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CODING FORM FOR GLOBAL INDEXING

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CHEMICAL MANUFACTURERS ASSOCIATION

OFFICE
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May 22, 1995

Langley A. Spurlock, Ph.D., CAE
Vice President, CHEMSTAR



FYI-94-031228
INIT 6/28/95

Dr. Lynn Goldman
Assistant Administrator
Office of Prevention, Pesticides and Toxic Substances TS-7101
Environmental Protection Agency
401 M Street, SW Room 637, East Tower
Washington, DC 20460



8495000021

Dear Dr. Goldman:

The Chemical Manufacturers Association makes public all final reports developed from research projects that it administers. The following reports, recently completed, are enclosed:

Solid Oxidizer Classification Test, conducted by the Chlorinated Pool Chemicals Panel.

Identification of Ethylene Glycol Metabolites in the Plasma of Female Sprague Dawley® Rats and CD-1® Mice, conducted by the Ethylene Glycol Panel.

Ethylene Glycol: Comparisons of *In Vitro* Skin Penetration Following a Single Application of Excised Skin of Humans and CD-1® Mice conducted by the Ethylene Glycol Panel.

Rat Whole Embryo Culture: Identification of Proximate Toxicant for Ethylene Glycol Development Toxicity Study conducted by the Ethylene Glycol Panel.

These reports do not include confidential information.

If you have any questions, please call Kathleen Roberts of my staff at 202/887-1146.

Sincerely,

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SUMMARY REPORT

IDENTIFICATION OF ETHYLENE GLYCOL METABOLITES IN THE PLASMA OF FEMALE SPRAGUE DAWLEY® RATS AND CD-1® MICE

PROJECT NUMBER: 93N1291

SPONSOR: Chemical Manufacturers Association (CMA)
2501 M Street, NW
Washington, DC 20037

TESTING FACILITY: Bushy Run Research Center (BRRC)
Union Carbide Corporation
6702 Mellon Road
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STUDY DIRECTOR: James D. Sun, Ph.D.

DATE: April 22, 1994

IDENTIFICATION OF ETHYLENE GLYCOL METABOLITES IN THE PLASMA OF FEMALE SPRAGUE DAWLEY® RATS AND CD-1® MICE

Introduction

Previous pharmacokinetic studies have yielded important information about the dose-dependent elimination of ethylene glycol (EG) in rodents (Marshall, 1982; Frantz *et al.*, 1992a). From these and other studies in the literature (Khera, 1990; 1991), an apparent mechanism for EG developmental toxicity appears to be related to the onset of metabolic acidosis conditions in exposed laboratory animals, presumably because of metabolism of EG to organic acid products. Furthermore, the rate of exposure (dose-rate) appears to be important, since the processes that control metabolic homeostasis appear to be overwhelmed by bolus doses of EG. There is evidence from the urine samples of rodents (Marshall, 1982; Frantz *et al.*, 1992a) that large amounts of glycolate are voided following bolus doses of 1000 mg/kg or higher by the intravenous (IV) or peroral routes. In contrast, ethylene glycol (EG) is absorbed through both rat and mouse skin as the unmetabolized moiety, and excreted mostly as unmetabolized EG in urine. Also, in both Fischer 344 (Marshall, 1982) and Sprague Dawley® (Frantz *et al.*, 1992a) male rats, small amounts of oxalate are detected at later intervals (12-24 hr after dosing) after both low and high doses given by these two routes. In these studies, the presence in plasma of glycoaldehyde and/or glyoxal, two reactive intermediates in the metabolism of EG, was not investigated. Marshall (1982) evaluated urine samples from Fischer 344 rats, but did not find either aldehyde metabolite and this was confirmed by Frantz *et al.* (1992b). Recently, Frantz *et al.* (1992c) have conducted an investigation of the plasma distribution of EG metabolites with time in the male Sprague Dawley® rat following IV and peroral doses. However, to date, the plasma distribution of metabolites in female Sprague Dawley® rats and CD-1® mice has not been investigated. The study reported here will build on this preliminary work.

Such information has important implications for the interpretation of developmental toxicity results in rats and mice. Khera (1991) has suggested that disruption of maternal homeostasis may play a key role in the mechanism of teratogenesis in rodents. It is known that glycolate is the major metabolite found in the urine, along with levels of unmetabolized EG, and that dose-dependent urinary elimination occurs for both rats and mice. It is not clear whether the formation and plasma distribution of the other intermediate metabolites play a role in developmental toxicity. Since plasma metabolite analysis was not conducted in previous studies by Frantz *et al.* (1992a), the objective of this investigation was to examine the time-course of metabolite formation and distribution in female rodents following 10 and 1000 mg EG/kg doses by the peroral administration route. It is hoped that this work will contribute important information towards our understanding of a mechanism of developmental toxicity for EG.

Methods

Test Material. A sample of [1,2-¹⁴C]-ethylene glycol was obtained from Wizard Laboratories (West Sacramento, CA) with a specific activity of 10 mCi/mmol and a radiochemical purity of >99%. Unlabeled EG (Polyester Grade) was obtained with a purity of >99% from Aldrich Chemical (Milwaukee, WI).

Dose Solution Analysis. The dosing solutions used in these studies contained both ¹⁴C-EG and unlabeled EG in amounts sufficient to produce 0.5% and 50% (w/w) solutions dissolved in water for oral studies. Dosing solution ¹⁴C concentrations were determined by liquid scintillation spectrometry (LSS) techniques and doses were delivered gravimetrically by weighing the syringe before and after dosing.

Test Animals. Young adult female Sprague Dawley® rats (approximately 9 weeks old upon arrival at the laboratory) were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Female CD-1® mice were ordered from Charles River Laboratories, Inc. (Portage, MI) and were approximately 5 weeks old upon arrival at the laboratory. These animals, which were non-pregnant and nulliparous, were acclimated to the laboratory environment for at least 5 days prior to use in the study.

Study Design. For each of the two species evaluated, a total of 12 animals/dose level (10 and 1000 mg/kg) were dosed perorally in duplicate in order to collect the maximum amount of plasma for individual sample analysis at each collection interval. Each animal was anesthetized with Metofane® and exsanguinated by a closed-chest cardiac puncture technique at the following times: the approximate t_{max} (see below), 4, 6, 8, 10, and 12 hr post-dosing; t_{max} is the time to C_{max} , or maximal plasma concentration. Total radioactivity values for t_{max} , at 10 and 1000 mg/kg peroral doses were 4.1 and 1.1 hr in female rats, and 0.4 and 1.2 hr in female mice, respectively. For mice, plasma samples were pooled from these duplicate animals for each interval to have sufficient sample for analysis. Plasma was processed as described in (Frantz et al., 1992a) and small aliquots (25-50 μ l) were counted by LSS; the remaining sample after LSS analysis was submitted for chromatographic analysis as outlined below. An additional three animals for each dose level were dosed and serially sampled for blood at 6, 12, 24, 48 and 72 hr post-dosing from the lateral tail veins (~200-300 μ l) in order to obtain samples for analysis of the possible incorporation of ¹⁴C into the C₁ pool at later time points (to correlate with the prior PK studies). This was done by taking an aliquot of each interval sample (~15-25 μ l) and analyzing by LSS, while the remaining sample was precipitated with trichloroacetic acid to determine the radioactivity in the precipitated macromolecules. The precipitated sample was centrifuged using a table top centrifuge and the supernatant was isolated from the pellet by drawing it off using a Pasteur pipet. The remaining pellet was washed three times, dried and then combusted using an R. J. Harvey Biological Materials Oxidizer prior to quantification.

by LSS. This last measurement should reveal the levels of ^{14}C in macromolecules potentially incorporated from the C_1 pool.

Chromatographic Data Analysis. The remaining volume of plasma was analyzed using HPLC techniques and refractive index (RI) detection and radiodetection, as described in Frantz *et al.* (1992c). Results for the pooled plasma samples were calculated using peak area methods and summarized per collection interval. Authentic standards for glycoaldehyde, glycolate, glyoxal and oxalate were used to compare retention times by RI for the principle metabolites; radiodetection was compared with RI retention times to identify major metabolite peaks. A time profile was constructed which summarizes the changes in metabolite presence and concentration in plasma with time for each of these two dose levels.

Results

I. Identification of EG Metabolites in Female Rat Plasma

When metabolite concentrations were determined over the initial 12 hr after oral dosing in female rat plasma after peroral administration of 1000 mg/kg (Figure 1), glycolate was the major metabolite measured. However, glyoxylate and glyoxal (not resolved in this HPLC system) concentrations were also detected in plasma samples from the 10 mg/kg dose. No attempt was made to resolve these last two aldehyde metabolites since this was not within the scope of the present study. Glycolate, glyoxylate/glyoxal, and unchanged EG constituted the largest percentages of ^{14}C detected in plasma for these peroral doses (Table 1). Oxalate and glycoaldehyde were not detected in the plasma during any collection periods for any dose level.

Elimination $t_{1/2}^B$ values for the disappearance of glycolate from plasma were 6.6 and 1.6 hr for the 1000 and 10 mg/kg doses, respectively (Figure 1). A $t_{1/2}^B$ value of 2.6 hr was also calculated for the glyoxylate/glyoxal plasma concentrations measured for the 10 mg/kg dose group. The corresponding $t_{1/2}^B$ values for the unmetabolized EG in this experiment were 2.3 and 1.3 hr for the 1000 and 10 mg/kg dose levels, respectively.

II. Precipitation of Macromolecules from Plasma of Female Rats

Plasma samples collected at 5, 12, 24, 48 and 72 hr after an oral 1000 mg/kg dose showed sustained levels of ^{14}C which were associated with the washed macromolecular pellet precipitated from the plasma (Figure 2). That is, as the level of ^{14}C diminished in the remaining plasma supernatant, the ^{14}C concentrations detected in the macromolecular pellet were 5- to 10-fold larger than the supernatant concentrations by 24 hr. Similarly, radioactivity levels in the pellet samples of rats

dosed at 10 mg/kg were an order of magnitude larger than that measured in the plasma supernatants at the later times after dosing.

These elevated ^{14}C levels associated with macromolecules probably indicate that EG doses may be metabolized so extensively that the ^{14}C radiolabel entered the C_1 pool and was subsequently incorporated into macromolecules. This may help explain why the plasma disposition of ^{14}C shows concentrations of radioactivity which remain at fairly high levels during the later times after dosing with ^{14}C -EG.

III. Identification of EG Metabolites in Female Mouse Plasma

Metabolite concentrations from female mice plasma were determined for 0-12 hr after peroral administration of 1000 mg/kg (Figure 3). As was observed for rats, glycolate was the major metabolite measured after this high dose was given. However, glyoxylate and glyoxal concentrations were also detected in plasma samples from this 1000 mg/kg dose. At the 10 mg/kg level, glycolate and glyoxylate/glyoxal, along with unchanged EG, showed the largest percentages of radioactivity detected in plasma for these peroral dose levels (Table 2). Oxalate and glycoaldehyde were not detected in the plasma during any collection periods for any dose level in mice.

Elimination $t_{1/2}^{\beta}$ values for the disappearance of glycolate from plasma were 4.1 and 7.4 hr for the 1000 and 10 mg/kg doses, respectively (Figure 3). Values for $t_{1/2}^{\beta}$ for the glyoxylate/glyoxal plasma concentrations were 4.5 hr and 1.3 hr, for the 1000 and 10 mg/kg dose groups, respectively. The corresponding $t_{1/2}^{\beta}$ values for the unmetabolized EG in this experiment were 2.0 and 1.2 hr for the 1000 and 10 mg/kg dose levels, respectively.

IV. Precipitation of Protein from Plasma of Female Mice

Plasma samples collected at the specified intervals (6, 12, 24, 48 and 72 hr) after oral 1000 or 10 mg/kg doses showed very similar ^{14}C levels to that for the washed macromolecular pellet precipitated from the plasma (Figure 4). While the level of ^{14}C diminished in the plasma supernatant, the ^{14}C concentrations in macromolecules were roughly parallel in the mouse throughout the 72 hr determination. This was in sharp contrast to the results from female rats, which showed higher levels in macromolecules than in plasma at the later times after ^{14}C -EG dosing.

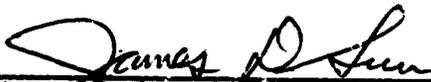
Conclusions

In summary, the results from this investigation will be used to strengthen the manuscript for the pharmacokinetics of EG in female rats and mice (Frantz et al., 1992a). Since the identification of metabolites in plasma had not been previously

reported for rodents, particularly those species for which extensive studies of developmental toxicity have been conducted with EG, these results have added considerably to our knowledge of the metabolic profile of this chemical following oral administration. Thus, it is presumed that further work to examine the mechanism of developmental toxicity for EG in rodents will be guided by the preliminary results derived from this investigation.

Review and Approval

Study Director:


James D. Sun, Ph.D.

4-22-94

Date

Director:


John P. Van Miller, Ph.D., DABT

4-25-94

Date

References

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S A F E T Y E N G I N E E R I N G L A B O R A T O R I E S , I N C .

**SAFETY ENGINEERING LABORATORIES, INC.
TEST REPORT**

SOLID OXIDIZER CLASSIFICATION TEST

TRICHLOR STORAGE FIRE TESTS

OCTOBER 28, 1994

0015



EXECUTIVE SUMMARY

Safety Engineering Laboratories, Inc. (SEL) contracted with the Chemical Manufacturers Association Chlorinated Pool Chemicals Panel (CMA) to conduct Trichlor storage fire tests. The tests were conducted in accordance with the test plan developed by SEL incorporated into the SEL/CMA agreement. The purpose of the tests was to identify differences in exposure fire performance between granular and tableted Trichloroisocyanuric acid (Trichlor) and provide baseline information with respect to the classification of solid oxidizers based on the increase in burning rate of combustibles in the fire.

