ADH was characterized. The polymer homologues of ethylene glycol from n = 1 to n = 8 were used as substrates. ADH catalyzed the oxidation of each of these PEGs. The oxidation of PEG was inhibited by the ADH inhibitor 4-methylpyrazole. With the exception of diethylene glycol, the K_m decreased as the homologue number increased, and the V_{max} decreased progressively through the series. The concentrations of PEG in the blood of poisoned humans and animals were 0.06 to 0.8 K_m of ADH for all the PEG homologues above the triethylene glycol. These investigations establish ADH as a candidate enzyme for mammalian metabolism of PEG and thus suggest that specific inhibitors of ADH may prove to be useful as tools to treat PEG poisoning.

We have described a fatal form of poisoning with polyethylene glycol (PEG); the clinical features of the toxicity are similar but not identical to those seen in poisonings with ethylene glycol [1]. The complete spectrum of findings in the poisoned patients has been reproduced in rabbits [2]. However, the mechanism of polyethylene glycol toxicity is not understood.

A clue to the mechanism of PEG toxicity was the unambiguous demonstration, by tandem quadrupole mass spectrometry, of diacid and hydroxy acid metabolites of PEG in the urine and serum of the PEG-treated rabbits and burn patients [3]. The enzymatic reactions involved in producing these products are unknown. A possible reaction sequence is sequential oxidation of PEG by alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). This sequence of reactions appears important in the metabolism of both ethylene glycol and other alcohols to toxic metabolites [4]. Knowledge of this system has been exploited to treat these poisonings by use of inhibitors of ADH.

The present study was undertaken to determine the ability of ADH to initiate this pathway for oxidation of PEG. Particular attention was directed at the dimer through octamer ethylene glycol homologues, since metabolites of these PEGs were demonstrated to be abundant in the poisoned patients and rabbits [3]. The oxidation of ethylene glycol (monomer) by ADH was studied for comparison to the PEGs.

MATERIALS AND METHODS

Materials. Liver alcohol dehydrogenase (equine), NAD^+, and glycine buffer were obtained from the Sigma Chemical Co., St. Louis, MO. One unit of ADH was defined as the amount of enzyme that will catalyze the oxidation of 1 μmol of ethanol to acetaldehyde per min at 25°. Polyethylene glycols were obtained from the Aldrich or Parish companies, and their purities were established with a gas chromatograph (Hewlett–Packard model 5890, Avondale, PA) using an Ultra 2 capillary column (Hewlett–Packard) temperature programmed from 60° to 300° at 15° per min. Ethylene glycol (99+%), diethylene glycol (99+%), triethylene glycol (99+%), tetraethylene glycol (98.8%), pentaethylene glycol (94%), hexaethylene glycol (98%) and semicarbazide HCl were obtained from the Aldrich Chemical Co., Milwaukee, WI. The heptaethylene glycol (95%) and octaethylene glycol (90%) were obtained from the Parish Co., Orem, UT. The pentaethylene, hexaethylene, heptaethylene and octaethylene glycols were contaminated only with high boiling materials. The tetraethylene glycol contained 1% triethylene and 0.2% pentaethylene glycols.

Apparatus. The assay was performed on an automated spectrophotometer (Cobas-Bio, Roche, Nutley, NJ). The addition of reagents and measurements of absorbance were performed under computer control.

Method. Reactions with glycols were characterized in the following concentration ranges in 0.1 M glycine buffer (pH 9.05): ethylene glycol, 110–1660 mM; diethylene glycol, 82–1230 mM; triethylene glycol, 58–870 mM; tetraethylene glycol, 45–670 mM; pentaethylene glycol, 37–550 mM; hexaethylene glycol, 25–380 mM; heptaethylene glycol, 27–410 mM; and octaethylene glycol, 14–211 mM. Reactions were performed at 37° in 0.1 M glycine, pH 9.05, containing 1.1 mM NAD^+, 8 mM semicarbazide HCl and 700 units/l ADH. The automated spectro-

