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SHORT COMMUNICATIONS

Metabolism of diethylene glycol in male rats

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Various incidents of diethylene glycol (2,2′-oxybisethanol; DEG) poisoning have occurred throughout the world [1–4]. Little is known about the metabolism and the mechanism of toxicity of DEG, a common industrial solvent that has recently been used illegally to sweeten wine. DEG has been suggested [5, 6] to follow the same metabolic pathway as ethylene glycol (EG), although this has not been demonstrated experimentally. Durand *et al.* [5] reported that acute intoxication with DEG in male rats is associated with considerable urinary oxalate excretion. Furthermore, they reported that the excretion of oxalate is decreased significantly by alkalinization of the urine and/or by injection of ethanol. Hebert *et al.* [6] reported the presence of calcium oxalate crystals in renal tubules following acute intoxication by DEG in male rats. Morris *et al.* [7] observed urinary calculi composed primarily of calcium oxalate in male rats following long-term ingestion of DEG which they considered almost conclusive proof that the ether linkage of DEG is broken in the rat and the products so formed are converted to oxalic acid.

Others have reported that calcium oxalate is not formed following DEG intoxication. Brown [8], based on the chemical structure of DEG, stated that the possibility of oxidizing DEG to oxalic acid does not exist. Wiley *et al.* [9] reported that DEG does not increase urinary oxalic acid, supporting the hypothesis that the ether linkage in DEG is not broken, since if it were, EG and, subsequently, glycolic acid and oxalic acid would have been formed. Similar conclusions have been drawn more recently. Balazs *et al.* [10] stated that calcium oxalate crystal deposition is not a feature of DEG nephrotoxicity in either humans or animals. Winek *et al.* [11] showed that the oxalate concentration in rat blood and kidneys after ingestion of EG is much higher than that following ingestion of DEG.

To determine the metabolic pathway for the oxidation of DEG, its mechanism of toxicity, and to resolve the dispute as to whether calcium oxalate is formed from DEG, studies were undertaken in male Wistar rats using radiolabeled DEG.

Methods

[1,2-^{14}C]Diethylene glycol was synthesized from [U-^{14}C]EG (4.7 mCi/mmol; New England Nuclear, Boston, MA) and ethylene oxide (provided by Dr. H. Sprecher) and purified by reversed phase high performance liquid chromatography (RPHPLC) as described previously [12]. Eight male Wistar Furth rats (WF/Hsd Br; 255–535 g; Harlan Sprague–Dawley, Inc., Indianapolis, IN) were divided into four groups. Each received 1.1 g/kg DEG (Fisher) containing 1 μCi [1,2-^{14}C]DEG. Groups I, III, and IV received this chemical by intragastric intubation and Group II by intravenous injection into the caudal vein. In addition, Group III received 3 mmol/kg pyrazole (K & K Laboratories, Inc., Plainview, NY) in normal saline i.p. 4 hr earlier, while Group IV received 4 mmol/kg diethyldithiocarbamate (Eastman) in water i.p. 16 hr earlier. All animals were fasted prior to the start of the experiment, although water was allowed *ad lib.* Each rat was placed in a metabolism chamber allowing for the collection of urine and respiratory $^{14}CO_2$ which was determined as described previously [13]. At the conclusion of the experiment, each rat was killed by ether overdose. The total radioactivity recovered in the urine was determined by liquid scintillation counting; radiolabeled urinary metabolites were determined by anion exclusion HPLC using the procedure of Marshall [14], and the elution profile was monitored using a Flow-One Beta Detector, model IC, and Flow-Scint III (Radiometric Instruments & Chemical Co., Tampa, FL) as the scintillation fluid.

Results and discussion

[^{14}C]Diethylene glycol is not available commercially in the United States, necessitating its synthesis from [U-^{14}C]EG and ethylene oxide. RPHPLC purification of the synthesized product yielded a labeled preparation that coeluted with unlabeled DEG and was radiochromatographically free of any EG as demonstrated by both anion exclusion HPLC and rechromatography by RPHPLC. The radiochemical purity was greater than 99.9%.