Three burn tests were conducted which were identical in configuration. The first test, a calibration burn, was conducted using an inert material in place of Trichlor. Tests two and three were conducted using tableted and granular materials respectively. The thermocouple data, weigh cell data and pyrometer data were analyzed along with video tapes from each of these tests. The results showed little to no enhancement of the burn rate (Rate of Heat Release) of the combustible packing materials and wood crib exposure fire by either the granular or tableted Trichlor.

In an overall assessment of the three tests from the data calibration test #1 (no active ingredient) is assessed to be somewhat more intense prior to wall collapse than test #3, granular Trichlor, but somewhat less intense than .1 inch tableted Trichlor. We did not observe any significant increase in the overall burn rate of the ordinary combustibles in any of the Trichlor

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tests. Therefore it is our assessment that these materials should be properly categorized as Class I oxidizers in accordance with the ratings from NFPA 43A (NFPA 430 in May 1995).



SOLID OXIDIZER CLASSIFICATION TESTS

Background

National Fire Protection Standard 43A assigns solid oxidizers to Classes I through IV. Although Class I through IV definitions provide some descriptive guidance about the anticipated behavior of oxidizers in fires, these definitions are quite subjective. Because there is no standard test used to assign materials into each Class, changing an oxidizer's classification needs to be supported by comparative information on how the oxidizer behaves in a realistic fire environment.

Anecdotal evidence and laboratory bench scale tests have indicated that tableted solid oxidizers react differently in fires than the same material in granular form. Safety Engineering Laboratories, Inc. was requested to develop a test plan which would identify the differences between and the contribution to burn rate enhancement for Trichloroisocyanuric acid (Trichlor). In the current edition of NFPA 43A all forms of Trichlor are listed as Class II oxidizers.

Safety Engineering Laboratories, Inc. prepared a draft test plan with the primary purpose of providing information about how solid oxidizers behave in fires. The test we proposed is a modification of the U.N. external fire test Type 6C. Since absolute pass/fail oxidizer classification assignment criteria do not exist, a comparative test with subjective assessment is the only way to assign individual materials to a class and to compare their behavior to similar fires not involving the solid oxidizers.

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The draft test plan was circulated to key members of the NFPA Hazardous Chemicals Committee including the committee chairman. Suggestions by various members were incorporated in the plan and a second draft prepared. The revised draft was submitted to Factory Mutual (FM) through their representative on the committee. FM's suggestions were incorporated into a final draft test plan. The test plan was reviewed by members of the CMA chlorinated Pool Chemicals Panel. Two members of this committee visited SEL's facilities in Warren, Michigan and reviewed the proposed test plan. They also visited SEL's remote test site in Sanilac County, Michigan. The approved test plan was incorporated into an agreement to perform the tests through the auspices of Chemical Manufacturers Association Chlorinated Pool Chemicals Panel.

The tests were conducted during October 1994 at SEL's remote test facility.

Tests

Materials tested were obtained from Biolabs, Conyers, GA. Test #1 used polyethylene containers with no active ingredient. Test #2 used 1" tablets of Trichlor and Test #3 used granulated Trichlor. Labeling on the container was: 99% Trichloro-s-triazinetriene 1% inert ingredients available chlorine 90%.

The tests were conducted in accordance with the written test plan provided in Appendix A. The basic instrumented test configuration shown in Figure 1 Appendix A used a simulated wood building corner with wood studs and plywood walls. In each test two stacked pallets of

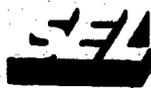


Trichlor (or inert material for calibration purposes) were placed within 12 inches of the corner on top of a low profile weigh cell. Thirty six inch high external wood cribs were placed 12 inches from the two open sides of the pallets with weigh cells under each crib. Thermocouples were located above the wall corner in an array, on the walls, on the pallets and the interior of several Trichlor containers. Flame height witness poles and markers were placed on and above the walls. A near field pyrometer was located to provide flux data from outside the fire plume. Video and still cameras were positioned in three locations to record the fire behavior of materials during the burn. Post-fire examination and measurements are designed to provide both qualitative and quantitative determination of decomposition, Trichlor consumption, combustibles consumption and radiation exposure. Appendix B photographs provide various views of the test set up and configuration including instrumentation.

Test #1 - Calibration Burn

Test #1 was a calibration burn to determine the contribution of the combustible building/packaging materials and to provide a base line from which an acceleration from the presence of Trichlor in tests #2 and #3 could be determined. Approximately 30 pounds of rocks were placed into each empty Trichlor container.

Crib Fuel -	340 lbs
72 PE Pails/Lids -	170 lbs
Wooden Pallets -	50 lbs
Rocks -	2160 lbs

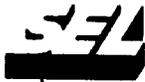


The test structure was oriented so that the wind would be blowing into the corner. At test time the actual wind was approximately 5 knots and varied between 5 and 10 knots throughout the test. The actual wind direction was parallel to the east corner wall or perpendicular to the north wall. In the first 90 seconds of this burn much of the convection energy from the west crib was directed away from the pallets and as can be seen in the video and was not impinging on the north wall.

The changing direction of the wind about 3 minutes into the test reduced the impact of wall involvement. This in fact delayed significant wall involvement until after approximately the 6 minute mark. The wall thermocouple data clearly show this.

Test #1 Description

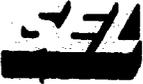
<u>Time</u>	<u>Observation</u>
0:00	Wood cribs ignition.
1:00	Established burning of cribs; light gray smoke above test structure.
1:40	Flames from south crib (cell #C) above top of pallets.
2:00	Flames from south crib impinging on palletted material.
2:15	Polyethylene pails burning along south face of lower pallet.
2:23	Several pails from upper pallet fall on south crib (weigh cell C) crib continues to burn; several pails fall free of fire from crib.
2:31	Additional pails fall from upper pallet.



<u>Time</u>	<u>Observation</u>
2:56	North wall of corner structure begins to burn.
3:20	The south wood crib fire is reestablished and is again making flame contact on the pallets. Polyethylene pails which had fallen on the load cell have melted and/or burned away.
4:00	Additional pile collapse to the west.
4:35	Additional pile collapse to the west on to load cell A.
4:41	Remainder of the pile collapsed.
6:00	Fire reestablished on north wall and in pile. Wind has shifted slightly to carry fire against north wall and to the west.
8:00	Wind velocity increased and was gusting to 10 knots.
9:20	The corner wall structure fell over not related to loss of integrity from the fire. It appeared that a gust of wind into the corner toppled the structure when the south support was pushed back.

Test #2 - Tableted Trichlor

Crib Fuel -	340 lbs
2 PE Pails/Lids -	170 lbs
1" Tableted Trichlor -	2160 lbs
Wooden Pallets -	50 lbs



The corner of the test structure was oriented so that the winds were blowing into the corner. At test time the actual winds were between 2 and 4 knots. The actual wind direction remained nearly into the corner throughout the test. This wind direction and light velocity materially aided the involvement of both walls early in the test. At approximately 3:30 into the test significant decomposition of Trichlor on and around the north crib was noted. No significant visual increase in the rate of burning was noted. Load cell data particularly from cell A under the north crib showed approximately the same rate of decrease as observed during the calibration burn when adjustments were made for weight changes due to the collapsing pile and subsequently the collapsing crib.

There were several periods during the fire particularly during 5:00 to 6:00 minutes into the fire when an increase in the burn rate of the combustibles is observed. After six minutes the size of the fire grows steadily as both sides of the corner structure are burning. The fire burned through the lower portions of both walls which allowed simultaneous upward flame spread on both sides of the wall.

By 11 minutes into the tests the plywood walls were consumed and the remaining fire in the pile consisting of polyethylene, wood and Trichlor burned unremarkably. Close up observations during this period did not reveal any vigorous burning even in the areas of rapidly decomposing Trichlor and large amounts of wood and melted polyethylene.

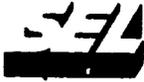


Test #2 Description

<u>Time</u>	<u>Observation</u>
0:00	Wood cribs fully involved; wind remain light with light smoke developing directly above crib fires.
1:50	Both crib fire flames extend above the top of pallets on a continuous basis no involvement yet of any Trichlor or any significant involvement of packaging materials.
2:25	Pails fall onto north crib but crib remains intact. No noticeable increase in burn rate during the next 15 seconds with significant amounts of tablets on an in the crib.
2:40	Several additional pails fall from pile onto north crib and load cell.
2:58	Additional pails of material fall from stack. Decomposition of Trichlor observed although no significant increase in combustion of adjacent combustibles noted.
3:00-3:40	The pallet stack continues to collapse with large amount of Trichlor decomposition noted in the smoke. Primary fire is the south wall fire with some contribution from the cribs. Wind remains into the corner and south wall at less than 4 knots.
3:20	North wall fully involved.
4:00	Two distinct fires noted. The north crib with numerous Trichlor pails and the pallet pile/south wall/south crib. Copious Trichlor decomposition noted from around the north crib, however no increase burn rate was noted.



<u>Time</u>	<u>Observation</u>
4:00-5:00	During this time copious quantities of black smoke appeared with little or no increase in the visible size of the fire. After 5 minutes the fire began to grow again involving the walls, wooden pallet and cribs.
4:20	Pile continues to collapse.
4:30	Wind has shifted slightly to a more westerly direction with an increasingly darker smoke and heavy involvement of the south wall.
5:00-6:00	During this period there appears to be a significant increase in the size of the fire and in the decomposition of the Trichlor. Full involvement of the wall shows clearly when the wind shifts directly into the corner.
6:00	Both walls fully involved; the south crib has remained intact; flame extension from the south wall/crib 12-16 feet.
7:00-8:00	Copious amounts of black smoke. Trichlor decomposition clearly observed on lower fire fringes.
10:00	The fire at this point is predominately a wood and structure fire. The crib fires and pile fires remain small even though significant Trichlor is present.
12:00	The wall structure was burned away and the pile fire continued to burn. The south crib remained intact and continued to burn as a crib fire.
12:40	South crib collapsed.
13:45	Test terminated and fire extinguished.

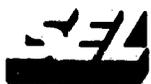


Test #3 - Granular Trichlor

Crib Fuel -	340 lbs
72 PE Pails/Lids -	170 lbs
Granular Trichlor -	2160 lbs
Wooden Pallets -	50 lbs

The corner of the burn structure was oriented so that the winds were into the corner. The actual winds varied from 3 to 12 knots throughout the tests with the predominant speed being 5 to 7 knots. The actual wind direction however varied from the west northwest over a range of ± 40 degrees. Up to the pile collapse at around 6 minutes into the test the crib fires showed normal growth causing some exposure to the lower pallet outside containers. Significant decomposition of Trichlor was occurring during this period although no significant growth in the fire was observed visually.

The pile collapse slowed the growth of the fire although the fire atmosphere was clearly primed with copious amounts of Trichlor decomposition products. The fire was allowed to reestablish and continue to burn to consume the test materials and structure. After the fire in the wood materials was reestablished the fire growth and appearance was that of wood and plastic and did not appear enhanced by the presence of the decomposing Trichlor.



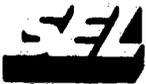
Test #3 Description

<u>Time</u>	<u>Observation</u>
1:15	Both crib fires were established. The wind was gusting and the north crib convective energy was vented past the east wall (similar to test #1).
2:20	The south crib flame extension was above the pallets periodically.
3:20	The upper wood pallet appeared to be burning along the west edge. The wind continued to shift to the north carrying most of the convective energy from the north crib away from the pallets.
3:30	When the wind shifted back into the corner it appears that both cribs now have well developed fires.
3:55	Pile collapse begins onto west crib and load cell.
4:00	Granular Trichlor that poured from holes melted into lower pails began to decompose producing copious quantities of smoke without any apparent increase in burn rate.
4:30	South crib fire begins to redevelop. Granular Trichlor which poured out of holes melted in the pails appeared to smother the fire at lower levels on the west crib. The north crib continued to burn with little effect on the remaining palletted material.



<u>Time</u>	<u>Observation</u>
5:00	Trichlor decomposition from the south crib area was evident in the smoke. This material from holes in the pails had little effect on the well established wood burning except where some extinguishing/smothering occurred.
5:50-6:00	The majority of the pile collapsed on to both cribs. Large amounts of white to gray smoke evolved.
7:00	Visible fire reestablished on the north crib. Despite the presence of large amounts of granular Trichlor the development of this area of the fire remained slow as did the entire fire.
7:10	The wind shift to a west to southwest direction during this period carried much of the convective energy from the north crib away from the structure.
9:00	Fire growth from the north crib to the pile with some involvement of the north wall.
11:00	Combustion of the east wall appears again as the pyrolysis products ignite in the plume from the crib fire.
12:30	The wind velocity dropped to 5 knots and remained into the corner. The fire was allowed to continue to burn.

Photographic Data. Appendix B contains captioned photographic data from each test.



Thermocouple Data

Appendix C and D contain graphs of all TC data collected during the tests. Wall, stack and pallet TC data were compiled as weighted averages to provide smoothing and these graphs are contained in Appendix E.