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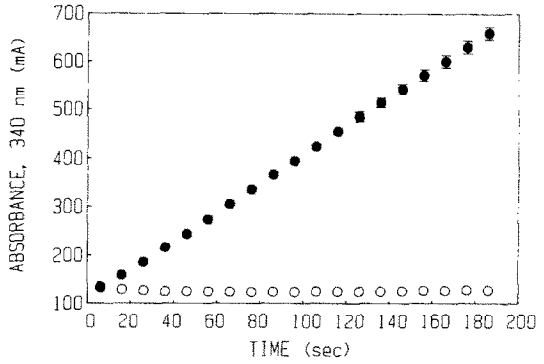

Fig. 1. Time course of ADH-catalyzed oxidation of tetraethylene glycol (●). The reaction was inhibited completely by the inclusion of 4-methylpyrazole, 10 μl/100 ml (○). Absorbances are means of triplicate determinations (100 mA equals 0.10 O.D.). The standard deviations of the means are not shown at most time points because they were smaller than the symbols.

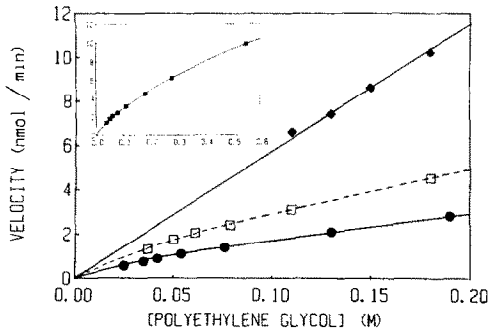

Fig. 2. Reaction velocities of pentaethylene glycol (□), hexaethylene glycol (●) and ethylene glycol (◆) shown as a function of substrate concentration in the range that occurred in the blood of humans and rabbits [3]. The inset shows the velocity versus substrate concentration curve for pentaethylene glycol (■) for the full range of substrate concentrations studied.

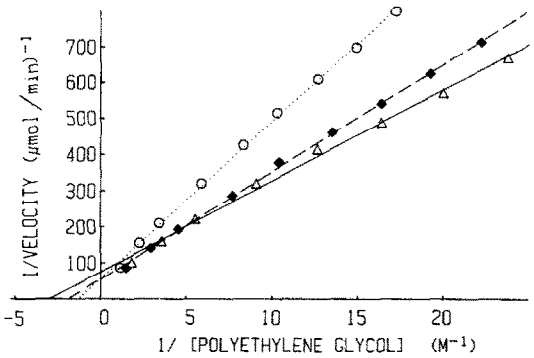

Fig. 3. Double-reciprocal plots of the oxidation of three representative polyethylene glycol homologues. The PEGs used were triethylene glycol (○) tetraethylene glycol (◆) and pentaethylene glycol (△). Each point is the mean of triplicate determinations. The drawn lines were derived by least squares fit of the data and are in agreement with values obtained by the non-parametric procedure used for data analysis.

photometer mixed 80 μl of glycol solution, 15 μl of distilled water and 250 μl of the NAD^+/ADH solution. This mixture without the ADH was used as a blank. The absorbance at 340 nm was measured at 10-sec intervals for 5 min and blank corrected, to determine the initial rate of the reaction. The apparent Michaelis–Menten constants (K_m) and the maximum velocities (V_{max}) were determined using a computer program [5], WILMAN4 (Instructional Media Center, Michigan State University, East Lansing, MI), to calculate K_m and V_{max} from substrate concentration and initial velocities according to a non-parametric procedure [6]. Rather than assume weighting factors, a robust method of parameter estimation which incorporates very few assumptions about the error structure was applied. The derived weighting factors are believed to be the best choice in the absence of detailed error structure analysis [5].

RESULTS

Each of the ethylene glycol homologues was oxidized by alcohol dehydrogenase. Reactions were linear with time as shown in Fig. 1 for tetraethylene glycol. The reaction was blocked by the ADH inhibitor 4-methylpyrazole (Fig. 1). No detectable reaction occurred in the absence of ADH.

Reaction velocities as a function of substrate concentration are shown in Fig. 2 for representative PEGs. Reaction rates were concentration dependent and saturable. Lineweaver–Burk double-reciprocal plots were linear (Fig. 3), indicating that the mechanism of the enzyme-catalyzed reaction was consistent with Michaelis–Menten kinetics.