Since Durand *et al.* [5] and Hebert *et al.* [6] observed increased urinary oxalate excretion and detected calcium oxalate crystals in renal tubules following acute intoxication by DEG, they hypothesized that DEG is first hydrolyzed to EG which is then oxidized to glycolaldehyde, glycolate, glyoxylate, and oxalate. However, their study did not confirm that DEG was the precursor of the urinary oxalate and kidney oxalate crystals observed. DEG does not appear to be oxidized by the same pathway as EG. In the present study, when [^{14}C]DEG was administered to male rats, no [^{14}C]oxalate could be detected in the excreted urine. The lower limit of detection for the anion exclusion HPLC procedure was approximately 0.25 nCi or 0.025% of the administered dose. Further, no radioactivity could be detected as EG, glycolaldehyde, glycolate, and glyoxylate, indicating that these compounds are not metabolic intermediates of DEG metabolism. This conclusion was supported by the absence of detectable $^{14}CO_2$, a normal product of EG oxidation in the rat [15]. The lower limit of $^{14}CO_2$ detection was approximately 10 nCi or 1% of the administered dose. The absence of radiolabeled EG, glycolaldehyde, glycolate, glyoxylate, and oxalate in the urine of rats receiving [^{14}C]DEG in this study indicates that the ether linkage of DEG was not hydrolyzed in the rat and that the oxalate produced following DEG ingestion, reported by Durand *et al.* [5] and Herbert *et al.* [6], was not derived from DEG. Since they administered unlabeled DEG, a definitive statement as to the source of the calcium oxalate reported in their studies cannot be made. The DEG administered by Durand *et al.* [5] and Hebert *et al.* [6] may have been contaminated with EG, a known precursor of oxalic acid. Since DEG is prepared on the industrial scale by the reaction of EG with ethylene oxide, the possibility of contamination with EG leading to oxalate synthesis would not be unreasonable.

In addition to unreacted DEG, a single radiolabeled peak was observed in the excreted urine by both anion exclusion and reversed phase HPLC; this was identified as (2-hydroxyethoxy)acetic acid (HEAA) since it coeluted with unlabeled HEAA (provided by Dr. A. M. Schumann, Mammalian and Environmental Research Laboratory, Dow Chemical U.S.A., Midland, MI) by both anion exclusion and reversed phase HPLC. Table 1 summarizes the results of the anion exclusion HPLC analysis of urine from rats receiving [^{14}C]DEG. The route of administration

Table 1. Metabolism of diethylene glycol (1.1 g/kg) by male Wistar rats

Group	Route of DEG administration	Pretreatment	Urine radioactivity* Percent DEG	Percent HEAA
I	Intragastric	None	79.5 ± 0.6	20.5 ± 0.6
II	Intravenous	None	80.3 ± 2.8	19.7 ± 2.8
III	Intragastric	Pyrazole	98.1 ± 1.5	1.9 ± 1.5
IV	Intragastric	Diethyldithiocarbamate	93.1 ± 0.6	7.0 ± 0.6

* Distribution of radioactivity recovered in the urine during the first 6 hr after DEG, as determined by anion exclusion HPLC [14]. Results are the average ± the arithmetic range of duplicate analyses of two animals.

did not alter the metabolic profiles. In male rats receiving DEG by intragastric intubation, 43 ± 7% of the administered radioactivity was recovered in their urine during the first 6 hr. For male rats receiving DEG by i.v. injection, 35 ± 5% of the administered radioactivity was recovered in the urine during the first 6 hr and 46% was recovered after 12 hr. After 6 hr approximately 20% of the radioactivity recovered in the urine was present as HEAA; this increased to approximately 32% at 12 hr following DEG administration.