Load Cell Data

Appendix G shows the raw load cell data obtained from each test. The early collapse of the pile which interfered with all three load cells compromised nearly all of the load cell data. Analysis of the data using various graphical and statistical techniques failed to yield any meaningful heat release rates for any tests.

Pyrometer Data

Pyrometer signal indicates that the fire size of the tableted test, test 2 was larger than the calibration test, test 1 but smaller than the granular test, test 3. Figure 4, Appendix E shows the pyrometer signals obtained from each test. Appendix F contains the raw pyrometer data for each test. As expected the radiation signal was the strongest during flaming involvement from the walls. The growth of the calibration test was similar to both the granular and tablet tests and it is expected that a similar signal would have been reached if flaming involvement of the wall occurred.



Discussion

Not all of the results from these tests were anticipated. Some of this is due to the nature of the test plan to conform to environmental requirements while others are related to the inability to predict large scale behavior from laboratory or bench scale tests.

These tests were of necessity conducted out of doors and therefore the biggest single variable affecting the growth and the appearance of the fires was the wind. Ideally each test would be conducted under no wind conditions. However because of the potential toxicity of the smoke some winds are desirable. For the test site it was necessary to have winds of 3-5 knots and a direction of west north west to east north east. This combination would move the smoke in a direction that permitted dispersion before the smoke reached the test site perimeter and/or provided more than 1 km to public roads and occupancy. It was also desirable to have some instability in the air to provide vertical as well as horizontal dispersion/dilution. Preliminary dispersion modeling indicated the above conditions would provide acceptable levels of carbon monoxide and chlorine containing species beyond the test site perimeter.

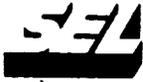
Thermocouple (TC) data from the walls and the stack were affected by the wind. The wall thermocouple did show some response to the wind conditions for each test. The averaging of all the wall thermocouple data however gives a relative smoothing of the data and indicates that the exposure fires on the wall occurred at different times for each test but were similar in size and strength in the three tests.



The growth of the fire was more rapid, in the early stages, in the calibration test. Figure 1 Appendix E shows the average wall thermocouple data for all these tests. Temperature increase during the first 3 minutes of burn was greater in test 1 (calibration) burn than tests 2 and 3. When stack collapse occurred in test 1 the fire growth was slowed which delayed the wall involvement until just prior to wall collapse.

The test 2 wall thermocouple growth is similar to test 1, however stack collapse did not adversely affect the growth of the wood crib fire and the wind remained into the wall corner. The wall fire growth in test 2 was faster and high temperatures were reached sooner than the calibration burn, test 1. The granular burn, test 3 showed a similar wall fire growth sizes as the calibration and tablet, but at a much longer time due to the stack collapse and from the addition of the granular trichlor which slowed the growth of the wood crib fires.

The stack thermocouples can be viewed as a relative rate of heat release, these indicate that the tablet and the granular tests showed similar peak rates of heat release and both occurred during flaming involvement of the plywood walls. The calibration test thermocouple show similar growth patterns but a lesser temperature than the granular and tablet tests. This temperature however did not include flaming involvement of the wall because the wall collapsed prior to its involvement. A review of the video in comparison to the thermocouple data shows that peak rate of heat release occurred during flaming involvement of the wall when the fire plume extended above the corner. No increased burning of the wall from the addition of the Trichlor was observed.



Pallet thermocouple data show that the calibration test had much faster involvement of the polyethylene pails than either the tablet or the granular tests. Figure 3 Appendix E shows the average pallet thermocouple data, discarding a failed thermocouple. The involvement of the tablet pails was faster than the granular pails. Both the granular and tablet thermocouple data indicate similar growth patterns and no enhancement or increased involvement due to the addition of the Trichlor. The calibration data collection was terminated due to the failure of the wall. However the data to nine minutes indicates that the pails were involved faster than with either Trichlor burn.

Conclusions

Based on all of the data obtained including the visual observations during each test no significant increase in the rate of burning when Trichlor was present was observed. The ordinary combustibles present in these tests were primarily polyethylene and wood. The presence of Trichlor and its thermal decomposition products did not materially affect the growth rate of the fires nor the peak rates of heat release. This data and the requirements of NFPA 43A demonstrate that Trichloroisocyanuric acid in tableted or granular form is a Class I oxidizer.



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STUDY TITLE

Ethylene Glycol: Species Comparisons of In Vitro Skin Penetration
Following a Single Application to the Excised Skin of Humans and CD-1[®] Mice

TEST SUBSTANCE

Ethylene Glycol

DATA REQUIREMENT

Not Applicable

AUTHORS

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STUDY COMPLETION DATE

May 20, 1994

PERFORMING LABORATORY

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**Ethylene Glycol: Species Comparisons of In Vitro Skin Penetration
Following a Single Application to the Excised Skin of Humans and CD-1[®] Mice**

COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

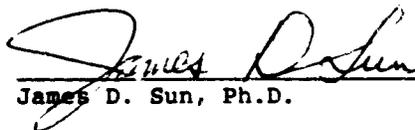
The portions of this study conducted by BRRR meet the requirements of the following Good Laboratory Practice Standards: Toxic Substances Control Act (TSCA), 40 CFR Part 792, with the following exceptions:

1. The reference substance (both radiolabeled and unlabeled) was not analyzed for chemical identity, purity or stability.
2. No experimental procedures from this study were audited by the BRRR Quality Assurance Unit. Other audits done on the raw data and report revealed no indications of GLP deviations having occurred during in-life procedures.

Other than radiochemical purity, the physical and chemical characterization of the test substance (both radiolabeled and unlabeled) were the responsibility of the Sponsor.

These exceptions are not expected to compromise the integrity of the results and conclusions of the study.

Study Director:


James D. Sun, Ph.D.

5-20-99
Date

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**Ethylene Glycol: Species Comparisons of In Vitro Skin Penetration
Following a Single Application to the Excised Skin of Humans and CD-1[®] Mice**

SUMMARY

Ethylene glycol (EG; CAS No. 107-21-1) was evaluated for its skin penetration characteristics using in vitro techniques. Full-thickness skin preparations from female CD-1[®] mice and female human abdominal skin were used. ¹⁴C-labeled EG was applied to skin preparations from each species in a flow-through in vitro skin penetration chamber. ¹⁴C-EG was applied both as undiluted chemical and as a 50% (w/w) aqueous solution in an "infinite dose" manner. For all experiments, a target dose of 22-28 mg EG/cm² of skin surface was used. This dose approximated the dose used in previous in vivo pharmacokinetic studies. The time course of ¹⁴C penetration through these skin samples was measured for 6 hr. The steady-state rate of ¹⁴C penetration through skin was determined from the linear portion of the penetration time course curve. This steady-state penetration rate was then used to calculate permeability constants (k_p) for both the undiluted EG and the 50% water solution of EG for both species.

For mouse skin, the steady-state rate of penetration for undiluted EG (0.52 mg/cm²/hr) was approximately twice that of the 50% aqueous EG solution (0.22 mg/cm²/hr). The permeability constants (k_p) for the undiluted EG and the 50% aqueous EG solution for mouse skin were approximately the same (4.68 cm/hr x 10⁻⁴ and 4.36 cm/hr x 10⁻⁴, respectively). For human skin, the steady-state rate of penetration for undiluted EG (0.013 mg/cm²/hr) was again approximately twice that of the 50% aqueous EG solution (0.007 mg/cm²/hr). The permeability constants (k_p) for the undiluted EG and the 50% aqueous solution of EG for human skin were also approximately the same (0.12 cm/hr x 10⁻⁴ and 0.14 cm/hr x 10⁻⁴, respectively).

When these results are compared between species, it is apparent that the penetration of EG through mouse skin is significantly greater than that through human skin. The steady-state rates of penetration of both the undiluted EG and the 50% aqueous solution of EG were 30 to 40 times greater for mouse skin than those for human skin. In addition, the lag times before these steady-state rates of penetration were attained were significantly shorter for mouse skin (0.90 - 1.02 hr) than that for human skin (3.07 - 3.10 hr). Species comparisons of the k_p values for both the undiluted EG and the 50% aqueous solution of EG showed that mouse skin was 30 to 40 times more permeable to EG than human skin. Therefore, based on these in vitro results, the potential toxicity resulting from cutaneous exposure to EG would likely be significantly less for humans than that predicted by experiments in mice. Additionally, since the rate of EG penetration decreased when diluted with water for both mouse and human skin, the total absorbed dose of EG would be less for cutaneous exposures to water solutions of EG than for similar cutaneous exposures to undiluted EG.

BACKGROUND INFORMATION

The percutaneous absorption of ethylene glycol (EG) represents an important potential route of entry which is relevant to the human health risk assessment for this chemical. EG has been shown by in vivo pharmacokinetic studies to penetrate the skin of male and female Sprague Dawley^o rats (Frantz et al., 1988) and female CD-1^o mice (Frantz et al., 1991a). These studies were conducted as single dose applications of either undiluted EG or a 50% aqueous EG solution. The 50% aqueous solution was chosen because it is a common concentration of EG used as a coolant in automobiles. Some data for EG penetration through human skin are available (Loden, 1986), but this study had been conducted using stored, frozen skin and was not conducted in comparison with skin from the rodent species used in previous developmental toxicity studies conducted on EG (Price et al., 1985; Tyl et al., 1989).

The data for absorption of EG by human skin can be readily obtained using in vitro skin penetration techniques and human skin preparations from donors obtained through tissue bank sources. Thus, a penetration time-course was assessed using excised skin preparations from CD-1^o mice, an animal species previously used for toxicity assessments of EG, in comparison with female human abdominal skin. The study was also designed to determine the recovery of radioactivity following a 6-hr application of the test substance to skin samples. As a reference, ¹⁴C-labeled ethanol penetration was also measured in each experiment to assess the suitability of each skin sample.

DOSE SELECTION

The present investigation measured the in vitro percutaneous penetration of EG in mice. This study also used these in vitro techniques to measure the percutaneous penetration of EG through human skin obtained from postmortem and abdominoplasty patients. For all experiments, a target dose of 22-28 mg/cm² was selected based on previous in vivo pharmacokinetic studies (Frantz et al., 1988, 1991a). The actual dose applied was 24-25 mg/cm².

MATERIALS AND METHODS

The protocol, protocol amendments, and protocol deviation detailing the design and conduct of this study are included in Appendix 2.

Unlabeled Test Substance

One 100 ml bottle of EG, Lot No. A107247AY, CAS No. 107-21-1, was received on February 2, 1993, from Aldrich Chemical Company, Milwaukee, WI, and assigned BRRC Sample No. 56-42. The test substance was a slightly viscous liquid. The test substance was stored at room temperature. The purity of the test substance was determined by the supplier to be 99%.

Labeled Test Substance

A sample of 10 mCi of ¹⁴C-EG, Lot No. 930106, CAS No. 107-21-1, was received on January 7, 1993, from Wizard Laboratories, Davis, CA, and assigned BRRC Sample No. 56-7. The specific activity was 10 mCi/mmo. The test substance

was a liquid. The test substance was stored frozen. The radiochemical purity of the test substance was determined by BRRC to be approximately 97.12% at the time of receipt.

Reference Radiochemical

¹⁴C-Labeled ethanol, CAS No. 64-17-5, was used as a reference radiochemical to assess the relative penetration and overall integrity of the skin preparations. A sample of 0.25 mCi of ¹⁴C-ethanol, Lot No. 101H9221, specific activity 8.0 mCi/mmol was received on January 13, 1993 from Sigma Chemical Co., St. Louis, MO and assigned BRRC Sample No. 56-14. The purity of this reference radiochemical was determined by the supplier to be >98%.

Analytical Chemistry

Analytical Instrumentation

The gas chromatographic (GC) operating parameters for the radiochemical purity determinations of ¹⁴C-labeled EG are listed in Table 1. The high performance liquid chromatographic (HPLC) operating parameters for the radiochemical purity determinations of ¹⁴C-labeled EG are listed in Table 2.

Radiochemical Purity Determinations

An aliquot of ¹⁴C-labeled EG was added to a GC or HPLC vial. An injection volume of 1 µl of the test chemical was injected into the GC. An injection volume of 10 µl of the test chemical was injected into the HPLC. The total injected radioactivity of each sample on the GC was at least 6,000 DPM for detection of a 1% impurity above the 30 DPM background. The total injected radioactivity of each sample on the HPLC was at least 40,000 DPM for detection of a 1% impurity above the 200 DPM background.

The radiochemical purity of the ¹⁴C-labeled EG was determined by integrating the amount of radioactivity that comigrated with an authentic EG standard. ¹⁴C-EG radioactivity as a percent of the total radioactivity analyzed was the reported purity value.

Table 3 contains a summary of the results for the radiochemical purity determination of the ¹⁴C-labeled EG. The results show that ¹⁴C-labeled EG was at least 97% pure throughout the study.

Animals and Husbandry

Adult female CD-1[®] mice were obtained from Charles River Laboratories, Portage, MI. They were approximately 8 weeks old at the time of testing. Only female mice were used because this study was designed to provide information that could be used in conjunction with results from previous developmental studies on EG that used female mice.

Mice were housed in Room 238 of the Radiation Building from arrival to sacrifice for obtaining skin samples. Within 2 days of receipt, the animals were examined by a Clinical Veterinarian, who indicated that the animals were in good health and suitable for use. The acclimation period was at least 1 week.

