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DESCRIPTIVE SUMMARY WITH CONCLUSIONS:

Previous research suggests that ethylene glycol (EG) developmental toxicity is due to a dose-rate dependent toxicokinetic shift leading to glycolate accumulation and metabolic acidosis. This study was conducted to determine the relative roles of these two factors *in vivo*. In Part I, carotid artery cannulated pregnant rats received 2500 mg/kg (40.3 mmol/kg) of EG or 650 mg/kg (8.5 mmol/kg) of free glycolic acid (GA) via gavage, or 833 mg/kg (8.5 mmol/kg) of sodium glycolate (NaG; pH 7.4) via subcutaneous (SC) injection. The EG dose was chosen to generate equimolar amounts of glycolate, based on an EG to glycolate conversion rate of 21% (Marshall, 1982). Following exposure, peak serum glycolate levels were nearly identical in all three groups (8.4-8.8 mmol/L). Glycolate area-under-the-curve (AUC) values also were similar for GA and NaG, but were three-fold higher in the EG group. EG and GA caused a clear, but mild, metabolic acidosis, while acid-base status was normal with NaG. In Part II, these three treatments were given on gd 6-15 to groups of 25 time-mated rats, followed by fetal evaluation on gestation day 21. A fourth group of rats was given distilled water via gavage and served as vehicle controls. NaG caused decreased fetal body weights and increased incidences of several minor skeletal variations, effects characteristic of the lowest-observable-effect level (LOEL) observed in previous developmental toxicity studies with EG (Yin et al., 1986; Neepor-Bradley et al., 1995). This result with NaG indicates that the glycolate anion is sufficient to cause developmental toxicity, even in the absence of metabolic acidosis. However, a much greater incidence and severity of fetal effects (including malformations) was observed with GA, indicating that metabolic acidosis is a major exacerbating factor leading to *in vivo* teratogenesis following large oral bolus exposures to EG. Finally, EG and GA both caused qualitatively similar (often identical) axial skeleton and abdominal wall defects, as well as dilated cerebral ventricles, providing further evidence that glycolate is the proximate developmental toxicant for EG. The only effects which were unique to the EG group were several external malformations involving the cranial neural tube and cranio-facial region. Considering that neural tube and cranio-facial effects were observed in a prior whole embryo culture with GA, but not with EG (Carney et al., 1996), the neural tube and cranio-facial effects found in this study were likely due to the three-fold higher glycolate AUC value seen in the EG group.

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Title: ETHYLENE GLYCOL DEVELOPMENTAL TOXICITY:
MECHANISTIC STUDY ON THE ROLE OF GLYCOLATE ANION AND
METABOLIC ACIDOSIS

All phases of this study were conducted in compliance with the Good Laboratory Practice Standards listed below, with the following exceptions:

Test material characterization (provided by commercial suppliers) was not audited for GLP compliance by The Dow Chemical Company.
Dose solutions were not analyzed in the preliminary (Part I) phase of this study.

Food and Drug Administration, Good Laboratory Practice Regulations for NonClinical Studies, Title 21 CFR, Part 58, Final Rule.

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Study Title

ETHYLENE GLYCOL DEVELOPMENTAL TOXICITY: MECHANISTIC STUDY
ON THE ROLE OF GLYCOLATE ANION AND METABOLIC ACIDOSIS

Data Requirement

There are no specific data requirements for this study.

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Study Completion Date

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QUALITY ASSURANCE STATEMENT

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METABOLIC ACIDOSIS

This study was examined for conformance with Good Laboratory Practices as published by the U.S. Environmental Protection Agency. The final report was determined to be an accurate reflection of the data obtained. The dates of Quality Assurance activities on this study are listed below.

Study Initiation Date: 2 October, 1995

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Protocol, study conduct	12 October, 1995	12 October, 1995
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SUMMARY

Previous research suggests that ethylene glycol (EG) developmental toxicity is due to a dose-rate dependent toxicokinetic shift leading to glycolate accumulation and metabolic acidosis. This study was conducted to determine the relative roles of these two factors *in vivo*. In Part I, carotid artery cannulated pregnant rats received 2500 mg/kg (40.3 mmol/kg) of EG or 650 mg/kg (8.5 mmol/kg) of free glycolic acid (GA) via gavage, or 833 mg/kg (8.5 mmol/kg) of sodium glycolate (NaG; pH 7.4) via subcutaneous (SC) injection. The EG dose was chosen to generate equimolar amounts of glycolate, based on an EG to glycolate conversion rate of 21% (Marshall, 1982). Following exposure, peak serum glycolate levels were nearly identical in all three groups (8.4-8.8 mmol/L). Glycolate area-under-the-curve (AUC) values also were similar for GA and NaG, but were three-fold higher in the EG group. EG and GA caused a clear, but mild, metabolic acidosis, while acid-base status was normal with NaG. In Part II, these three treatments were given on gd 6-15 to groups of 25 time-mated rats, followed by fetal evaluation on gestation day 21. A fourth group of rats was given distilled water via gavage and served as vehicle controls. NaG caused decreased fetal body weights and increased incidences of several minor skeletal variations, effects characteristic of the lowest-observable-effect level (LOEL) observed in previous developmental toxicity studies with EG (Yin et al., 1986; Neepser-Bradley et al., 1995). This result with NaG indicates that the glycolate anion is sufficient to cause developmental toxicity, even in the absence of metabolic acidosis. However, a much greater incidence and severity of fetal effects (including malformations) was observed with GA, indicating that metabolic acidosis is a major exacerbating factor leading to *in vivo* teratogenesis following large oral bolus exposures to EG. Finally, EG and GA both caused qualitatively similar (often identical) axial skeleton and abdominal wall defects, as well as dilated cerebral ventricles, providing further evidence that glycolate is the proximate developmental toxicant for EG. The only effects which were unique to the EG group were several external malformations involving the cranial neural tube and cranio-facial region. Considering that neural tube and cranio-facial effects were observed in a prior whole embryo culture with GA, but not with EG (Carney et al., 1996), the neural tube and cranio-facial effects found in this study were likely due to the three-fold higher glycolate AUC value seen in the EG group.

INTRODUCTION

Background. It is well known that large oral doses of EG in rats and mice can lead to systemic and developmental toxicity. In contrast, potential human exposures from typical industrial and consumer uses of EG are more likely to involve the dermal or inhalation routes. In order to address questions regarding the developmental toxicity potential of dermal and inhalation exposures to EG, a working hypothesis (Fig. 1) was developed by the EG Toxicology Research Task Group of the Chemical Manufacturers Association, which integrates the animal developmental toxicity data with the extensive data base describing the absorption, metabolism, pharmacokinetics and mechanisms of systemic toxicity for EG (Carney, 1994).