Although EG and its metabolites are not intermediates in DEG metabolism, the same or similar enzymes appear to be involved in both pathways. The postulated pathway for the oxidation of DEG is presented in Fig. 1. DEG is first oxidized by alcohol dehydrogenase producing (2-hydroxyethoxy)acetaldehyde which is rapidly oxidized by aldehyde dehydrogenase to HEAA in a four electron oxidation process. Both enzymatic reactions require NAD as their cofactor. The formation of HEAA in male Wistar rats receiving pyrazole, an alcohol dehydrogenase inhibitor, was decreased by approximately 91% ($P < 0.01$). Treatment with diethyldithiocarbamate, at a dose reported to inhibit aldehyde dehydrogenase by 75% [16], decreased HEAA production by approximately 66% ($P < 0.001$).

Further oxidation of HEAA to diglycolic acid was not observed. This is consistent with the work of Braun and Young [17] who reported the presence of radiolabeled HEAA and DEG in the urine of rats following the administration of [U-^{14}C]-1,4-dioxane and suggested that HEAA does not undergo further oxidation since, under acid conditions, HEAA forms a cyclic compound, 1,4-dioxanone, that is not oxidized by alcohol dehydrogenase and aldehyde dehydrogenase.

The toxicity of EG is not due to EG *per se*, but to the metabolic products of its oxidation, specifically glycolic acid [15]. To determine if the toxicity of DEG were due to its oxidation products and could, therefore, be treated using alcohol dehydrogenase inhibitors, studies were conducted in which male rats received pyrazole and the LD_{50} dosage of DEG. Pyrazole pretreatment was found to lessen the lethality of an LD_{50} dose of DEG in male Wistar rats (data not shown), indicating that the HEAA produced from DEG contributes to its toxicity.

In summary, this study investigated the metabolism of DEG in male Wistar Furth rats using [1,2-^{14}C]DEG which was synthesized from [U-^{14}C]EG and ethylene oxide and purified by reversed phase high performance liquid chromatography. 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,

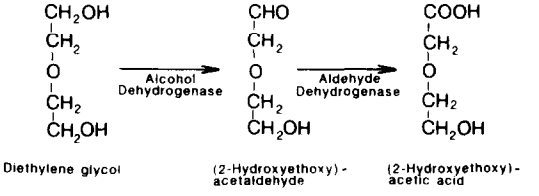

Fig. 1. Proposed metabolic pathway for the oxidation of diethylene glycol.

glycolaldehyde, glycolate, glyoxylate, oxalate, or carbon dioxide could be detected. The oxidation of DEG in male rats was inhibited by pyrazole, an alcohol dehydrogenase inhibitor, and diethyldithiocarbamate, an aldehyde dehydrogenase inhibitor. These results suggest that the treatment for DEG poisoning should follow a regimen similar to the current treatment for EG poisoning, employing alcohol dehydrogenase inhibitors.

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Inhibition of human dihydrofolate reductase by antifolyl polyglutamates

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Methotrexate (MTX*), 10-deazaaminopterin (10-DAM), 10-ethyl-10-deazaaminopterin (10-EDAM) and aminopterin (AMT) are potent inhibitors of dihydrofolate reductase (DHFR; EC 1.5.1.3) [1, 2]. Polyglutamate derivatives of these analogs have a prolonged intracellular half-life [3, 4] and have an increased affinity for folate requiring enzymes such as thymidylate synthase (EC 2.1.1.45) [5] and aminoimidazolecarboxamide ribonucleotide formyltransferase (EC 2.1.2.3) [6]. Folate substrates also exist intracellularly as polyglutamates, and many folate enzymes show a higher affinity for folate polyglutamate substrates than for their monoglutamate counterparts [5]. While binding studies in cell extracts indicate that MTX polyglutamates have an affinity for DHFR as high [3, 7, 8] or higher [9, 10] than MTX, the effect of antifolylpolyglutamates on mammalian DHFR activity has not been studied extensively. However, increasing inhibition of DHFR from sheep, beef and chicken liver is observed as the MTX glutamate chain is lengthened [11]. Another indication that polyglutamate chain length is a factor in DHFR activity is that, when $H_2PteGlu_5$ is substituted for $H_2PteGlu_1$ as substrate, the inhibitory potency of MTX derivatives is decreased [11]. The effect of polyglutamylation on the inhibitory potency of MTX is much less pronounced for *Lactobacillus casei* DHFR [12] than for animal DHFRs [11].