All animals were assigned unique numbers. Mice were identified by ear tags and housed in plastic shoe boxes containing ALPHA-dri™ bedding (Shepherd Specialty Papers, Inc.). An automatic timer was set to provide fluorescent lighting for a 12-hour photoperiod (approximately 0500 to 1700 hours for the light phase). Temperature and relative humidity measurements (Cole-Parmer Hygrothermograph® Seven-Day Continuous Recorder, Model No. 8368-00, Cole-Parmer Instrument Co., Chicago, IL) were recorded during the study.

Tap water (Municipal Authority of Westmoreland County, Greensburg, PA) was available ad libitum and was delivered by water bottles. Water analyses were provided by the supplier, Halliburton NUS Environmental Laboratories (contaminants), Professional Services Industries, Inc. (asbestos), and Lancaster Laboratories, Inc. (aflatoxin) at regular intervals. EPA standards for maximum levels of contaminants were not exceeded. Pelleted, certified AGWAY® PROLAB® Animal Diet Rat, Mouse, Hamster 3000 (Agway Inc.) was available ad libitum. Analyses for chemical composition and possible contaminants of each feed lot were performed by Agway Inc., and the results were included in the raw data.

Human Skin Samples

Samples of fresh human female abdominal skin, from donors between the ages of 20 and 60 years of age, were obtained from The International Institute for the Advancement of Medicine (IIAM), Exton, PA. In obtaining these tissues, an abbreviated patient history was supplied and the specimens were tested for and shown to be free of Hepatitis B Virus (HBV) and Human Immunodeficiency Virus (HIV). The tissue was perfused in situ with saline and shipped on wet ice by a "same day" carrier. Upon receipt, specimens were prepared in the same manner as skin from mice (see below) and maintained in minimum essential medium (MEM/d-Valine) until placed into the skin penetration apparatus chamber. MEM is Eagle's medium with Earle's salts (Eagle, 1959), 25mM HEPES buffer (Gibco), and penicillin/streptomycin as antimicrobial agents.

Study Organization

No formal randomization procedure was used to assign animals to individual test groups because of the small number of animals used in each experiment. Animal selection was based upon relative uniformity of body weight. Animals not used for the study were euthanized or used for other testing. The fate of all animals not selected for use on this study was documented in the raw data.

The initial experiment was conducted on February 23, 1993 and the final experiment on October 2, 1993.

Administration of Test Substance and Reference Radiochemical

Preparation of Skin

Skin discs were prepared by a modification of the method described by Kao et al. (1983) for excised, full-thickness skin preparations. The mice were given an anesthetic overdose of Metofane® just prior to removal of the skin samples. The fur of these animals was clipped from the dorsal trunk in the thoracic region. A piece of clipped skin was removed and placed in a Petri dish

containing MEM. Skin pieces were gently scraped with a spatula or scalpel to remove fat and connective tissue. Two 1-inch discs from each mouse skin sample and 6, 1-inch discs from each human skin specimen were placed in a Petri dish containing MEM prior to positioning in the chamber.

Application of Dose

¹⁴C-EG was applied to skin under a gauze covering as either an undiluted liquid or a 50% (w/w) aqueous solution. The volume used was an amount that covered the entire exposed surface of the skin. In addition, an "infinite dose" or an amount of EG that would not be entirely absorbed by the skin during the 6-hr exposure period was used. The opening in the chamber was sealed with a glass stopper during experiments to prevent evaporative losses. A target radioactivity level of 5-10 µCi and a target amount of 22-28 ng/cm² of skin surface was applied to each skin disc. The concentrations of EG and the levels of radioactivity in dosing solutions were determined by liquid scintillation spectrometry (LSS).

The ¹⁴C-ethanol was radiodiluted with unlabeled ethanol and 250 µl applied as a 25% (v/v) aqueous solution in the same manner as the radiolabeled test substance to 1 skin disc from each animal skin preparation and to 2 skin discs from each human skin preparation. The concentrations of ethanol and the levels of radioactivity in dosing solutions were determined by LSS.

In Vitro Methodology

The techniques and apparatus used were a modification of the methods and skin chamber design previously described by Holland *et al.* (1984). A schematic representation of the chamber design used in this study is shown in Figure 1. The use of this chamber in a standardized method has been previously described and evaluated (Frantz *et al.*, 1990), and has been successfully used in previous *in vitro* studies (Tallant *et al.*, 1990; Frantz *et al.*, 1991b). The dermal surface of the skin preparations was bathed with the MEM medium described by Kao *et al.* (1983) at a flow rate of approximately 2.5 ml/hr for at least 30 minutes prior to application of test substance. ¹⁴C-EG was applied to the exposed epidermal surface (1.77 cm²) of each skin disc through the openings in the upper plate of the chamber. During the sampling period, media effluent was collected directly into empty scintillation vials, mixed with LSS cocktail and analyzed by LSS. At the end of 6 hr, skin pieces were removed from the chamber, placed in a Petri dish, and any unabsorbed dose was removed from the skin using water-wetted cotton swabs, which were placed in scintillation vials. Skin discs were then rinsed with water and rinse samples analyzed by LSS. The remaining skin samples were combusted in a biological oxidizer for inclusion in the calculation of total recovery. Similar methods were used to measure the skin penetration and mass balance of the reference chemical, ¹⁴C-ethanol.

Data Processing

Mass Balance Determinations

The recovery of the applied radioactive dose of both ¹⁴C-EG and ¹⁴C-ethanol was conducted at the termination of each experiment. This was done to determine a mass balance, which consisted of the amount that penetrated the

skin and into effluent media, the amount of unabsorbed dose which could be removed from the skin surface, and the amount of residual chemical left in the skin which was inaccessible to removal by the water rinse.

Determination of Permeability Constants

The fraction of ^{14}C -EG and ^{14}C -ethanol that penetrated skin (cumulative percent absorbed) was determined from the sum of effluent radioactivity divided by the mean amount of radioactivity applied to skin samples. The pseudo-steady-state penetration rate was computed by plotting interval ^{14}C values (normalized to mass per unit surface area) versus time and then taking the slope from the linear portion of the curve. A correlation coefficient of >0.99 was used as the criteria for including data in this linear regression calculation. From these results, the comparative rates of penetration for the 2 species were determined. These steady-state rate constants were also used to predict the percent penetration of both undiluted and the 50% aqueous solution of EG after 12 and 24 hr of continuous contact with both mouse and human skin.

Permeability constant (k_p) values were calculated as described by Bronaugh et al. (1982) for ^{14}C -EG and ^{14}C -ethanol applied to mouse and human skin. This was done by dividing the calculated steady-state penetration rates by the initial concentrations of the ^{14}C -EG or ^{14}C -ethanol applied to the skin.

RETENTION OF RECORDS

All raw data, documentation, the protocol, amendments, and a copy of the final report generated as a result of this study will be retained in the BRRC Archives for at least 10 years. A reserve sample of the unlabeled test substance was stored in the BRRC Archives. A reserve sample of the radiolabeled test substance was not retained due to the instability of the radiochemical.

RESULTS

Reference Radiochemical Skin Penetration

A 25% aqueous solution of ^{14}C -labeled ethanol was used as a reference radiochemical to evaluate the relative penetration and overall integrity of each skin preparation. The results for this chemical on mouse and human skin (Table 6 and Figure 2) showed that mouse skin penetration by ethanol was approximately 15% of the applied dose after 6 hr, while the penetration of ethanol through human skin averaged 7.5% over the same period. These results showed that the penetration of this reference radiochemical was in the normal BRRC historical range of 4-43% and 3-35% for mouse and human skin preparations, respectively. Therefore, the overall integrity of each skin preparation was normal and the skin preparations were suitable for use in this study. Individual data of these results are included in Appendix 1.

Mass Balance Determinations

Mouse Skin Preparations

An undiluted EG dose application to mouse skin preparations resulted in 10.8% of the applied ^{14}C dose being recovered in effluent samples (Table 4) after 6 hr. The largest recovery fraction for this undiluted dose was the unabsorbed dose residue (73.2%) removed from the skin surface at the end of the experiment. More of the dose was also recovered from the skin disc after it was rinsed (4.7%) and combusted (1.9%). The average recovery was $91.9 \pm 0.9\%$ for the 3 mouse skin samples evaluated.

For the 50% aqueous dose application of EG to mouse skin, about 4.4% of the ^{14}C was recovered in effluent samples by the end of the 6-hr measurement period (Table 4). Most of this dose was unabsorbed dose residue (62.6%) removed from the skin at the end of the experiment. An additional 5.8% was rinsed from the skin after removal of the unabsorbed dose and 1.6% was recovered after combusting the skin sample. An average of $75.0 \pm 1.6\%$ of the applied radioactivity was recovered. Individual data of these results are included in Appendix 1.

Human Skin Preparations

Of the undiluted ^{14}C -EG dose applied to human skin preparations, 0.14% was recovered in effluents (Tables 4 and 7). The largest recovery fraction was found in the unabsorbed dose residue (68.9%) that was removed from the skin surface at termination. More of this dose was recovered in the water rinse of the skin disc (5.3%) and in the skin after combustion (2.1%). The total recovery was $76.5 \pm 10.3\%$ for the 6 human skin samples evaluated.

When a 50% aqueous EG solution was applied to human skin preparations, 0.08% of the ^{14}C dose was recovered in the effluents (Tables 4 and 7). Most of the dose was recovered in the unabsorbed dose residue (83.6%) with 3.7% recovered in the skin rinse and 1.4% in the skin after combustion. Overall, $88.7 \pm 0.7\%$ of the original ^{14}C dose applied was recovered at the end of this 6-hr determination. Individual data of these results are included in Appendix 1.

Ethylene Glycol Skin Penetration

Mouse Skin Preparations

For the undiluted ^{14}C -EG applied to mouse skin, the time-course for the appearance of ^{14}C in effluent samples is illustrated in Figure 3. The steady-state (linear) phase of this curve extended from 3 through 6 hr. The lag time to reach a steady-state penetration rate was calculated to be 1.0 hr. The penetration rate was calculated to be $0.5204 \text{ mg/cm}^2/\text{hr}$ (Table 5). In addition, steady-state projections of a 12- and 24-hr absorbed dose were 24% and 50%, respectively (Table 6). The k_p for mouse skin penetration of undiluted EG was calculated to be $4.68 \times 10^{-4} \text{ cm/hr}$.

For the 50% aqueous ^{14}C -EG dose applied to mouse skin, the time-course for the appearance of ^{14}C in effluent samples is illustrated in Figure 4. The steady-state (linear) phase of this curve extended from about 3 hr through the end of the 6-hr measurement. The lag time to reach a steady-state penetration rate

was calculated to be 0.9 hr. The penetration rate was calculated to be 0.2179 mg/cm²/hr (Table 5). Steady-state projections of a 12- and 24-hr absorbed dose were 9.6% and 20.0%, respectively (Table 6). The k_p for mouse skin penetration of the 50% aqueous solution of EG was calculated to be 4.36×10^{-4} cm/hr. Individual data of these results are included in Appendix 1.

Human Skin Preparations

For the undiluted ¹⁴C-EG applied to human skin, the time-course for the appearance of ¹⁴C in effluent samples is illustrated in Figure 3. The steady-state (linear) phase of this curve extended from 4.5 through 6 hr in this measurement. The lag time to reach a steady-state penetration rate was calculated to be 3.1 hr. The penetration rate was calculated to be 0.0130 mg/cm²/hr (Table 5). Steady-state projections of a 12- and 24-hr absorbed dose were 0.48% and 1.13%, respectively (Table 6). The k_p for human skin penetration of undiluted EG was calculated to be 0.12×10^{-4} cm/hr.

For the 50% aqueous ¹⁴C-EG dose applied to human skin, the time-course for the appearance of ¹⁴C in effluent samples is illustrated in Figure 4. The steady-state (linear) phase of this curve extended from 4 hr through the end of the 6-hr measurement. The lag time to reach a steady-state penetration rate was calculated to be 3.1 hr. The penetration rate was calculated to be 0.0070 mg/cm²/hr (Table 5). Steady-state projections of a 12- and 24-hr absorbed dose were 0.26% and 0.60%, respectively (Table 6). The k_p for human skin penetration of the 50% aqueous solution of EG was calculated to be 0.14×10^{-4} cm/hr. Individual data of these results are included in Appendix 1.

DISCUSSION

The steady-state rate of undiluted EG absorption through mouse skin was approximately twice the rate of penetration of a 50% aqueous EG solution. This result was expected since the rate of chemical penetration through skin is known to be directly related to its concentration. The permeability constant (k_p) for both undiluted EG and the 50% aqueous solution of EG for mouse skin was approximately the same. Again, this latter result was expected since k_p is the measure of skin permeability of a chemical for a given species that is independent of chemical concentration. Similar differences were found for the penetration of undiluted EG and the 50% aqueous solution of EG through human skin. However, when these results are compared across species, it is apparent that the penetration of EG through mouse skin is significantly greater than that through human skin. The rates of penetration of both the undiluted EG and the 50% aqueous solution of EG were 30 to 40 times faster for mouse skin than for human skin. When comparing the k_p values for both the undiluted EG and the 50% aqueous solution of EG, again mouse skin was found to be 30 to 40 times more permeable to EG than human skin. The differences in the lag times of penetration before steady-state penetration rates were achieved (approximately 1 hr for mouse skin and 3 hr for human skin) again suggest that mouse skin is significantly more permeable to EG than human skin. Also, because of this difference in lag times, the amount of EG absorbed following a relatively short-term exposure situation would be additionally reduced for humans as compared to mice.