One of the key tenets of the model is that developmental toxicity is caused by a metabolite of EG, called glycolic acid (GA), and not parent EG. Under most conditions of EG exposure the GA metabolite is present in the blood at very low levels. However, it can become a major metabolite following large doses of EG due to saturation of GA oxidation and/or elimination (Marshall, 1982; Frantz et al., 1996). When levels of this acidic metabolite exceed the capacity of maternal blood buffers to neutralize it, a maternal metabolic acidosis ensues, which has been hypothesized to be the true agent responsible for EG-induced developmental toxicity (Khera, 1991).

The focus on GA and acidosis in the model is based on the following experimental evidence:

- (1) The oral dose of EG at which GA kinetics begin to shift corresponds with the lowest-observable-effect level (LOEL) for developmental toxicity (Marshall, 1982; Nepper-Bradley et al., 1995; Frantz et al., 1996).
- (2) Doses given rapidly are much more effective than similar doses administered over longer time periods, again suggesting that saturation kinetics are important. For example, a gavage dose of 750 mg/kg/day in CD-1 mice resulted in significant developmental toxicity, while a slightly higher dose (800 mg/kg/day) of EG given in drinking water for 14 weeks was a no-effect level.

In rats, 1000 mg/kg/day by gavage resulted in an increased incidence of axial skeleton malformations, but when this same dose was administered through the animals' feed, developmental effects were limited to delayed ossification of vertebral centra (Text Table 1).

- (3) Co-administration of oral sodium bicarbonate with EG almost entirely prevented metabolic acidosis and also partially ameliorated many developmental effects (Khera, 1991).
- (4) In rat whole embryo culture, 12.5 mM GA (at pH 6.7) induced abnormal embryo development, whereas parent EG at up to 50 mM had little effect on embryo development (Carney et al., 1996).
- (5) GA has recently been shown to cause developmental toxicity consistent with EG in rats (Munley, 1996).

TEXT TABLE 1. Summary of development and maternal toxicity studies with EG¹

Species	Developmental		Fetal effects at LOEL
	NOEL	LOEL	
<i>Oral - gavage (mg/kg/day):</i>			
Mouse	n.d.	11,090	Decr. number of viable litters
Mouse	n.d.	750	Decr. body wts, axial skel. malforms.
Mouse	150	500	Extra 14 th rib (variation)
Rat	n.d.	1250	Axial skel. and viscera ^l malforms.
Rat	500	1000	Decr. body wts, axial skel. malforms./ vars.
Rat	638	858	Decr. body wts, sternum anomalies
Rabbit	2000	n.d.	None
<i>Oral - feed/ drinking water (mg/kg/day):</i>			
Mouse	800	1600	Decr. body wts, facial, skull and toe anomalies
Rat	1000	n.d.	Delayed, unossified vertebral centra
<i>Inhalation (mg/m³):</i>			
Mouse-whole body	150	1000	Decr. body wts, axial skel. And facial malforms.
Mouse-nose only	1000	2500	Decr. body wts, fused ribs, skel. vars.
Rat-whole body	150	1000	Decr. Skel. ossification
<i>Dermal (mg/kg/day):</i>			
Mouse	3549	n.d.	Decr. ossification-parietals, hindlimb phalanges - only effects noted at 3549 mg/kg

¹References cited within Carney (1994).

n.d. = not determined; vars. = variations, malforms.=malformations

The above results clearly indicate that GA kinetics are an important determinant for EG-induced developmental toxicity. However, the role of metabolic acidosis in such toxicity is not as certain. In the rat whole embryo culture study mentioned above (Carney et al., 1996), 12.5 mM sodium glycolate at pH 7.4 caused 58% of the embryos to be malformed, which was similar to the 67% malformation rate for embryos cultured in 12.5 mM GA at acidic pH (pH 6.7). However, only 8% of the embryos cultured in control medium acidified with HCl to pH 6.7 were malformed. Several possibilities are available to explain this *in vitro* result in light

of Khera's (1991) *in vivo* EG/bicarbonate study. First, GA could be the true developmental toxicant, but because it is a weak acid, bicarbonate treatment could have altered its ionization and thus decreased its transfer across the yolk sac/placenta. Alternatively, metabolic acidosis could still be involved through effects on the placenta, as suggested by Khera (1991), or through other physiological parameters which are altered in acidosis (e.g. PCO_2 , HCO_3^- , PO_2 , lactate). In this regard, it is important to note that the placenta is not represented in whole embryo culture, nor does simply lowering medium pH adequately mimic the complex changes which characterize metabolic acidosis *in vivo*.

Objective. This study addressed the question of whether developmental toxicity following high dose exposure to EG *in vivo* was caused by (1) an intrinsic toxicity of the glycolate anion, (2) metabolic acidosis or (3) an additive effect of the two.

Statement of GLP Compliance. Although this is a mechanistic study for which no specific regulatory guidelines exist, the study was conducted in accordance with the Food and Drug Administration Good Laboratory Practice Regulations for NonClinical Studies (Title 21 CFR, Part 58, Final Rule), the Environmental Protection Agency TSCA Good Laboratory Practice Standards (Title 40 CFR, Part 792, Final Rule), the Organisation for Economic Co-Operation and Development Principles for Good Laboratory Practice (European Economic Community, Council Directive 87/18 EEC), the Japan Ministry of International Trade and Industry, Good Laboratory Practice Standards Applied to Industrial Chemicals and the Standard Operating Procedures of The Toxicology Research Laboratory of The Dow Chemical Company.

In addition, in response to the Final Rules amending the U.S. Animal Welfare Act that were promulgated by the U.S. Department of Agriculture effective October 30, 1989, the Animal Care and Use Activity (ACUA) that was required for the conduct of this study were reviewed and given full approval by the Institutional Animal Care and Use Committee (IACUC). The IACUC determined that the proposed Activity was in full accordance with these Final Rules. The IACUC assigned Activity No. Reproductive Toxicology 02, Metabolism 02 and Animal ID 01 to this Animal Care and Use Activity.

MATERIALS AND METHODS

Test Materials:

Ethylene glycol (EG)

Formula: HO-CH₂-CH₂-OH

Formula weight: 62.07

CAS 107-21-1

Source: Aldrich Chemical Company

Lot: 07053JZ

Purity: 99.98%

Glycolic acid (GA)

Formula: HO-CH₂-COOH

Formula weight: 76.05

CAS 79-14-1

Source: Aldrich Chemical Company

Lot: 06608MZ

Purity: 99.7%

Sodium glycolate (NaG)

Formula: HO-CH₂-COO⁻ Na⁺

Formula weight: 98.03

CAS 2836-32-0

Source: Pfaltz & Bauer, Inc.