The kinetics of the ADH-catalyzed reactions were affected markedly by the sizes of the glycols. As shown in Table 1, the experimental conditions allowed reasonably precise estimates of kinetic parameters for most homologues, with coefficients of variation generally less than 10% for K_m and V_{max}. The K_m for all glycols was markedly higher than the K_m for ethanol (Table 1). However, the larger homologues (n > 3) exhibited higher apparent affinities for the enzyme than did the monomer which is thought to be metabolized by ADH *in vivo* [7]. Only an imprecise estimate of K_m could be achieved for the intermediate homologue (diethylene glycol) because the apparent K_m exceeded the highest concentration of diethylene glycol that could be tested (see below). Nonetheless, the estimated K_m was significantly higher than the apparent K_m for any of the other substrates, suggesting a low affinity of ADH for diethylene glycol.

The maximum velocities of the reactions decreased markedly with increasing size of the homologues (Table 1). Under the conditions of the assay, the calculated V_{max} of the reaction with ethylene glycol as the substrate was 2–32 times greater than those with the higher homologues.

DISCUSSION

The present studies indicated that ADH catalyzed

Table 1. Kinetic parameters of alcohol dehydrogenase with ethanol and ethylene glycol homologues as substrate

Substrate	No. of determinations	Substrate concentrations studied (% of K_m)	K_m (mM)	V_{max} (nmol/min)
Ethanol	3	30–500	8 ± 0.6	12 ± 0.4
Ethylene glycol	3	10–170	1000 ± 60	64 ± 1
Diethylene glycol	4	5–70	1900 ± 300	27 ± 5
Triethylene glycol	4	7–115	810 ± 50	19 ± 2
Tetraethylene glycol	3	10–140	490 ± 20	17 ± 1
Pentaethylene glycol	4	10–180	340 ± 20	12 ± 2
Hexaethylene glycol	3	10–140	280 ± 20	6 ± 0.7
Heptaethylene glycol	4	15–210	200 ± 10	3 ± 0.1
Octaethylene glycol	3	15–225	90 ± 10	2 ± 0.1

Values are means ± SD.

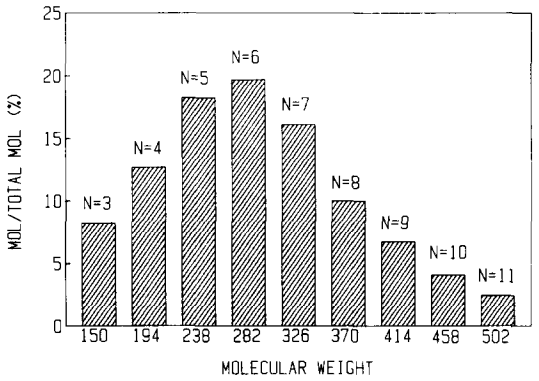

Fig. 4. Composition of the various homologues of PEG-300 ranging from n = 3 through n = 8. The vertical axis indidates the relative molecular contribution of each homologue.

the oxidation of all PEG homologues studied. The homologues n = 1 through 8 were studied because oxidation products of the homologues n = 1 through 7 were found in the serum and urine of patients who exhibited the syndrome of PEG toxicity [3]. This distribution reflected the composition of the PEG mixture to which the patients had been exposed. The patients were burn victims who had been treated with a PEG-based antimicrobial cream. The vehicle for this antimicrobial contained a mixture of three PEG fractions with average molecular weights of 300, 1000 and 4000. The so-called PEG-300 constituted 63% of the total weight of the vehicle or more than 95% of the polyethylene glycol molecules. Figure 4 shows that the homologues n = 3 through 8 accounted for about 85% of the PEG molecules in PEG-300 (data were obtained from a Union Carbide Corp. publication, "Carbowax polyethylene glycols", New York, 1976). The commercial availability of these compounds enabled us to focus our present efforts on the study of these homologues.