In summary, for both mouse and human skin, when EG was diluted 50% with water, the rate of EG penetration through skin was approximately half that of the undiluted EG. However, mouse skin was found to be 30 to 40 times more permeable to EG than human skin. Thus, results from percutaneous toxicity studies using the mouse may significantly overestimate the potential human health risk from cutaneous exposure to EG. In addition, since EG is commonly used as a solution in water, for example, as an engine coolant in automobiles, the total dose absorbed for a given cutaneous exposure to a water solution of EG would be less than that if the same exposure involved undiluted EG.

CONCLUSIONS

Human skin was found to be 30 to 40 times less permeable to EG than mouse skin in vitro and the lag time before steady-state rates of penetration were achieved was approximately 3 times longer for human skin than that for mouse skin. In addition, the rate of EG penetration through both mouse and human skin was reduced to approximately half when the EG was diluted to 50% with water. Based on these in vitro results, the potential toxicity resulting from cutaneous exposure to EG would probably be significantly less for humans than for mice and the total absorbed dose of EG in either species would be less for cutaneous exposures to water solutions of EG than for similar cutaneous exposures to undiluted EG.

REVIEW AND APPROVAL

Study Director: James D. Sun 5-20-94
 James D. Sun, Ph.D. Date

Director: John P. Van Miller 5-20-94
 John P. Van Miller, Ph.D., DABT Date

KEY PERSONNEL

Study Coordinator: M. J. Tallant

Scientist: S. W. Frantz

Additional personnel are listed in the raw data.

REFERENCES

Bronaugh, R. L., Stewart, R. F., Congdon, E. R. and Giles, A. L. (1982). Methods for in vitro percutaneous absorption. I. Comparison with in vivo results. Toxicol. Appl. Pharmacol. 62, 474-480.

Eagle, H. (1959). Amino acid metabolism in mammalian cell culture. Science, 130, 432.

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- Frantz, S. W., Tallant, M. J., and Ballantyne, B. (1991b). In vitro skin penetration of Polymer JR400: species comparisons following a single application to the excised skin of humans, Fischer 344 rats, B₆C₃F₁ mice, Hartley guinea pigs, and New Zealand White rabbits. *J. Toxicology: Cutaneous & Ocular Toxicol.* 10(3), 175-186.
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- Tallant, M. J., Frantz, S. W., and Ballantyne, B. (1990). Evaluation of the in vitro skin penetration potential of glutaraldehyde using rat, mouse, rabbit, guinea pig, and human skin. *The Toxicologist* 10, 256.
- Tyl, R. W., Fisher, L. C., Kubena, M. F., Losco, P. E., and Urbanic, M. A. (1989). Determination of a developmental toxicity "No Observable Effect Level" (NOEL) for ethylene glycol (EG) by gavage in CD-1[®] mice. *Teratology* 39(5), 487.

**Ethylene Glycol: Species Comparisons of In Vitro Skin Penetration
Following a Single Application to the Excised Skin of Humans and CD-1® Mice**

Protocol, Protocol Amendments and Protocol Deviation

(20 Pages)

**The Material Safety Data Sheet (Attachment 2)
that was included in the protocol has been removed due to
illegibility as a result of being reproduced for the report.**



BUSHY RUN RESEARCH CENTER

6702 Mellon Road, Export, Pennsylvania 15632-8902

Telephone (412) 733-5200
Telecopier (412) 733-4804

PROTOCOL

TITLE: Ethylene Glycol: Species Comparisons of In Vitro Skin Penetration Following a Single Application to the Excised Skin of Humans and CD-1[®] Mice

BRRRC PROJECT NUMBER: 92N1191

SPONSOR: Chemical Manufacturers Association
2501 M Street, NW
Washington, DC 20037

TESTING FACILITY: Bushy Run Research Center (BRRRC)
Union Carbide Chemicals
and Plastics Company Inc.
6702 Mellon Road
Export, PA 15632-8902

Reviewed and Approved by:

Bushy Run Research Center:


Stephen W. Frantz, Ph.D., DABT Date 12/31/92
Study Director


James D. Sun, Ph.D. Date 12-31-92
Associate Director


Linda J. Calisti, B.S. Date 1/20/93
Manager, Good Laboratory
Practices/Quality Assurance


John P. Van Miller, Ph.D., DABT Date 1/21/93
Director

Sponsor's Representative:


Kathleen M. Roberts Date 1-24-93
Manager, Ethylene Glycol Panel
Chemical Manufacturers Association

Union Carbide Chemicals and Plastics Company Inc.
Excellence Through Quality

EQ

Test Animals

Species and Strain CD-1[®] mice

Supplier Charles River Laboratories, Portage, MI

Rationale This species and strain has been chosen because of prior cutaneous testing experience in this laboratory.

Number and Sex At least 6 females.

Age The mice will be approximately 7-10 weeks at the time of testing.

Acclimation and Pretest Evaluations Shortly after their arrival at the laboratory, the animals will be transported to the room selected for holding prior to the study. Once in the room, the animals will be removed from the shipping cartons and examined. All animals with evidence of disease or physical abnormalities will be discarded and the reason for rejection will be recorded. If an unusually large number of animals shows evidence of disease or physical abnormalities, the entire shipment of animals will be rejected for use in the study.

The animals will be examined for health status by the BRRC clinical veterinarian. The animals will be acclimated to the laboratory environment for at least 3 days prior to obtaining the skin. Due to the short duration of the experiments, animals will only be inspected for signs of general good health.

Identification Each animal will be assigned a unique identification number and identified by metal ear tag prior to the initiation of the study. No formal randomization procedure will be used to assign animals to test groups because of the small number of animals used in each experiment. Records will be kept documenting the fate of all animals received for the study.

Husbandry

Conditions Prior to use in the study, animals will be housed in plastic shoe boxes containing ALPHA-driSM (Shepherd Specialty Papers, Inc). Temperature and humidity in the holding room will be recorded continuously using an automatic recorder. Temperature will be maintained at 66-77°F and relative humidity will be maintained at 40-70%. Temperature and humidity will be checked by a technician at each room check and a record will be kept indicating that it was done. Appropriate corrective action will be taken whenever readings outside the specified limits are observed.

The accuracy of the temperature and humidity recording devices will be checked periodically and calibrated when necessary. The verification and calibration data will be recorded. Any time the continuous recording equipment is found to be malfunctioning, the temperature and humidity of the animal room will be manually recorded at each room check.

An automatic timer will be set to provide fluorescent lighting for a 12-hour photoperiod (approximately 0500 to 1700 hours for the light phase). In the animal room, there will be at least 10 air changes each hour.

Diet Pelleted, certified AGWAY® PROLAB® Animal Diet Rat, Mouse, Hamster 3000 (Agway Inc.) will be available ad libitum. The analyses of chemical composition and possible contaminants of each batch of diet will be performed by Agway Inc. and the results of the analyses will be reviewed by the Study Director.

Water Tap water (Municipal Authority of Westmoreland County, Greensburg, PA) will be available ad libitum by an automatic watering system with demand control valves mounted on each rack. Water pressure and function of the individual cage rack systems will be checked at each room check, and a record will be kept indicating it was done. Drinking water contaminant levels will be measured at regular intervals according to EPA specifications, to include the "priority" pollutants, and will comply with human drinking water requirements. The results of the analyses will be reviewed by the Study Director.

Human Skin Samples Samples of fresh human female abdominal skin will be obtained from a suitable source. In obtaining these tissues, a complete patient history will be supplied. Tissues will be tested for and shown to be free of Hepatitis B Virus (HBV) and Human Immunodeficiency Virus (HIV). It will also be specified that the tissues must be fresh with regard to metabolic capacity. This will include that the tissue will be perfused in situ with saline and shipped on wet ice by a "same day" carrier. Upon receipt, specimens will be prepared as for skin from other species and maintained in minimum essential medium (MEM/d-Valine)* until placed into the chamber.

* Eagle's medium with Earle's salts (Eagle, H. (1959, Science, 130, 432) and 25mM HEPES buffer (Gibco); with penicillin/streptomycin as antimicrobial agents.

Administration of Test Substance

**Preparation
of Skin**

The mice will be given an overdose of anesthesia. The fur will be clipped from the dorsal trunk of the animal in the thoracic region. Care will be taken to avoid abrading the skin. For processing of animal skin, a piece of clipped skin (approximately 6 x 6 cm) will be removed and placed in a petri dish containing MEM to keep it moist; human skin will be received in MEM from the hospital and processed similarly to animal skin. The skin piece will be placed on a dissecting board and scraped with a spatula to remove fat and connective tissue. Two 1-inch discs from the animal skin piece and six 1-inch discs from human skin specimens will be removed and placed in a petri dish with several drops of MEM to keep moist before placing in the chamber.

**Application
of Dose**

The ^{14}C -EG will be applied as either undiluted or as a 50% aqueous solution with Milli-Q[®]-filtered water (CAS No. 7732-18-5) in an amount sufficient to produce a target radioactivity level of at least 5-10 $\mu\text{Ci/skin}$ preparation and a target concentration of approximately 215-230 mg/cm^2 of skin surface; the actual amounts dosed will correspond to the previous in vivo cutaneous doses (Frantz et al. 1988, 1991b) and will be documented in the study records and final report. Aqueous doses will also be applied in the same mg/cm^2 amounts as in the previous in vivo studies. Dosing solutions will be analyzed only by quantification of radioactivity by liquid scintillation spectrometry.

Study Design

The following methodology will be used to conduct the experiments defined in the study design summary table contained in Attachment 1 to this protocol.

In Vitro Methodology

Discs of skin will be prepared by a modification of the method described by Kao et al. (1983) for full-thickness excised skin preparations. The technique used is a modification of the methods and skin chamber described by Holland et al. (1984) which has been verified by Frantz et al. (1990) and successfully used in previous studies (Frantz et al., 1991a; Tallant et al., 1990). The skin specimens will be placed into the chamber and the dermal surface of the skin preparations will be perfused with the MEM described by Kao et al. (1983) at a flow rate of approximately 2.5 ml/hr for at least 30 minutes prior to application of test substance. Test substances will be applied either as a neat material or in an aqueous solution to the exposed epidermal surface (1.77 cm^2) of each skin

disc through the openings in the upper plate of the chamber. Media effluent will be voided directly into empty scintillation vials and an aliquot of the effluent or the entire effluent will be dissolved into liquid scintillation cocktail. These samples will then be counted for ^{14}C activity in a liquid scintillation spectrometer. The amount of test substance which penetrates will be presented as cumulative percent absorbed radioactivity determined from the sum of counts found in the effluent media divided by the mean dosing solution counts. The penetration rate will be computed by normalizing interval radioactivity values to an hourly rate and plotting these values at the midpoint of the measurement interval.

Upon termination of the experiment, the skin pieces will be removed from the chamber and placed in a petri dish for further removal of the remaining dose; any unabsorbed dose will be removed (using water-wetted, cotton swabs) from the skin and placed in a scintillation vial. These samples will be radioassayed by liquid scintillation spectrometry for inclusion in the calculation of total balance. The skin samples will be stored frozen until analyzed (combusted directly in a biological oxidizer or pulverized at liquid N_2 temperatures in a freezermill).

Data Processing

The amount of test substance which penetrates skin will be presented as cumulative percent absorbed EG and will be determined from the amount of test substance found in the effluent media divided by the amount of test substance applied to each skin preparation. The pseudo steady-state penetration rate will be determined from interval test substance values, calculated as the cumulative mg/cm^2 absorbed, and an hourly rate will be taken from the linear segment of the curve after plotting these values versus time (data plotted at the endpoint of the measurement interval). Permeability constants (k_p) for H_2O and ethanol will be calculated using the following formula described by Bronaugh *et al.* (1982):

$$k_p \text{ (in cm/hr)} = \frac{\text{Steady State Absorption Rate (mg/cm}^2\text{/hr)}}{\text{Initial Dose Concentration (mg/cm}^3\text{)}}$$

Additional calculations of the projected amount of EG which would penetrate the skin preparation at contact times beyond the 6-hr determination will be calculated using linear regression and corrected for surface area of absorption to derive the amount absorbed at the projected time point.

BTRC Project 92N1191
Page 8

Analytical Chemistry Adaptation of an analytical method from Frantz et al. (1991b) for the characterization/identification of the chemical which penetrates into effluent media will be included. The number of samples for analysis will be selected at the discretion of the Study Director, and a detailed description of the methods employed will be documented in the study record and the final report.

ALTERATION OF PROTOCOL

Alterations to this protocol may be made as the study progresses. No changes in the protocol will be made without the specific written request or consent of the Sponsor. In the event that the Sponsor authorizes a protocol change verbally, such change will be honored. However, it then becomes the responsibility of the Sponsor to follow such verbal change with a written verification. BTRC reserves the right to revise the protocol or deviate therefrom solely at the discretion of the Study Director if prior approval of the Sponsor cannot be obtained and the integrity of the study is considered in jeopardy. In this event, the Sponsor will be notified of the alteration as soon as possible, and documentation of the change will be the responsibility of the Study Director.

RECORDS

All raw data, the protocol and any amendments, and a copy of the final report generated as a result of this study will be retained in the BTRC Archives for at least 10 years.

Prior to discarding any of the above data or materials, the Sponsor will be contacted and given the option of obtaining it or arranging for continued storage. All data and materials mentioned above will remain the sole property of the Sponsor and can be removed from BTRC at the Sponsor's discretion.

DISPOSAL OF RADIOACTIVE SAMPLES

Samples will be disposed of by laboratory SOP when the final report is issued.