Lot: S05530

Purity: ≥ 98%

Purity data was limited to that provided by the commercial supplier and was not analyzed further by the testing laboratory.

Test Animals. Time-mated CD® (Sprague-Dawley derived) rats were obtained from Charles River Breeding Laboratories (CRBL; Portage, MI). The CD® rat was selected as a test species based on its general acceptance for developmental toxicity testing, the availability of a reliable commercial source, the availability of historical control data and previous EG developmental toxicity and acid/base studies using this species/strain.

Adult virgin female CD rats, approximately 10 weeks old and weighing approximately 180 - 300 grams (Part I) or 205 - 245 grams (Part II) were naturally mated with one male of the same strain at CRBL. Females were checked for vaginal plugs the following morning and those found with plugs were removed from the males' cage. The day on which a vaginal plug was detected was considered day 0 of gestation.

Upon receipt in the laboratory¹, all animals were examined for health status by a veterinarian. Only healthy animals were used for the study. The animal rooms of the facility were designed to maintain humidity at approximately 40-70%, temperature at approximately 22 °C, photoperiod at 12 hrs light:12 hrs dark and air flow at 10 changes/hour.

Rats used in Part I of the study (preliminary blood glycolate/acid-base study) were randomized on gestation day 10 using a random numbers table, while those in the main study were randomized based on gestation day 0 body weights (provided by CRBL) using a computer-generated procedure designed to increase the probability of uniform body weights across treatment groups. In the preliminary study, the rats were housed in plastic cages provided with ground corn cob bedding material, while rats used for Part II of the study (main study) were housed individually in wire bottom cages. All rats were identified using uniquely coded alphanumeric metal ear tags. Certified Laboratory Rodent Chow No. 5002, Purina Mills, Inc. (St. Louis, MO) and municipal tap water were available *ad libitum*. Analysis of the chow was performed by Purina Mills, Inc. to confirm that the diet provided adequate nutrition and to quantify the levels of selected contaminants associated with the formulation process. Drinking water obtained from the City of Midland was analyzed for chemical constituents and biological contaminants by the City of Midland Water Department. In addition, specific analyses for chemical contaminants were conducted at periodic intervals by an independent laboratory as stated in the Standard Operating Procedures of the Toxicology Research Laboratory, The Dow Chemical Company. The results of the feed and water analyses indicated no contaminants at levels that would interfere with the conduct of this study or interpretation of the results.

Compound Preparation and Administration. EG, GA and NaG were prepared as aqueous solutions in deionized water. GA was prepared as the free acid with no adjustment of pH, while solutions of NaG were titrated to approximately pH 7.4 using HCl. No pH adjustments were made to the EG solutions. In Part I, EG and GA were administered via gavage, such that a dose volume of 10 ml/kg body

¹ Fully accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC).

weight yielded the appropriate dose. NaG was administered via subcutaneous (SC) injection using a dose volume of 10 ml/kg body weight. Subcutaneous injections were made in the mid-dorsal region. Part II exposures were conducted as in Part I, except that the dose volume was 4 ml/kg body weight. This reduction in dose volume was done to preclude any problems with repeated SC injections.

The dose solutions used in Part I were not analyzed. However, solutions used in Part II were analyzed prior to the start of dosing to confirm that the targeted concentrations were achieved and at the end of dosing to confirm stability.

Rationale for routes of exposure. Gavage administration was chosen as the route of EG exposure based on previous studies (Table 1) demonstrating the effectiveness of this route for induction of metabolic acidosis and terata. GA given by gavage was also anticipated to cause metabolic acidosis. A pH 7.4 solution of NaG (dose equimolar to GA) was given via subcutaneous injection in order to generate equivalent blood glycolate concentrations, but *without* inducing metabolic acidosis. Through application of the Henderson-Hasselbach equation, one can see that theoretically >99.97% of the glycolate would remain in the ionized form and only <0.03% of the dose would be present as the free acid if NaG were to be administered at a neutral pH site such as the subcutaneous space. Thus, an insignificant amount of acid would be added to the system. Administration of NaG via gavage would not be desirable because the acidic stomach environment would shift the ionization of NaG toward the free acid, potentially increasing the amount of free acid absorbed into the systemic circulation. Calculations regarding the ionization of GA are shown below:

$$\begin{aligned}
 \text{pH} &= \text{pK}_a + \log \frac{[\text{A}^-]}{[\text{HA}]} && \text{where pK}_a \text{ of GA} = 3.83 \\
 7.4 &= 3.83 + \log \frac{[\text{A}^-]}{[\text{HA}]} && [\text{A}^-] = \text{glycolate concentration,} \\
 3.57 &= \log \frac{[\text{A}^-]}{[\text{HA}]} && [\text{HA}] = \text{glycolic acid concentration} \\
 3715 &= \frac{[\text{A}^-]}{1} \\
 & && 1 \quad [\text{HA}] \\
 \\
 \% \text{ as A}^- &= \frac{[\text{A}^-]}{[\text{A}^-] + [\text{HA}]} \times 100 = \frac{3715}{(3715 + 1)} \times 100 = 99.97\%
 \end{aligned}$$

Experimental Design

1. Part I - Preliminary blood glycolate and acid/base homeostasis study.

Preliminary work was necessary to establish if the dosing strategies described above accomplished the desired objectives and to make any adjustments in dosing regimens if necessary. This was done using cannulated pregnant rats as follows:

Cannulation and dosing. Time-mated rats were implanted with a chronic carotid artery cannula on gestation day 8. The carotid artery was chosen based on Khera's (1991) previous acid-based homeostasis study and the fact that arterial sample is generally preferred for blood pH and blood gas determinations (Brun-Pascaud et al., 1982). Enough rats were prepared to yield at least five rats/group with patent catheters during the majority of sample time points. Under methoxyflurane anesthesia, the right common carotid artery was isolated, clamped and incised with a fine scalpel blade. A cannula containing heparinized saline was then inserted approximately 15 mm into the vessel and ligated in place. The cannula was then tunneled through the subcutaneous space and exteriorized through an incision in the nape of the neck. The distal end of the cannula was fitted with a small length of flexible medical tubing and sealed with a copper wire plug. In the first few rats used in the study, the cannula was composed of polyethylene tubing. Due to problems with blocked cannulae, a switch was made to a softer and more pliable material (Silastic) in an effort to reduce irritancy to the arterial wall and thus improve patency. The rats were allowed to recover for two days following surgery. The cannulae did not interfere with normal mobility of the rats and they were periodically flushed with heparinized saline in an effort to maintain patency.