We report here on the inhibitory potency of polyglutamates of MTX, 10-DAM, 10-EDAM and AMT for pure human DHFR using $H_2PteGlu_1$ or $H_2PteGlu_5$ as substrate. We show that the polyglutamate chain length of both inhibitor and substrate are determinants of human DHFR inhibition.

Materials and methods

Poly-gamma-glutamyl derivates of folate and folate analogs were synthesized by the solid phase method [13]. All were analyzed by HPLC [11] to verify their authenticity and purity. None of the compounds showed significant contamination. $H_2PteGlu_5$ was prepared from $PteGlu_5$ by dithionite reduction [14]. The concentrations of $H_2PteGlu_1$ and $H_2PteGlu_5$ were determined by their absorbance at 282 nm in 0.2 M 2-mercaptoethanol using a molar absorbance coefficient of 19,000 [15]. Both substrates were stoichiometrically reduced to tetrahydro forms in the presence of DHFR. The concentrations of antifolates and antifolate polyglutamates were determined by their absorbance at 256 nm in 0.1 N KOH. The molar absorbance coefficients used were 23,000, 28,500, 33,000 and 31,000 for MTX [15], AMT [15], 10-DAM [13] and 10-EDAM [13] respectively.

The DHFR reaction was monitored in a Gilford model 250 spectrophotometer at 30° using the decrease in absorbance at 340 nm that occurs when NADPH and $H_2PteGlu_1$ are converted to $NADP^+$ and $H_4PteGlu_1$. The incubation mixture contained: NADPH, 0.14 mM; $H_2PteGlu_1$ or $H_2PteGlu_5$, 0.1 mM; Tris–HCl buffer, pH 7.2, 50 mM. The reactions were initiated by addition of an amount of enzyme sufficient to obtain a change of absorbance of 0.027/min. NADPH, $H_2PteGlu_1$ and $H_2PteGlu_5$ were present at saturating concentrations. Control rates obtained with $H_2PteGlu_1$ and $H_2PteGlu_5$ were the same. The reaction velocities obtained at various inhibitor concentrations were plotted as described [11] to determine the IC_{50} values.

Human DHFR was obtained by recombinant DNA techniques using an overproducing plasmid vector carried by *Escherichia coli* [16]. The K_m values for NADPH and dihydrofolate, the isoelectric point, and the N-terminal amino acid sequence for this preparation were in close agreement with those of the enzyme isolated from WIL2/M4 human lymphoblastoid cells [17].

Results and discussion

Polyglutamylation of MTX, 10-DAM, 10-EDAM and AMT resulted in a progressive decrease in IC_{50} values (Fig. 1) with $H_2PteGlu_1$ as substrate. For example, the IC_{50} values decreased 2-,2-,3- and 2-fold when comparing the Glu_1 forms to the Glu_4 forms for MTX, 10-DAM, 10-EDAM and AMT respectively. The largest overall effect was seen in the 10-DAM series where the IC_{50} decreased 5-fold

* Abbreviations: MTX, methotrexate; DHFR, dihydrofolate reductase; AMT, aminopterin; 10-DAM, 10-deazaaminopterin; 10-EDAM, 10-ethyl-10-deazaaminopterin; and $PteGu_1$, folic acid with subscript numeral indicating the total number of Glu residues.