REPORT

Draft Report

An unaudited draft of the final report will be submitted to the Sponsor approximately 4 months after the completion of the last experiment. This report will be a comprehensive report which will include all information necessary to provide a complete and accurate description and evaluation of the test procedures and results. It will include: a summary; appropriate text discussions of the experimental design, materials and methods and results; and summary mean and individual tables of radioactive recoveries and graphic representation of skin penetration. Two copies of the draft report will be provided to the Sponsor.

Final Report

The draft final report will be reviewed by the Sponsor, and comments on the report will be provided to BRRC within 8 weeks from the date of submission of the draft version. BRRC will consider these comments in preparing the final report. Assuming the Sponsor's comments are received at the specified time and no major revisions are required, BRRC will submit a final report within 12 weeks of issuance of the draft report. The final report will be audited by the Quality Assurance Unit and contain a signed quality assurance statement. One original and one copy of the final report will be submitted to the Sponsor.

ANIMAL USE POLICY

It is the goal of BRRC, through the establishment and activities of the Institutional Animal Care and Use Committee, to comply with the U.S. Animal Welfare Act and the subsequent rules promulgated by the U.S. Department of Agriculture and in effect on the date of this protocol. It has been determined that the work described herein minimizes the number of animals used, is necessary, and uses the most appropriate species and strain in order to provide meaningful results and the most useful information for comparative purposes relative to previous studies. Furthermore, this study will be conducted humanely, and to the best of our knowledge, neither unnecessarily duplicates any previous work, nor can it be accomplished using currently available, validated nonanimal models.

GOOD LABORATORY PRACTICE COMPLIANCE

BRRC, through the administration of a quality assurance program by the Good Laboratory Practice Committee and Quality Assurance Unit, assures compliance of all phases of studies conducted at BRRC with existing regulations and generally accepted good laboratory practices.

The study will be subjected to periodic inspections and the final report will be reviewed by the BRRC Quality Assurance Unit.

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- Bronaugh, R. L., Stewart, R. F., Congdon, E. P., and Giles, A. L. (1982). Methods for in vitro percutaneous absorption studies. I. Comparison with in vivo results. Toxicol. Appl. Pharm. 62, 474-480.
- Frantz, S. W., Jensen, C. B., Grosse, C. N., Tallant, N. C., and Baskitt J. L. (1989). Ethylene Glycol: Studies for comparison of pharmacokinetics and material balance following single intravenous, oral and cutaneous administration to male and female Sprague-Dawley rats. BRRC Project Report No. 51-543.
- Frantz, S. W., Dittenger, D. A., Eisenbrandt, D. L., and Watanabe, P. G. (1990). Evaluation of a flow-through in vitro skin penetration chamber method using acetone-deposited organic solids. J. Toxicology: Cutaneous & Ocular Toxicol. 9(4), 377-389.

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Frantz, S. W., Tallant, M. J., and Beskitt J. L. (1991b). Ethylene Glycol: Comparisons of pharmacokinetic and material balance studies following single intravenous, peroral and percutaneous administrations to female CD-1 mice. BRRC Project Report No. 53-550.

Holland, J. M., Kao, J. Y., and Whitaker, M. J. (1984). A multi-sample apparatus for kinetic evaluation of skin penetration in vitro: The influence of variability and metabolic status of the skin. *Toxicol. Appl. Pharmacol.* 72, 272-280.

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Loden, M. (1986). The in vitro permeability of human skin to benzene, ethylene glycol, formaldehyde, and n-hexane. *Acta Pharmacol. et Toxicol.* 58, 382-389.

Price, C. J., Kimmel, C. A., Tyl, R. W., and Marr, M. C. (1985). The developmental toxicity of ethylene glycol in rats and mice. *Toxicol. Appl. Pharmacol.* 81, 113-127.

Tallant, M. J., Frantz, S. W., and Ballantyne, B. (1990). Evaluation of the in vitro skin penetration potential of glutaraldehyde using rat, mouse, rabbit, guinea pig, and human skin. *The Toxicologist* 10, 256.

Tyl, R. W., Fisher, L. C., Kubena, M. F., Louco, P. E., and Urbanic, M. A. (1989). Determination of a developmental toxicity "No Observable Effect Level" (NOEL) for ethylene glycol (EG) by gavage in CD-1 mice. *Teratology* 39(5), 487.

Ethylene Glycol In Vitro Skin Protocol

<u>Experiment</u>	<u>Number of Animals On Study</u>	<u>Description</u>
1. Probe Mouse Skin Study Segment	3*	Preliminary evaluation of 50% aqueous solution (215-280 mg/cm ²) to determine <u>in vitro</u> time-course; 6 hr duration, 15 minute collection intervals.
2. Definitive Mouse Skin Investigation	3*	Definitive study with 50% aqueous EG solution.
3. Definitive Mouse Skin Investigation	3*	Definitive study with undiluted EG.

<u>Experiment</u>	<u>Numbers of Human Skin Samples</u>	<u>Description</u>
4. First Female Human Skin Sample	6 skin preparations	Evaluation of aqueous and undiluted EG (plus positive controls).**
5. Second Female Human Skin Sample	6 skin preparations	Evaluation of aqueous and undiluted EG (plus positive controls).**
6. Third Female Human Skin Sample	6 skin preparations	Evaluation of aqueous and undiluted EG (plus positive controls).**

* Females only to be used; 2 skin preparations/animal.

** N=2 skin preparations for aqueous and undiluted EG, plus n=2 controls.

Note: This design may be modified following consultation with the Sponsor regarding the results of the probe mouse skin investigation. Such modifications will be formally amended to the study protocol.

The Material Safety Data Sheet (Attachment 2; Pages 12-17)
that was included in the protocol has been removed due to
illegibility as a result of being reproduced for the report.



The Dow Chemical Company

Developmental & Reproductive Toxicology

December 1, 1994

Tel: 517/636-2580

Fax: 517/638-9863

Division of C&E
CHEMSTAR DIVISION
From Kathleen Roberts
Ref. No. EG TRTG
Date 12/6/94

Kathleen Roberts, Manager
Ethylene Glycol Panel
Chemical Manufacturer's Association
2501 M Street NW
Washington, DC 20037

Dear Kathleen,

Enclosed please find a final report for the rat whole embryo culture study (EG-15.0-EMB-DOW) for review by the EG TRTG. As we discussed on the telephone, I would appreciate if comments could be sent to me by December 16, 1994 so that I can wrap things up by the the years' end.

Thanks for your assistance.

Sincerely,

Edward W. Carney, Ph.D
Sr. Research Toxicologist

*****DRAFT*****

THE DOW CHEMICAL COMPANY
STUDY ID: K-002558-010
PAGE 1 OF

RAT WHOLE EMBRYO CULTURE: IDENTIFICATION OF
PROXIMATE TOXICANT FOR ETHYLENE GLYCOL
DEVELOPMENTAL TOXICITY STUDY

Data Requirement

There are no specific data requirements for this study

Author(s)

E. W. Carney, A. B. Liberacki, M. Bartels and W. J. Breslin

Study Completion Date

December , 1994

Performing Laboratory

The Toxicology Research Laboratory
Health and Environmental Sciences
The Dow Chemical Company
Midland, Michigan 48674

Laboratory Project Study ID

K-002558-010

CMA Project #

EC-15.0-EMB-DOW

*****DRAFT*****

THE DOW CHEMICAL COMPANY
STUDY ID: K-002558-010
PAGE 2

Compound: ETHYLENE GLYCOL

**Title: RAT WHOLE EMBRYO CULTURE: IDENTIFICATION OF
PROXIMATE TOXICANT FOR ETHYLENE GLYCOL
DEVELOPMENTAL TOXICITY STUDY**

**Page Two is reserved for the inclusion of a Statement of Data Confidentiality
Claims as required by the United States Environmental Protection Agency
(EPA) under the Provisions of PR NOTICE 86-5 issued July 29, 1986.**

*****DEP A-T*****

THE DOW CHEMICAL COMPANY
STUDY ID: K-002558-010
PAGE 3

COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

Compound: ETHYLENE GLYCOL

Title: RAT WHOLE EMBRYO CULTURE IDENTIFICATION OF
PROMOTE TOXICANT FOR ETHYLENE GLYCOL
DEVELOPMENTAL TOXICITY STUDY

At the time this study was conducted, it was not subject to Good Laboratory Practice Standards, but was, nevertheless, conducted to the extent possible in accordance with existing and proposed standards.

United States Environmental Protection Agency, Title 40 Code of Federal Regulations, Part 160, FEDERAL REGISTER, August 17, 1989.

Japan Ministry of Agriculture, Forestry and Fisheries,
59 NohSan, Notification No. 3350
Agricultural Production Bureau
10 August 1984

Organisation for Economic Co-Operation and Development,
ISBN 92-64-12367-9, Paris 1982

E. W. Carney, Ph.D. (Date)
Study Director

J. T. Young, D.V.M., M.S. (Date)
Director
The Toxicology Research Laboratory

Sponsored by:

K. Roberts (Date)
Manager, Ethylene Glycol Panel
Chemical Manufacturer's Association

*****DRAFT*****

THE DOW CHEMICAL COMPANY
STUDY ID: K-002558-010
PAGE 4

Compound: ETHYLENE GLYCOL

Title: RAT WHOLE EMBRYO CULTURE: IDENTIFICATION OF
PROXIMATE TOXICANT FOR ETHYLENE GLYCOL
DEVELOPMENTAL TOXICITY STUDY

Page Four is reserved for the inclusion of a Flagging Statement as required by the United States Environmental Protection Agency (EPA) under the provisions of PR Notice 86-5 issued July 29, 1986 and the Code of Federal Regulations, Title 40, Part 158.34.

QUALITY ASSURANCE STATEMENT

Compound: RAT WHOLE EMBRYO CULTURE

Title: RAT WHOLE EMBRYO CULTURE: IDENTIFICATION OF
PROXIMATE TOXICANT FOR ETHYLENE GLYCOL
DEVELOPMENTAL TOXICITY STUDY

This study was examined for conformance with Good Laboratory Practices as published by the U.S. Environmental Protection Agency, and the Food and Drug Administration. 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:

Study Completion Date:

TYPE OF AUDIT:	DATE OF AUDIT:	DATE FINDINGS REPORTED TO STUDY DIRECTOR/MANAGEMENT:
Preliminary protocol	02/10/94	02/10/94
Final protocol	08/25/94	08/25/94
Protocol, study conduct	08/01/94	08/04/94
Draft report	12/05/94	

ARCHIVING: In accordance with the above regulations, all raw data and the final report will be filed in the testing facility archives by the indicated study completion date.

K. T. Haut, B.S. (Date)
Quality Assurance
Health and Environmental Sciences
The Dow Chemical Company
1803 Building
Midland, Michigan 48674

SIGNATURE PAGE

Compound: RAT WHOLE EMBRYO CULTURE

**Title: RAT WHOLE EMBRYO CULTURE: IDENTIFICATION OF
PROXIMATE TOXICANT FOR ETHYLENE GLYCOL
DEVELOPMENTAL TOXICITY STUDY**

E. W. Carney, Ph.D. (Date)
Study Director

A. B. Liberacki, B.S. (Date)
Project monitor

M. J. Bartels, Ph.D. (Date)
Analytical chemist

W. J. Breslin, Ph.D. (Date)
Co-investigator

Reviewed by:

R. A. Corley, Ph.D. (Date)

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SUMMARY

Previous studies have shown that the onset of ethylene glycol (EG)-induced developmental toxicity corresponds with a dose-dependent shift in EG metabolism, leading to accumulation of a weak acid metabolite, glycolic acid (GA), and a resultant metabolic acidosis. Although neutralization of acidosis ameliorates some of EG's developmental toxicity, it was not known if other factors were also involved. To gain more insight as to the proximate toxicant for EG-induced developmental toxicity, the effects of GA vs. parent EG were compared in whole embryo culture. Day 10.5 rat embryos were cultured for 46 h in media containing 0.5, 2.5, 12.5, 25 or 50 mM EG (all pH 7.5) or with GA at these same concentrations (pH 7.5-4.3). These concentrations spanned the range of peak maternal plasma levels observed at a NOEL dose (11 mM EG, < 2 mM GA), LOEL dose (21 mM EG, 2 mM GA) or dose causing malformations (43 mM EG, >13 mM GA). EG up to 50 mM and GA at ≤ 2.5 mM were essentially without effect, while 12.5 mM GA significantly inhibited embryo growth, protein content and morphology score. Higher GA levels were lethal. Dysmorphogenesis, mainly of the craniofacial region, was found in 70% of the 12.5 mM GA embryos vs. 0% in controls. To determine the role of reduced pH in vitro, a second experiment was conducted in which embryos were cultured in 12.5 mM GA (pH 6.7), 12.5 mM sodium glycolate (pH 7.4) or in control medium (pH 7.4 or 6.7). Similar to Experiment 1, 12.5 mM glycolic acid (pH 6.7) inhibited embryo growth, protein content and morphology score, with 67% of the embryos exhibiting dysmorphogenesis. Glycolate at pH 7.4 similarly affected growth and development, with 58% of the embryos exhibiting dysmorphogenesis. Control medium at pH 6.7 caused significant, but less severe, effects on growth and development and an 8% dysmorphogenesis rate. These results support the hypothesis that EG developmental toxicity requires a shift in metabolism leading to high levels of GA. However, GA appears to be acting both through its own intrinsic toxicity and via induction of maternal metabolic acidosis.

INTRODUCTION

Background.