On gestation day 10, all rats with a patent cannula were randomly allocated into one of four groups until there were a total of five rats/group:

- (1) deionized water via gavage
- (2) 2500 mg/kg (40.3 mmol/kg) of EG via gavage
- (3) 650 mg/kg (8.5 mmol/kg) of GA via gavage

(4) 833 mg/kg (8.5 mmol/kg) of NaG via subcutaneous injection

The EG dose was shown to induce terata in previous studies (reviewed in Carney, 1994), while the GA and NaG doses were based on published pharmacokinetic data in rats indicating that 21% of a 2000 mg/kg I.V. bolus dose of EG is converted to GA (see Marshall, 1982).

Blood sampling. Blood samples (200-300 μ l) were withdrawn from the cannulae into 1 cc syringes at 0 (pre-treatment), 1, 3, 6, 9 and 24 hours post-dosing. These time points were based on the fact that peak plasma glycolate levels and maximum acid/base disturbance were achieved at approximately 4-6 hours post-EG (Hewlett et al., 1989; Khera, 1991; Frantz et al., 1996). The first 200 μ l of blood was transferred into Baxter heparinized blood gas analysis capillary tubes and were used for analysis of acid/base parameters. The remainder of the sample was transferred to a microcentrifuge tube and stored at approximately 4°C to allow the blood to clot. The samples were then centrifuged and the serum was removed and used for serum glycolate analysis.

Acid/base parameters. After each sample collection, the capillary tube was immediately sealed to prevent sample contact with air and was placed on ice until analysis. The sample was transported to the MidMichigan Regional Medical Center (Midland, MI) and analyzed on a Corning Model 278 blood gas analyzer within 90 minutes of collection. The analyzer reports data for pH, PCO₂, PO₂, oxygen saturation, bicarbonate and base excess. Preliminary methods development work verified the stability of the acid-base determinations for up to 90 minutes post-sampling.

Blood glycolate analysis. Blood serum was stored at -80° C for subsequent analysis for total glycolate concentration (i.e., the method quantitated the sum of both ionized and non-ionized forms). Serum samples were thawed to room temperature, then mixed briefly using a vortex mixer. An aliquot (25- μ l) of each serum sample was placed in a vial and 0.5 ml of 0.1 N o-phosphoric acid in acetonitrile (approximately pH 1.5) was added to convert glycolate ions present to the protonated glycolic acid form for analysis. The samples were

extracted with the acidified acetonitrile for 15 minutes using an automated vortex mixer. The samples were then placed in a centrifuge for 5 minutes at 3000 rpm. The acetonitrile supernatant extracts were analyzed for total glycolate concentration (detected as glycolic acid) by reversed-phase high pressure liquid chromatography (HPLC) using ultraviolet detection (UV) at 200 nm. The analytical column employed was a 250 x 4.6 mm YMC ODS-AQ column (YMC, Inc. Wilmington, DE) with a corresponding guard column cartridge. The mobile phase was 0.02 M monobasic sodium phosphate (aq.) adjusted to pH 2.8 with o-phosphoric acid at a flow rate of 0.5 ml/min. Sample injection size was 50 microliters.

External standards, prepared in extraction solvent and bracketing expected study sample concentrations, were used to quantitate the glycolic acid peak in the sample extracts. The mean response factor for the UV response in the linear portion of the response curve was used to calculate results. Fortified serum samples (spikes) were prepared covering the expected concentration range of the study samples. They were analyzed with each sample set to determine recovery of glycolic acid. The recovery was non-linear over the concentration range of the study samples; study sample concentrations were adjusted for spike recovery using the recovery factor derived from the spike that most closely matched sample peak area.

Animal observations, body weights and disposition. Animals were observed daily for alterations in behavior or demeanor. Body weights were taken on gestation day 8, 10 and 11. Feed consumption was not determined. Following collection of the last blood sample on gestation day 11, the rats were sacrificed via CO₂ inhalation, cannula placement was verified, and the uterine implantation sites were examined grossly. Animals found not pregnant were excluded from the analyses. A gross necropsy was performed by study personnel for any animal which died or appeared moribund prior to the scheduled sacrifice.

Data evaluation and interpretation. Means and standard deviations were calculated for serum glycolate concentrations, arterial pH, PCO₂, and bicarbonate. The serum glycolate concentration time curve was then plotted

on a log scale to determine peak concentration (C_{max}) and time to peak concentration (T_{max}). Area under the serum glycolate concentration curve (AUC) was calculated using the trapezoidal rule, and elimination constant (K_{elim}) and elimination half-life ($t_{1/2\ elim}$) estimates were derived from slope analyses as described by Gibaldi and Perrier (1982).

Part I study data were submitted to the Sponsor upon completion. After reviewing these data, approval to proceed with Part II of the study was granted.

2. Part II - Developmental toxicity study

Groups of 25 (non-cannulated), time-mated CD rats were exposed on gestation day 6-15 to one of the following: (1) deionized water via gavage (2) 650 mg/kg/day of GA via gavage (3) 833 mg/kg/day of NaG via SC injection or (4) 2500 mg/kg/day of EG via gavage. These dosing regimens were based on the results of Part I.

All animals were observed daily during the study for alterations in behavior or demeanor. Any animal which died, appeared moribund or showed indications of early termination of pregnancy was submitted for a gross pathological examination conducted by a veterinary pathologist. Body weights were recorded on day 0 (provided by CRBL), 6-16, 19 and 21 (terminal body weight) of gestation. Dose volumes were adjusted daily based on body weights. Feed consumption was determined at 3-4 day intervals beginning on day 3 of gestation.

On day 21 of gestation, all surviving animals were euthanized by carbon dioxide inhalation and given a limited necropsy. Any obvious structural or pathologic changes noted in the adult were recorded and the weights of the liver, kidneys and gravid uteri were recorded. The uterus with attached placentae (fetuses removed), liver, kidneys and any gross lesions were preserved in neutral, phosphate-buffered 10% formalin, but microscopic examination of tissues was not conducted.

Fetal Observations. At necropsy, the uterine horns were exteriorized through an abdominal incision and the following data were recorded: 1) the number and position of fetuses *in utero*, 2) the number of live and dead fetuses, 3) the number and position of resorptions, 4) the number of corpora lutea, 5) the sex and body weight of each fetus, and 6) any gross external alterations. The uteri of animals which appeared non-pregnant were stained with a 10% aqueous solution of sodium sulfide (Kopf et al., 1964) and examined for evidence of early resorptions. Corpora lutea were not counted for females that were not visibly pregnant at Cesarean-section or for females that were submitted for necropsy prior to day 21. At least one-half of the fetuses in each litter, selected using a computer-generated randomization procedure, were immediately examined by dissection under a low power stereomicroscope for evidence of visceral alterations (Staples, 1974). The heads of rat fetuses examined by dissection were removed, placed in Bouin's fixative and examined by the serial sectioning technique of Wilson (1965). All fetuses were then preserved in alcohol, eviscerated and stained with alizarin red-S (Dawson, 1926). Skeletal examinations were conducted on all fetuses that were not given visceral examinations.