The presence of diacid and hydroxy acid metabolites of PEG in the serum and urine of PEG-treated rabbits and burn patients indicated that PEG was oxidized *in vivo*. The oxidation of PEG to these metabolites provided an explanation for the metabolic acidosis, renal failure and other signs of PEG toxicity [1]. However, the enzymes or cofactors responsible for the oxidation of PEG have not been identified. Studies of other alcohols such as ethanol and methanol have indicated the possibility of oxidations by catalase [8] or cytochrome P-450 [9]. However, the major oxidative pathway of primary alcohols *in vivo* is thought to be the alcohol dehydrogenase/aldehyde dehydrogenase pathway [10].

The present studies were designed to characterize accurately the ADH-catalyzed oxidation of PEG in a range that spanned the concentrations observed *in vivo*. Although the most accurate values of K_m are obtained when the reaction velocities are measured at substrate concentrations that span the range from 0.2 to 5 times the K_m value [11], the maximum substrate concentration in these studies was limited to 2% (v/v) to avoid decreases in free water and potential alterations of enzyme conformation at high concentrations of PEG. The effect of this substrate limitation on the determination of V_{max} was minimized by the use of non-parametric calculations and the automated spectrophotometric analysis of the enzyme reaction. The assays described here were performed on a centrifugal spectrophotometric analyzer which allowed the simultaneous analysis of up to twenty-eight samples at constant temperature. Consequently, nine different substrate concentrations could be analyzed in triplicate during one determination. The automated analyzer also ensured precision pipetting, rapid mixing and subsequent multiple absorbance readings over a specified reaction time period. The method provided reasonably precise estimates of kinetic values.

With the exception of diethylene glycol, the K_m values decreased as the homologue number increased. In a study of the Michaelis–Menten constants of equine ADH for unbranched alkane diols, it was similarly found that the K_m values decreased with increasing chain length and with increasing distance between the hydroxyl groups [12]. No studies with PEG were reported. The relatively high K_m found in the present study for diethylene glycol sug-

gests that the ADH has a lower affinity for diethylene glycol than for other homologues. The behavior of diethylene glycol likely reflects the intramolecular hydrogen bonds of diethylene glycol which alter the configuration of the molecule, making it less compatible with the active site of ADH. In addition, the specific distance between the hydroxyl groups of the diethylene glycol may produce a negative interaction with a portion of the active site.

The V_{max} values also decreased with increased chain length. The two most striking changes occurred between ethylene glycol and diethylene glycol and between triethylene glycol and diethylene glycol.

To evaluate the potential role of ADH in PEG toxicity, it is important to consider the reaction rates at PEG concentrations found *in vivo*. Our studies of humans and rabbits poisoned with PEG suggested that PEG was present in blood at concentrations between 30 and 70 mM [1, 2]. These values are 0.06 to 0.77 K_m of ADH found in the present study for all PEG homologues above triethylene glycol. Figure 2 shows reaction velocities for two representative PEGs, pentaethylene glycol and hexaethylene glycol, at low substrate concentrations. The figure also shows, for comparison, velocities for ethylene glycol which is known to be metabolized by ADH *in vivo*. The figure shows that these glycols were oxidized *in vitro* at concentrations that occurred in the blood of the humans and rabbits. Moreover, the reaction velocities were at least 30–50% of those found for ethylene glycol. Thus, ADH is a candidate enzyme for metabolism of PEG *in vivo*. The role of ADH in the *in vivo* metabolism of diethylene glycol is less clear than for the other homologues. In the present study, detectable oxidation of diethylene glycol was observed at 82 mM (the lowest concentration tested). Thus, metabolism of diethylene glycol may be at least partially catalyzed by ADH *in vivo*. Alternatively, enzymes such as catalase may play a more important role in the oxidation of this homologue.

The present studies clearly established alcohol dehydrogenases as candidate enzymes for mammalian metabolism of polyethylene glycols. This finding raises the possibility that poisonings with PEG may be amenable to treatment by inhibitors of ADH. We have shown (Fig. 1) that 4-methylpyrazole markedly inhibited the oxidation of PEG by mammalian ADH. Alternatively, ethanol would appear to be an excellent choice of competitive inhibitor since its apparent K_m is only 0.5 to 9% of the K_m values for PEGs (Table 1). Animal studies of such potentially life-saving therapies appear warranted.

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