It is well known that large oral bolus doses of EG can lead to systemic and developmental toxicity. However, potential exposures from typical industrial and consumer uses of EG are much more likely to occur via the dermal or inhalation routes. In order to address questions regarding the developmental toxicity potential of various exposures to EG, a working model was developed by the EG Toxicology Research Task Group of the Chemical Manufacturer's Association which integrated the animal developmental toxicity data with the extensive data base describing the absorption, metabolism, pharmacokinetics and mechanisms of systemic toxicity for EG in both animals and humans (Carney, 1994).

One of the critical features of the hypothesized model is that developmental toxicity is *not* caused by EG directly, but by its weak acid metabolite, glycolic acid (GA). Generally this metabolite is present at low levels. However, the oxidation of GA by glycolic acid oxidase is a rate limiting step in the EG metabolism pathway and exhibits saturation kinetics at high substrate concentrations. Thus, large bolus doses of EG lead to accumulation of this metabolite (see table below). Interest in GA as the possible developmental toxicant is based on the following evidence: (1) a high degree of correspondence exists between the onset of the metabolic shift leading to GA accumulation and the occurrence of developmental toxicity (2) doses given by gavage (high dose-rate) are much more effective at inducing developmental toxicity than the same dose administered in the diet (i.e. lower dose-rate) and (3) GA is known to be the proximate toxicant for EG-induced acute systemic toxicity.

Peak plasma levels of EG and GA following single oral doses of EG in female Sprague-Dawley rats.

Dose (mg/kg)	Developmental effects <i>in vivo</i>	C _{max} EG ¹ (mM/L)	C _{max} GA (mM/L)
10	None	0.2	0.001 ²
500	NOEL	(10.7)	na
1000	LOEL (decr. fetal weight)	21.4	2 ²
2500	Malformations	(53.6)	>13 ³

¹ From Frantz et al. (1994). Values in parentheses are interpolated via linear regression analysis.

² From Frantz et al. (1994). na=data not available.

³ From Hewlett et al. (1989). C_{max} for 2000 mg/kg oral dose, male Sprague-Dawley rat = 13 mM/L.

Another factor which comes into play upon accumulation of GA is the induction of a maternal metabolic acidosis. Metabolic acidosis has been amply shown to be the cause of the major systemic manifestations of acute EG poisoning in animals and humans. Treatments which either inhibit glycolic acid formation (e.g. 4-methylpyrazole) or neutralize the acidosis (bicarbonate infusion) are effective in preventing toxicity (Clay and Murphy, 1977; Jacobsen et al., 1984; Hewlett et al., 1989; Karlson-Stiber and Persson, 1992). Khera (1991) infused bicarbonate into pregnant rats injected subcutaneously with 3,333 mg/kg of EG on gestation day 11 and found that many, but not all, developmental effects were ameliorated. This indicated that metabolic acidosis was responsible for at least some of the developmental effects following high dose EG exposure.

Incorporating this evidence, the working model hypothesized that large oral bolus doses of EG caused developmental toxicity due to rapid absorption by this route (high dose-rate), leading to high substrate concentrations saturating GA oxidation, increased blood levels of GA and induction of maternal

metabolic acidosis. Conversely, the lack of developmental toxicity following most dermal and inhalation exposures was considered to be due to the slower dose rate associated with these routes. Under such conditions, plasma levels of EG would be low, allowing for complete oxidation to carbon dioxide without accumulation of GA or induction of metabolic acidosis.

Areas of uncertainty.

Although metabolic acidosis was shown to be responsible for some of EG's effects, the fact that bicarbonate co-administration did not completely ameliorate the developmental toxicity associated with EG administration left open the possibility that other factors were also involved. It has been suggested that serum hyperosmolality (Khera, 1991), a direct toxicity of parent EG (Grafton and Hansen, 1987) or a direct toxicity of GA (Carney, 1994) might also be responsible for some of EG's toxicity. The effect of hyperosmolality on embryo development had not been tested, nor had there been any studies on the developmental toxicity of GA. Although one study (Grafton and Hansen, 1987) tested the effects of parent EG on rat postimplantation embryos developing in culture, this study was not considered relevant for risk assessment due to the extremely high exposure concentrations used in the culture medium (531 and 710 mM/L). These concentrations were 25-33 times higher than peak blood concentrations following an acutely toxic gavage dose of 1000 mg/kg in rats. Lower exposures which might reflect plausible blood levels in vivo were not tested.

Objectives.

This study addressed the above areas of uncertainty through the use of rat whole embryo culture. Experiment 1 compared the effects of EG and GA on rat gestation day 10.5 whole embryo cultures in order to test the hypothesis that GA, not parent EG, is the proximate developmental toxicant. Concentrations tested spanned the peak plasma concentrations of EG and GA associated with NOEL, LOEL and high doses of EG given in vivo, thus linking the in vitro data with in vivo pharmacokinetics. Experiment 2 addressed the contribution of reduced medium pH in causing effects following GA exposure

in culture. Finally, measurement of medium osmolality was used to gain perspective on the effects of hyperosmolality on embryo development.

Whole embryo culture was chosen as the appropriate test system for a number of reasons. First, the use of an *in vitro* system allows one to control variables such as pH and test material concentration. Secondly, embryos at this stage of development lack the enzymes necessary to metabolize EG (this is accomplished by the alcohol/aldehyde dehydrogenase system of maternal liver). Therefore, the confounding effects of maternal metabolism are avoided (parent EG in the culture medium remains as EG). Third, the whole embryo culture system utilized intact conceptuses cultured within their visceral yolk sac and amniotic membranes. Development in this system closely mimics *in vivo* development during the period of culture (Brown, 1990; Carney, unpublished data).

Statement of GLP practice.

This study was conducted to the greatest extent possible in accordance with the Good Laboratory Practice (GLP) guidelines listed below. However, as it was designed as an exploratory study for which there are no specific regulatory testing guidelines, it was not intended to meet all specific GLP requirements listed therein:

- (1) United States Environmental Protection Agency (USEPA): FIFRA Good Laboratory Practice Standards (Final Rule); Toxic Substances Control Act; Good Laboratory Standards (Final Rule)
- (2) Organisation for Economic Co-Operation and Development (OECD): Good Laboratory Practice Procedures (OECD, 1982)
- (3) United States Food and Drug Administration (USFDA): Good Laboratory Practice Regulations for NonClinical Studies (FDA, 1988)

(4) Standard Operating Procedures (SOPs) of The Toxicology Research Laboratory of the 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 is required for the conduct of this study was reviewed and given full approval by the Institutional Animal Care and Use Committee (IACUC). The IACUC determined that the proposed Activity is in full accordance with these Final Rules. The IACUC assigned File No. Metabolism 02 to this ACUA.

Quality assurance statement.

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

MATERIALS AND METHODS

Culture methods.

On gestation day 10.5 (day of sperm-positive vaginal smear or copulation plug designated day 0.5), pregnant rats were anesthetized with methoxyflurane and exsanguinated via the abdominal aorta. Gestation day 10.5 was chosen to correspond with previously described whole embryo culture (Grafton and Hansen, 1987) and single dose teratology/maternal homeostasis (Khera, 1991) studies. Rat serum was prepared by immediate centrifugation followed by heat-inactivation of complement proteins for 30 minutes at 56° C. Serum was stored at -80 C for use in subsequent experiments. Serum from male rats was also used when necessary. Previous work in this laboratory and others has shown no difference between these serum sources in supporting embryo development (Carney, unpublished data; Cockroft, 1990).

Dissection and culture of embryos generally was conducted as described by Cockroft (1990). Briefly, conceptuses were dissected free of decidual tissue and Reichert's membrane, leaving the visceral yolk sac and ectoplacental cone intact. Early somite stage embryos were then transferred to culture bottles containing pre-warmed, pre-gassed test or control culture media. Pre-somite or advanced somite stage embryos were not cultured. The culture bottles were maintained in a continuous gas flow rotating culture unit (BTC Engineering, Cambridge, England) at 37° C for approximately 46 hours. Among the embryologic structures undergoing major development during this period included the yolk sac, allantois, heart, brain, ears, eyes, olfactory system, facial structures, neural tube, limb buds and vertebral column (somites).

Culture medium.

The culture medium was composed of 75% immediately centrifuged, heat-inactivated serum collected from adult rats (Cockroft, 1990) and 25% Dulbecco's Modified Eagle's Medium (D-MEM).

Test materials.

Chemical name:	Ethylene glycol (EG)	Glycolic acid (GA)
CAS number:	107-21-1	79-14-1
Formula:	$\text{OH-CH}_2\text{-CH}_2\text{-OH}$	$\text{OH-CH}_2\text{-COOH}$
Formula weight:	62.01 daltons	76.05 daltons
Source:	Aldrich Chemical Co.	Aldrich Chemical Co.
Lot:	07053JZ	06608MZ
Purity:	99.98%	99.7%
Chemical name:	Sodium glycolate (Na-GA)	
CAS number:	2836-32-0	
Formula:	$\text{OH-CH}_2\text{-COO}^- \text{Na}^+$	
Formula weight:	98.03 daltons	
Source:	Pfaltz & Bauer, Inc.	
Lot:	S05530	
Purity:	>98+%	

Positive control material.

Chemical name: Sodium valproate (VPA)
CAS number: 1069-66-5
Formula: $\text{CH}_3\text{-CH}_2\text{-CH}_2\text{-CH}(\text{CH}_2\text{-CH}_2\text{-CH}_3)\text{-COO}^- \text{Na}^+$
Formula weight: 166.2 daltons
Source: Sigma Chemical Co.
Lot: 067F0250
Purity: 99.4%

Concentrated (100x) stock solutions of these test materials were prepared in D-MEM and added directly to the appropriate culture bottles at a volume of 10 $\mu\text{l}/\text{ml}$ to achieve the desired final concentrations.

Experimental design.

This study was conducted as two separate experiments. Experiment 1 compared the toxicity of EG with that of its weak organic acid metabolite, GA. Embryo development in media containing 0.5, 2.5, 12.5, 25 or 50 mM/L of EG or GA was compared to embryos grown in control media. Valproic acid (1 mM) was used a positive control (Kao et al, 1981). Ten embryos were cultured per group with exposure to test materials continuous throughout the culture period.

Experiment 2 was designed to determine if GA caused effects on cultured embryos via acidification of medium pH or via direct chemical toxicity. Embryos were cultured for 46 h in one of four experimental media: (1) control medium at pH 7.41, (2) control medium titrated to a pH 6.74 with HCl, (3) medium containing 12.5 mM glycolic acid (pH 6.74) or (4) medium containing 12.5 mM sodium glycolate (pH 7.42). The 12.5 mM concentration represented the lowest-observed-effect concentration (LOEC) from Experiment 1. There were twelve embryos per group and exposure was continuous throughout the culture period.

To minimize the influence of litter effects, embryos from the same donor rat were distributed as equally as possible amongst the treatment groups. Due to limitations in the number of embryos which could be dissected on a given

day, five replicates of Experiment 1 and two replicates of Experiment 2 were necessary to achieve the desired number of embryos. All experimental treatments were equally represented in each of these replicates. An additional two replicates of Expt. 1 (replicates 1 and 2) were excluded from the study due to technical problems. In one case a microbial contamination occurred in the cultures while in another, animals of the incorrect gestational age were inadvertently shipped by the supplier.

End points.

Evaluation of development was essentially as described by Brown (1990). Upon completion of the culture period, embryos were evaluated for the presence of a beating heart and an active visceral yolk sac circulation. Embryos with a beating heart were considered viable embryos. To ensure a thorough examination for any developmental abnormalities, morphology was assessed using the Brown-Fabro scoring system (Brown and Fabro, 1981). Growth was assessed by measurement of visceral yolk sac diameter, crown-rump length and head length with the aid of a calibrated eyepiece reticle. Protein contents of embryo and visceral yolk sac were determined by the Bradford method as described by Brown (1990) and modified for microtiter plate reader as described by Stark et al. (1987).

Statistical analysis.

Embryo growth measures, protein contents, somite number and morphological score 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 ANOVA was significant, analysis by Dunnett's test or the Wilcoxon Rank-Sum test with Bonferroni's correction was performed, respectively. Non-viable embryos were excluded from the statistical analyses for growth measures and morphological score. Percentage data were analyzed using Fisher's exact test.

The nominal alpha levels were as follows:

Bartlett's Test (Winer, 1971)	a= 0.01
Parametric ANOVA (Steel and Torrie, 1960)	a= 0.10
Nonparametric ANOVA (Hollander and Wolfe, 1973)	a= 0.10
Dunnett's Test (Winer, 1971)	a= 0.05, two-sided
Wilcoxon Rank-Sum Test (Hollander and Wolfe, 1973)	a= 0.05, two-sided with Bonferroni's correction (Miller, 1966)
Fisher's Test (Siegel, 1956)	a= 0.05, one-sided

Test animals and husbandry.

Adult female time-mated CD (Sprague-Dawley) rats were obtained from Charles River Breeding Laboratory (Portage, MI). The day on which a copulation plug was observed by the supplier was designated gestation day 0.5. This strain of rat was selected as a test species based on its general acceptance for developmental toxicity testing, the availability of reliable commercial source and the use of this strain in previous whole embryo culture and in vivo homeostasis/teratology studies with EG. Upon arrival at the laboratory¹, animals were examined by a veterinarian and acclimated to the laboratory environment for at least five days prior to use.