Statistical Evaluation. In the methods development study, only descriptive statistics (means and standard deviations) for glycolate and acid/base parameters were calculated. In the main study, maternal body weights, body weight gains, organ weights, reproductive parameters and mean fetal body weights were evaluated by Bartlett's test for equality of variances. Based on the outcome of Bartlett's test, a parametric or nonparametric analysis of variance (ANOVA) was performed. If the parametric or nonparametric ANOVA were significant, analysis by Dunnett's test or the Wilcoxon Rank-Sum test with Bonferroni's correction were performed, respectively. Statistical evaluation of the frequency of pre-implantation loss, resorptions and fetal alterations among litters and the fetal population were performed using a censored Wilcoxon test with Bonferroni's correction. The number of corpora lutea and implants, and litter size were evaluated using a nonparametric ANOVA followed by the Wilcoxon Rank-Sum test with Bonferroni's correction. Pregnancy rates were analyzed using the Fisher exact probability test. Descriptive statistics for feed consumption data were calculated with no further statistical analysis. Nonpregnant females, females pregnant following staining, or females having totally resorbed litters were excluded from the appropriate analyses. Fetal sex ratios were evaluated using a

binomial distribution test. Statistical outliers were identified using a sequential method, but only values for feed consumption were routinely excluded unless justified by sound scientific reasons unrelated to treatment.

The nominal alpha levels were as follows:

Bartlett's Test (Winer, 1971)	$\alpha = 0.01$
Parametric ANOVA (Steel and Torrie, 1960)	$\alpha = 0.10$
Nonparametric ANOVA (Hollander and Wolfe, 1973)	$\alpha = 0.10$
Dunnett's Test (Winer, 1971)	$\alpha = 0.05$, two-sided
Wilcoxon Rank-Sum Test (Hollander and Wolfe, 1973)	$\alpha = 0.05$, two-sided with Bonferroni's correction (Miller, 1966)
Fisher's Test (Siegel, 1956)	$\alpha = 0.05$, two-sided
Censored Wilcoxon Test (Haseman and Hoel, 1974)	$\alpha = 0.05$, two-sided
Sequential Outliers Test (Grubbs, 1969)	$\alpha = 0.02$, two-sided
Binomial Distribution Test (Steel and Torrie, 1960)	$\alpha = 0.05$, two-sided

Because numerous measurements were statistically compared in the same group of animals, the overall false positive rate (Type I errors) was expected to be much greater than the cited alpha levels suggested. Therefore, the final interpretation of the numerical data also took into consideration whether or not the results were significant in light of other biologic and pathologic findings.

Quality Assurance. The conduct and data generated during this study were reviewed according to the procedures of the Quality Assurance Unit of The Dow Chemical Company, Health and Environmental Research Laboratories. Permanent records of all data generated during the course of the study, the protocol, any addenda, and the final report were available for inspection by the Quality Assurance Unit. All data generated, including the protocol, addenda, and final report are archived at Health and Environmental Research Laboratories, The Dow Chemical Company, Midland, Michigan.

RESULTS AND DISCUSSION

Part I

Acid-base balance. As shown in Figure 2 and Table 1, both EG and GA produced a mild metabolic acidosis, with a maximal decrease in blood pH of approximately 0.1 unit and which persisted for at least 9 hours, but was completely resolved by 24 hours post-dosing. The time course and magnitude of these changes were nearly identical in these two groups. These changes also were very similar to those observed by Khera (1991) in rats following an oral dose of 2500 mg/kg/day on gestation day 11. Corresponding with the mild metabolic acidosis in the EG and GA groups was a slight decrease in blood bicarbonate level. PCO₂ also was decreased slightly in the EG group, and to a barely perceptible degree in the GA group. In contrast, treatment with NaG did not cause any alterations in blood pH, PCO₂ or bicarbonate relative to control values.

Blood glycolate analysis. Figure 3 and Table 2 show the serum glycolate concentration data for gestation day 10 rats given EG, GA or NaG. Serum glycolate was expressed in Figure 3 on a log scale so that a slope of the elimination phase of each curve could be calculated. Pharmacokinetic values derived from the curves are summarized in Table 3.

Administration of 2500 mg/kg of EG resulted in serum glycolate levels which generally were in the millimolar range, with a peak concentration of 8.8 mmol/L of glycolate occurring at the 3 hour blood-sampling interval. This value corresponds with the previously mentioned whole embryo culture study (Carney et al., 1996), in which embryo development was affected at ≥ 12.5 mmol of glycolic acid/L, while 2.5 mmol/L was the no-effect-concentration. In male rats gavaged with 2000 mg/kg of EG, peak plasma glycolate was approximately 13 mmol/L (Hewlett et al., 1989). The lower value in pregnant rats relative to male rats may possibly be due to the expansion of blood volume which occurs during pregnancy, thus resulting in hemodilution. In this study with pregnant rats, AUC for serum glycolate was calculated to be 146.6 mmol L⁻¹ h. Extrapolation of the elimination phase of the curve indicated a half-life of approximately 10 hours for

elimination of glycolate from blood. Serum glycolate levels were undetectable by 24 hours after dosing with EG.

Both GA administered by oral gavage and NaG given via SC injection resulted in blood glycolate levels which were in a very similar range as for the 2500 mg/kg dose of EG. In fact, peak glycolate levels in all three groups were within 5% of one another (8.4-8.8 mmol/L). In comparing the GA and NaG groups to one another, it was found that most pharmacokinetic values were also very similar. The major difference between the two curves was the earlier T_{max} of NaG, reflecting the more rapid rate of absorption associated with its SC route of administration. Most likely, blood glycolate levels in the NaG group peaked prior to the first sampling point of 1 hour, which, if determined, would have increased the C_{max} and AUC values slightly. In any case, the two curves appeared remarkably similar, almost overlapping one another if one accounts for the differences in absorption time. There was no detectable glycolate in any of the 24 hour samples from the GA or NaG groups, nor was there any in the 9 h samples in the NaG group.

While peak glycolate levels were very similar in the EG, GA and NaG groups, glycolate tended to persist in the serum for longer amounts of time when EG was administered vs. when GA or NaG was given. This resulted in an $AUC_{glycolate}$ value for the EG group which was three-fold higher than that following GA or NaG exposure. Several factors may be responsible for this phenomenon. One likely explanation is a greater competition for rate-limiting oxidation potential when all of the reactions in the EC pathway are operative (EG group) compared to when the initial reactions are by-passed when administering the glycolate metabolite (GA, NaG groups). EG presumably persists in the blood for a period of time, during which conversion of EG to GA continuously takes place. In contrast, there is no opportunity for continued GA production in the GA group. Finally, renal elimination of glycolate also could differ slightly between the EG and GA/NaG groups.

In-life observations, body weights and gross necropsy There were no clinical observations which were related to treatment with any of the test compounds (Table 4). Observations apparently related to complications of the arterial catheter occurred in two control rats and one rat in the NaG group. Control group

rat 95A8122 exhibited convulsions during an attempt to sample blood and was euthanized shortly thereafter. No visible lesions were detected at necropsy (Table 5). Control group rat 95A8124 exhibited decreased activity and was later found dead. Necropsy revealed a mass of clotted blood at the surgical incision site, suggesting a leakage of blood at this site. Clotted blood was also found in one other rat in this group, but without any associated clinical signs. Rat 95A8133 from the NaG group exhibited decreased activity (lying on side) and was later euthanized. No gross lesions were found at necropsy. Correct cannula placement within the lumen of the right common carotid artery was confirmed at necropsy in all rats.

Mean body weights decreased between surgery on gestation 8 and the start of dosing on gestation day 10 (Table 6). However, mean body weight loss during this interval was less than 9% in all groups. Control rats then gained an average of 1.3 grams of body weight from gestation day 10 to 11, while the rats in the treated groups exhibited a loss of several grams body weight during this time.

Part I Summary. The following are the salient findings of Part I:

- (1) Doses of 650 mg/kg (8.5 mmol/kg) of GA, 833 mg/kg (8.5 mmol/kg) of NaG or 2500 mg/kg (40.3 mmol/kg) EG resulted in peak blood glycolate concentrations which were within 5% of one another.
- (2) In comparing GA and NaG kinetics, the elimination constants and elimination half lives were essentially identical, and the AUC values were very similar to one another. The major difference between the two curves was the more rapid absorption of NaG, due its SC route of administration. If one accounted for the differences in the initial absorption phase, the remaining portion of the curves would overlap one another almost identically.
- (3) The GA dose caused a mild metabolic acidosis in pregnant rats which was nearly identical to that caused by 2500 mg/kg of EG, while administration of subcutaneous NaG did not induce any detectable acidosis.
- (4) AUC values and serum elimination half-lives were three-fold higher in the EG group compared to those of the GA and NaG groups. These differences were considered potential modifying factors important in the final interpretation of the study data.

Part II

The treatment regimens developed and validated in Part I were then applied in a developmental toxicity study design in which groups of 25 time-mated rats were dosed on gestation days 6-15 with (1) distilled water via gavage, (2) 650 mg/kg/day of GA via gavage, (3) 833 mg/kg/day of NaG via SC injection, or (4) 2500 mg/kg/day of EG via gavage. As mentioned earlier, these dose levels were originally based on a study in male rats indicating that 21% of a 2000 mg/kg IV bolus dose was converted to GA (Marshall, 1982). Parameters of maternal and developmental toxicity were evaluated as detailed in the Materials and Methods section.

Analysis of dosing solutions. All dose solutions assayed from 99-107% of target concentrations and were stable throughout the dosing period (Table 7). As the materials were in solution, homogeneity analyses were not indicated.

In-life observations. Several dams in the 650 mg/kg GA group were noted with mouth breathing, noisy or deep respirations, facial soiling, salivation and/or excessive chromorhinorrhea (Table 8). Three of these dams (96A0857, 96A0874, 96A0878) were sacrificed due to their respiratory difficulties. Another dam (96A0862) was found dead, with no prior clinical symptoms observed. One dam (96A0923) in the EG group exhibited vaginal bleeding on gestation day 19 and 20. The latter dam was found with a completely resorbed litter on gestation day 21, suggesting that the prior vaginal bleeding was related to fetal demise.

Gross pathology - spontaneous deaths/moribund animals. Gross pathological findings among the four GA group dams that either died or were sacrificed in moribund condition included mucoid exudate in the nasal turbinates, soiling of the face/nose region with blood, clear fluid and/or porphyrin, gas in the stomach, congestion of the liver, lungs and kidneys and dilated renal pelvis (Table 9). Based on both the in-life clinical history of respiratory difficulty and the gross pathology findings of the nasal region along with gas in the stomach, the most probable cause of death/moribundity was considered to be obstruction of the nasal turbinates. Considering the acidic nature of the test material and the absence of any findings in the remaining GA rats, these respiratory effects may

have been due to nasal reflux of small amounts of test material as a complication of oral gavage dosing.

Feed consumption. Mean feed consumption values were slightly decreased during the treatment and post-treatment periods (Table 10). Percent decreases from controls ranged from 1-7% for the NaG group, 4-12% for the GA group and 6-13% for the EG group.

Body weights/body weight gains. Maternal body weights were significantly decreased on gestation days 16 and 21 in the GA dams and on gestation days 12, 16 and 21 in the EG group (Table 11). Similarly, body weight gains were significantly decreased for the gestation day 9-12, 12-16, 0-21 and 6-16 intervals in the GA group, and all intervals (except the 0-6 pre-dosing period) in the EG group (Table 12). Body weights and body weight gains in the NaG dams were not significantly different from controls.

Organ weights. Weights of the kidney, a known target organ for EG, were significantly increased in both the EG (absolute and relative weight) and GA (relative weight only) groups (Table 13). Mean absolute and relative kidney weights in the NaG rats were slightly higher than controls, but the differences did not achieve statistical significance. Liver weights were also increased in all three groups, with statistical differences from controls being identified for absolute liver weight in the NaG and EG groups, and for relative liver weights in all three treatment groups.

Gross necropsy - scheduled necropsy. There were no visible lesions observed in any of the dams surviving to the scheduled necropsy (Table 14).

Reproductive/fetal observations made at necropsy. Pregnancy rate across all groups was uniform, with only one of the 100 rats placed on test being found not pregnant (Table 15). There were no effects on the number of corpora lutea or implantations, percent preimplantation loss, or sex ratio. Resorption rate tended to be elevated slightly in all groups relative to controls, but none of the differences reached the predetermined level of statistical significance. While resorption rate in the GA and NaG groups was within the range of historical control, in the EG

group, values for mean resorptions/litter, percent of implantations resorbed and fetuses per litter slightly exceeded the maximum limit of historical control values (Table 16). This suggests that the increased resorption rate in the EG group was due to the test material. Contributing to the elevated resorption rate in the EG group was one totally resorbed litter. Fetal body weights were significantly decreased in all three experimental groups, with this parameter being most severely affected in the EG group, followed by the GA group and then the NaG group. Gravid uterine weights also were significantly decreased in the GA and EG groups.