The rats were housed in stainless steel cages in rooms designed to maintain humidity at approximately 40-60%, temperature at approximately 22°C, photoperiod at 12 L:12 D and air flow at 12-15 changes per hour. A feed crock and a pressure-activated stainless steel water nipple were components of all cages. A basal diet of Purina Certified Rodent Chow No. 5002 (Purina Mills, Inc., St. Louis, MO) and municipal drinking water was available *ad libitum*

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

throughout the prestudy and study periods. Analysis of the chow was performed by Purina Mills, Inc. to confirm that the diet provides adequate nutrition and to quantify the levels of selected contaminants. Drinking water was analyzed by the City of Midland and an independent laboratory in accordance with Laboratory SOPs.

Analytical chemistry.

Purity information for all test materials provided by the commercial suppliers indicated that all test materials were at least 98% pure.

For one replicate of each experiment, representative samples of culture media from each treatment group were submitted for analysis to the Analytical Chemistry Department of the Dow Chemical Company. Samples were taken both at the start and completion of the culture period to determine (1) if the targeted concentrations were achieved and (2) to verify that EG was not metabolized to GA by the embryos.

pH stability.

The pH stability of the test material-containing media was monitored in the culture system. In Experiment 1, this was accomplished by measuring the pH of representative test material-containing media samples which did not contain embryos. The pH was measured at preparation and after approximately 46 hours incubation in the culture apparatus. To insure accurate readings, temperature and gas phase conditions were maintained during pH determinations. In Experiment 2, the pH of media containing embryos was similarly measured to determine if the presence of embryos influenced pH under the conditions of the experiment.

Osmolality.

Hyperosmolality has been suggested as a possible contributory factor in ethylene glycol-induced developmental toxicity. Therefore, the osmolality of selected culture media samples was measured to determine if hyperosmolality played a role in the present whole embryo culture study.

Culture media containing 50 mM ethylene glycol, 12.5 mM glycolic acid or 12.5 mM sodium glycolate and vehicle control media were prepared as described previously. These samples of media represented the highest concentration of ethylene glycol tested and the lowest-observable-effect levels for glycolic acid and sodium glycolate. Osmolality of the samples was determined using a freezing point depression osmometer (Osmette A).

RESULTS AND DISCUSSION

EXPERIMENT 1

Analytical chemistry (Table 1)

During replicate 4 of Experiment 1, samples of culture media taken at the start and end of culture were analyzed to verify proper test material preparation methods and also to determine stability during culture. As identical test material preparation procedures were used during all replicates of Experiment 1 and 2, these samples were considered representative of all the other (unanalyzed) replicates. Analysis of medium samples taken at the start of culture verified that the test material/medium preparation methods were adequate. However, a slight serial dilution error was indicated by the slightly lower percent of target levels (71-86%) in the 0.5 - 25 mM/L EG groups. The 50 mM EG sample was 103% of target and all GA groups were within 90-94% of target. Analysis of samples taken at the end of culture (Day 2) indicated that both test materials were stable throughout the 46 h culture period and that there was no detectable conversion of EG to GA during this period. There was one sampling error involving the Day 2 EG 0.5 mM sample which was inadvertently placed in the Day 2 GA 0.5 mM vial. This did not compromise the interpretation of the data, as all higher concentrations showed no conversion of EG to GA.

Experiment 1 - pH stability without embryos (Table ...)

Samples of media were maintained under normal culture conditions, but without embryos, to determine the pH stability of the various media. Control

medium, all EG media and the GA 0.5 mM medium showed no appreciable change in medium pH throughout the culture period. The pH of the GA \geq 2.5 mM groups initially was acidic, but became less so during culture. A virtually identical result was obtained by Andrews et al (1993) in their study of pH/formate interactions in WEC. Most likely this was due to a shift in the equilibrium between CO₂ and bicarbonate ion driven by excess weak acid in the medium.

Experiment 1 - Viability (Table 3)

Viability was assessed by the presence of a beating heart at the end of culture. No embryos survived exposure to 50 mM GA and only 50% were viable in the 25 mM GA group. This was compared with 100% viability for the control and remaining GA groups. There was no effect of EG on viability at any concentration tested (viability 90-100%).

The presence of red blood cells actively circulating through yolk sac vessels provided an additional measure of embryo function, as this is the site of gas/nutrient exchange for the conceptus. Yolk sac circulation was inhibited in the GA \geq 25 and 50 mM groups, in parallel with the effects on heart beat. Yolk sac circulation was also inhibited in the VPA-treated embryos, although this appeared to be due to abnormal development of the visceral yolk sac vasculature, rather than an effect on viability *per se*. There was no effect of EG on visceral yolk sac circulation at any concentration tested.

Experiment 1 - Growth & development (Table 4)

There were no effects on the various measures of embryonic growth and development for any of the EG groups, with the exception of a very slight decrease in morphological score for the 50 mM EG group. Based on published data (Brown, 1990) describing morphology scores for normally developed Sprague-Dawley rat embryos, this decreased score is equivalent to a developmental delay of approximately 2.5 hours (relative to controls). This small delay was considered biologically insignificant, as embryos *in vivo* would be expected to recover from such a small delay quite easily. In contrast

to the general lack of effects with even very high concentrations of EG, exposure to GA at concentrations ≥ 12.5 mM adversely affected nearly all parameters of growth and development. VPA treatment also caused decreases in embryonic growth measures, as shown previously by others (Kao et al, 1981).

Experiment 1 - Morphology (Table 5)

There was no evidence of dysmorphogenesis due to EG at concentrations up to and including 50 mM. All EG-treated embryos appeared morphologically normal, with the exception of one embryo in each of the 2.5 and 25 mM groups with minor abnormalities of the yolk sac. The nature of these changes, their low incidence and the lack of a dose response relationship indicated that these effects were spurious and unrelated to treatment.

In contrast, the percentage of morphologically abnormal embryos was significantly increased in the GA 12.5 and 25 mM groups. These abnormalities were predominantly craniofacial and appeared to correspond with many of the EG-induced malformations seen in vivo. The most commonly observed embryological alteration was a cystic enlargement of the maxillary process. Also observed in numerous embryos were abnormalities involving the rostral tip of the embryo in the region of the olfactory ridge. These structures give rise to the maxilla (upper jaw), secondary palate and lip. Thus, effects on these structures in culture are consistent with increased incidences of cleft palate, cleft lip and micrognathia observed in vivo (Price et al., 1985). Other effects seen in embryos cultured with GA included dysmorphogenesis of the optic vesicles, corresponding with anophthalmia observed in vivo, and abnormal differentiation of the somites, which is consistent with axial skeleton malformations seen in term fetuses.

Exposure of embryos to the positive control agent, VPA, resulted in abnormal somite segmentation, altered neural tube development characterized by a wavy or kinked neural suture line, incomplete closure of the otic vesicles, disorganized vitelline (visceral yolk sac) vessels and other abnormalities. The

effects observed in this study were highly consistent with those reported previously (Kao et al., 1981).

The results of Experiment 1 clearly show that parent EG is not responsible for the developmental effects observed following large oral gavage doses of EG and provide strong evidence against conclusions drawn from a previously discussed whole embryo culture study (Grafton and Hanson, 1987). The present study demonstrates that EG is essentially innocuous at concentrations which could be realistically attained in vivo. Furthermore, the data indicate that the EG metabolite, GA, is the proximate toxicant for EG-induced developmental toxicity. Whether or not the effects of GA in culture were due to an inherent toxicity of this metabolite, or instead, an indirect effect of culture medium acidification was addressed in Experiment 2.

EXPERIMENT 2

Experiment 2 - pH stability with embryos (Table 6)

In Experiment 2, embryos were cultured for 46 h in control medium at neutral (pH 7.41) or acidic pH (pH 6.74) or in medium containing 12.5 mM glycolic acid (pH 6.74) or 12.5 mM sodium glycolate (pH 7.42). To determine the effect embryos had on medium pH during culture, the pH of a representative set of cultures was checked at the beginning and end of culture. Only slight changes (≤ 0.24 units) in pH occurred over the course of the culture period in the glycolic acid, sodium glycolate and acidic control groups. However, pH decreased by 0.5 units in the neutral pH control group. Changes in the pH of media with embryos present may be influenced by the metabolic activity of the embryos, as larger, more metabolically active embryos would be expected to produce more CO₂ and thus acidify the medium to a greater extent than smaller, less metabolically active embryos. While these pH changes indicate a degree of pH instability when embryos are present, the changes were not of sufficient magnitude to compromise interpretation of the data.

Experiment 2 - Viability (Table 7)

All embryos in all treatment groups were viable. There were no significant alterations in yolk sac circulation due to any of the experimental treatments.

Experiment 2 - Growth & development (Table 8)

The effects observed in embryos cultured in 12.5 mM glycolic acid (initial pH 6.74) were generally very similar to those observed in Experiment 1, with all seven parameters of growth and development significantly different from control (pH 7.41). Growth and development parameters for embryos cultured in medium containing 12.5 mM sodium glycolate at pH 7.42 or in control medium at pH 6.74 were generally intermediate between the neutral pH control and the glycolic acid groups. However, while all seven parameters were significantly affected by sodium glycolate at neutral pH, only embryo protein, yolk sac protein and head length were significantly affected in the acidic pH control group. Even for these three parameters, glycolate at neutral pH was more inhibitory than control medium at acidic pH. These data indicate that most of the effects observed in Experiment 1 were due to an inherent toxicity of glycolate, although an additive contribution from decreased medium pH was also apparent.

Experiment 2 - Morphology (Table 9)

Both treatment with 12.5 mM glycolic acid and 12.5 mM sodium glycolate resulted in a significant increase in the percentage of embryos containing morphologic abnormalities. The type of alterations in the latter two groups were nearly identical and, as seen in experiment 1, were predominantly craniofacial. Only one embryo in the acidic control group exhibited craniofacial dysmorphogenesis. As discussed above, these results suggest a predominant role for an inherent glycolate toxicity, with a minor contribution from culture medium acidification.

Although these results suggest that acidic pH plays a minor role in altering embryological development, the results do not rule out an important role for metabolic acidosis in causing teratogenesis in vivo. As shown by Khera

(1991), other critical changes occur during metabolic acidosis, including decreased CO₂ and bicarbonate levels, increased glucose, etc. These changes were not represented in our whole embryo culture system. Thus, the possibility remains that these factors may still play a contributory role in EG's effects in vivo.

Experiment 2 - Osmolality (Table 10)

One other factor suggested to be involved in EG-induced teratogenesis is maternal serum hyperosmolality (Khera, 1991). In Khera's study, a dose of 3,333 mg/kg of EG caused osmolality to rise as high as 359 mosmol/kg H₂O (control value=285). To gain perspective on the role of hyperosmolality on embryo development, the osmolality of selected media samples was determined. Culture medium containing 50 mM EG had an osmolality of 418 mosmol/kg H₂O, well in excess of what Khera observed in his EG study. Although one might expect this degree of hyperosmolality to have adverse consequences, embryos in this medium were essentially normal. This finding indicates that embryos during this stage of development are resistant to the hyperosmolality induced by high concentrations of EG.

CONCLUSIONS

The results of this study show clearly that parent EG has little impact on embryos, even when exposed continuously for 46 h to peak concentrations associated with a dose causing malformations (as well as maternal mortality). This finding supercedes previous suggestions (Grafton and Hansen, 1987), based on a more limited rat whole embryo culture study, that EG acts directly on embryos. It is also interesting to note that culture medium containing the highest concentration of EG (50 mM) was severely hyperosmotic (418 mosmol/kg H₂O), yet did not adversely affect embryo development. This would appear to rule out maternal hyperosmolality as a contributing factor in EG-induced developmental toxicity.

To the contrary, the weak acid metabolite GA at ≥ 12.5 mM/L caused abnormalities of the developing maxillary process, midfacial anlagen, optic vesicles and somites, effects bearing strong embryological correspondence with cleft palate and lip, micrognathia, anophthalmia and axial skeleton malformations observed following large oral EG doses in vivo. This result provides strong evidence that GA is the proximate developmental toxicant for EG-induced developmental toxicity. This result also supports the hypothesis that a dose-rate dependent shift in EG metabolism is prerequisite to developmental toxicity following EG exposure, in that GA accumulation is a high dose-rate, metabolic saturation phenomenon.

The results of Experiment 2 indicate that GA effects in culture were due in large part to an inherent chemical toxicity of this metabolite. This was shown by the fact that sodium glycolate (12.5 mM) at neutral pH was nearly as potent and caused essentially identical morphological alterations as did 12.5 mM GA (free acid) at pH 6.74. Lesser, but statistically significant effects of control medium at pH 6.74 indicated an additive contribution of medium acidity. A direct toxicity of glycolate could explain why correction of metabolic acidosis with sodium bicarbonate infusion was only partially effective in ameliorating EG-induced developmental toxicity (Khera, 1991), as plasma GA levels would be expected to remain high with this treatment. However, the relatively minor effect of acidic culture medium does not contradict a contributory role for metabolic acidosis. Metabolic acidosis is a complex condition involving dramatic changes in carbon dioxide, bicarbonate and glucose, none of which were represented in the whole embryo culture system.

In conclusion, this study provides direct evidence that GA is the proximate toxicant for EG-induced development toxicity. This is important for risk assessment because this metabolite accumulates only under high dose-rate scenarios, such as oral exposure to large doses. Integration of the present results with previous in vivo homeostasis findings (Khera, 1991) indicate that GA acts both through an inherent chemical toxicity as well as via induction of metabolic acidosis.

*****DRAFT*****

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