Fetal morphologic alterations. Treatment with 2500 mg/kg/day of EG resulted in significantly increased incidences of numerous fetal variations and malformations (Table 17), the nature of which was consistent with the previously reported findings of Price et al. (1985) and Neeper-Bradley et al. (1995). The malformations observed in the present study following oral EG exposure consisted primarily of axial skeleton defects (primarily fused, extra, missing or incorrectly paired bones of the vertebral column and ribs), cranial neural tube defects (exencephaly, meningoencephalocele, dilated cerebral ventricles), cranio-facial abnormalities (cleft lip, cleft palate, anophthalmia, microphthalmia) and abdominal wall defects (omphalocele, gastroschisis, umbilical hernia). Limb rotations were also seen, although these appeared in the majority of cases to be related to physical obstructions associated with abdominal wall alterations. A number of isolated visceral malformations occurred in the EG group, but no clear pattern of these alterations was readily identifiable.

In the GA group fetuses, all of the skeletal malformations and 10 of the 11 skeletal variations which were significantly increased following EG treatment were also seen in the GA group, albeit at lesser frequencies (Tables 17, 18). Dilated cerebral ventricles, limb rotations and the abdominal wall defects were additional findings common to both the EG and GA groups. The only malformations found at an increased incidence in the EG group which were not represented in the GA group were several external malformations involving the cranial neural tube and craniofacial region. GA exposure did not result in any unique fetal alterations above and beyond those observed with EG.

NaG treatment caused a statistically significant increase in six skeletal variations, all of which were also seen in the EG group. Also, two axial skeletal malformations consistent with the EG group (hemivertebra and missing ribs) were observed in single NaG group fetuses. Overall, however, the incidence and severity of the fetal alterations in the NaG group was much less than that of either the EG or GA groups. The character and incidence of fetal effects in the NaG group resembled those seen at the LOEL in several other EG developmental toxicity studies (Yin et al., 1986; Neeper-Bradley et al., 1995).

In addressing the primary study objective of determining the relative contributions of the glycolate anion vs. metabolic acidosis in EG developmental toxicity, there are two major conclusions supported by the data. The first conclusion deals with the effects of the glycolate anion *per se*. The data clearly show that some of the developmental effects of EG can be induced by the glycolate anion in the absence of maternal metabolic acidosis. However, significant fetal effects observed under such conditions consisted primarily of decreased fetal body weights and increased incidences of several skeletal variations. This profile of developmental effects is typically seen at the threshold or LOEL of the EG dose response curve as observed in prior developmental toxicity studies (Yin et al., 1986; Neeper-Bradley et al., 1995). This result indicates that metabolic acidosis is not an absolute requirement for these less severe manifestations of developmental toxicity, with glycolate kinetics instead being the major mechanistic determinant for these effects.

The second conclusion regards the role of metabolic acidosis. In contrast to the relatively minor effects caused by NaG, the incidence and severity of developmental effects was much greater in the GA group. Given that serum glycolate levels were very similar following GA and NaG administration, but only GA induced a mild metabolic acidosis, it is concluded that metabolic acidosis is a major exacerbating factor leading to the teratogenic effects of EG at high dose levels. This result was consistent with data obtained by Khera (1991), who greatly ameliorated most of EG's teratogenic effects by co-administering sodium bicarbonate with EG to reduce the degree of acidosis.

A related question for which the current study offers some critical insight is whether or not the evidence is sufficient to conclude that the EG metabolite, GA, is the proximate toxicant for all EG developmental effects *in vivo*. As discussed earlier, exposure of gestation day 10.5 rat embryos in a previous whole rat whole embryo culture study using up to 50 mmol/L EG was essentially without effect, whereas GA at 12.5 mmol/L caused embryonic dysmorphogenesis consistent with EG effects *in vivo* (Carney et al., 1996). Also, numerous studies have shown that the acute toxic effects of EG are due to the GA metabolite and that parent EG has an extremely low intrinsic toxicity (reviewed in Carney, 1994). The present *in vivo* study results indicate that GA is the key metabolite responsible for EG's effects on fetal body weights, axial skeleton variations and malformations, abdominal wall defects and dilated cerebral ventricles, based on the common occurrence of these in both the EG and GA groups. The only effects for which the present study leaves some uncertainty as to the role of parent EG are the external malformations involving the cranial neural tube (meningoencephalocele, exencephaly) and cranio-facial region (cleft lip, cleft palate, anophthalmia, microphthalmia), as these were only seen in the EG group.

There are two possibilities for explaining these results. One, this subset of effects which incidentally, is restricted to the upper extreme of the EG dose response curve (Price et al., 1985; Neepser-Bradley et al., 1995), may be due directly to parent EG. Again, this is highly unlikely given the lack of effects on neural tube and cranio-facial development in whole embryo culture. Furthermore, cranio-facial defects were induced by GA in this system. The alternate hypothesis is that these effects resulted from the three-fold higher AUC_{glycolate} observed after EG dosing vs. dosing with GA. This interpretation is more consistent with the larger body of data on EG and appears to be a much more plausible explanation.

CONCLUSIONS

In summary, the present study incorporated a novel experimental protocol in which treatment with EG, GA or NaG induced very similar peak serum glycolate levels associated either with metabolic acidosis (EG, GA) or without metabolic acidosis (NaG) to determine the relative contributions of these two factors to EG-induced developmental toxicity *in vivo*. Decreased fetal body weights and

increased incidences of six minor skeletal variations were observed with NaG, showing that the glycolate anion is sufficient to cause these most subtle of EG developmental effects in the absence of metabolic acidosis. Treatment with GA resulted in a much greater severity of developmental effects, which also included a variety of malformations characteristic of EG, indicating that metabolic acidosis is a major exacerbating factor leading to *in vivo* teratogenesis following large oral bolus exposures to EG. The high degree of correspondence between the GA and EG fetal alterations supports previous *in vitro* data indicating that EG's developmental effects *in vivo* are due to its metabolite, GA.

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Figure 1

ETHYLENE GLYCOL DEVELOPMENTAL TOXICITY: MECHANISTIC STUDY
ON THE ROLE OF GLYCOLATE ANION AND METABOLIC ACIDOSIS

PROPOSED MODE OF ACTION FOR EG INDUCED DEVELOPMENTAL TOXICITY

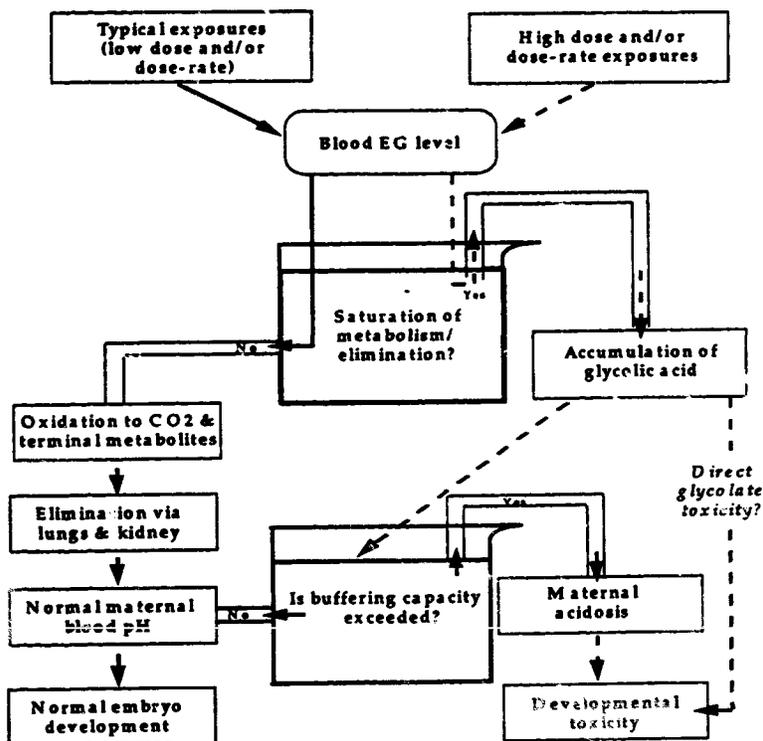


Figure 2
ETHYLENE GLYCOL DEVELOPMENTAL TOXICITY: MECHANISTIC STUDY
ON THE ROLE OF GLYCOLATE ANION AND METABOLIC ACIDOSIS

MEAN ARTERIAL BLOOD PH CHANGES FOLLOWING SINGLE DOSE OF EG, GA, NaG or Vehicle-Part I

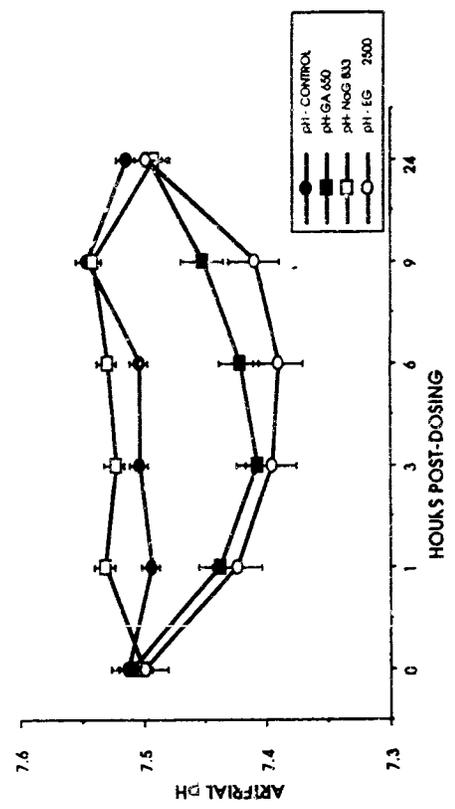
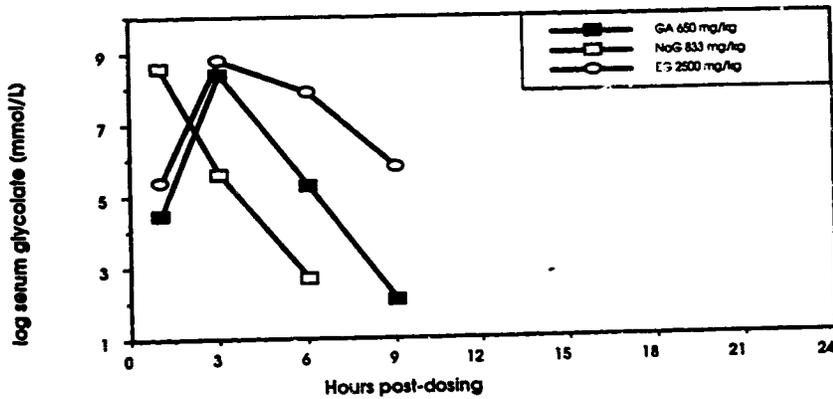


Figure 3

ETHYLENE GLYCOL DEVELOPMENTAL TOXICITY: MECHANISTIC STUDY
 ON THE ROLE OF GLYCOLATE ANION AND METABOLIC ACIDOSIS

SERUM GLYCOLATE IN GD 10 RATS DOSED WITH GA (PO) OR NaG (SC)-PART I



Serum glycolate expressed in mmol/L (mmol/L = $\frac{\text{ug glycolate}}{\text{ml}} \times \frac{1 \text{ mmol}}{76.05 \text{ mg}} \times \frac{1000 \text{ ml}}{\text{L}} \times \frac{\text{mg}}{1000 \text{ ug}}$)

Where formula wt of glycolic acid=76.05

Table 1
 ETHYLENE GLYCOL DEVELOPMENTAL TOXICITY: MECHANISTIC STUDY
 ON THE ROLE OF GLYCOLATE ANION AND METABOLIC ACIDOSIS

Acid-Base Values Summary-Part I-^a

GROUP	BLOOD pH					
	0h	1h	3h	6h	9h	24h
CONTROL	7.514	7.495	7.488	7.503	7.544	7.514
MEAN:	0.031	0.044	0.021	0.040	0.029	0.023
SD:	5	5	5	5	3	2
N:	7.511	7.437	7.408	7.422	7.451	7.496
GA 650	0.018	0.033	0.049	0.031	0.024	0.049
MEAN:	5	5	5	5	4	3
SD:	7.504	7.533	7.522	7.535	7.546	7.498
N:	0.013	0.019	0.017	0.032	0.013	0.050
NaG 833	4	4	4	4	3	3
MEAN:	7.499	7.424	7.393	7.386	7.413	7.500
SD:	0.032	0.045	0.046	0.074	0.059	0.032
N:	5	5	5	5	5	5

CHANGES IN N DUE TO BLOCKED CANNULA OR PREMATURE DEATHS
^a VALUES FROM NON-PREGNANT ANIMALS WERE EXCLUDED

FYI - 1197-001314

December 17, 1997

To the FYI File:

The attached report "Ethylene Glycol developmental Toxicity: mechanistic Study on the Role of Glycolate Anion and Metabolic Acidosis" from Dow Chemical Company was received by the EPA Assistant Administrator for Prevention, Pesticides, and Toxic Substances on 11/7/97 without cover letter.

Terry O'Bryan/OPPT
FYI Coordinator