

MICROFICHE LABEL -- SPECIAL TITLE

ATTENTION DATA ENTRY

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UNION CARBIDE -

DIETHYLENETRIAMINE

Microfiche # 206423

Document ID # _____

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OFFICE OF TOXIC SUBSTANCES
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REV. 7/27/82

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	TABLES OF PROTOCOLS ATTACHED WITH			
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Chemical Name (300 per name)	25	CAS No. (10)	24	
DIETHYLENETRIAMINE		111-4φ-φ		

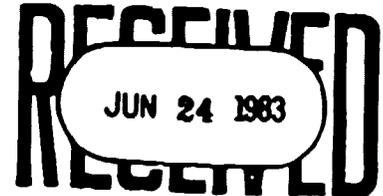
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**CERTIFIED MAIL
RETURN RECEIPT
REQUESTED**

UNION CARBIDE CORPORATION OLD RIDGEBURY ROAD, DANBURY, CT 06817
Corporate Health, Safety and Environmental Affairs Department

June 28, 1983



U.S. Environmental Protection Agency
TSCA 8D-1
P.O. Box 2060
Rockville, Maryland 20852

Subject: Union Carbide TSCA Sec. 8(d) Report
40 CFR 716.6 & 716.7

Sirs:

With respect to:

Chapter I of Title 40 of the Code of Federal Regulations;
Subpart A, Secs. 716.6 and 716.7;
As amended 716.17(a)(3), Federal Register Vol. 48, p. 13181,
March 30, 1983;
Sec. 8(d), Pub. L. 94-469, Stat. 2029 (15 U.S.C. 2607 (d));

Union Carbide Corporation herewith submits the attached copies of studies and lists of studies in compliance with the above-identified regulation.

There is no information in the enclosed copies of studies or lists of studies for which Union Carbide asserts claims of confidentiality. The printed words "BUSINESS CONFIDENTIAL" or "Confidential" at the top of pages for some reports was for the internal guidance of Union Carbide personnel at the time of report issuance and does not represent a Union Carbide Corporation claim for confidential handling of the information, submitted pursuant to TSCA Sec. 8(d) rules.

Where some lines are deleted from certain reports, especially earlier ones, it is solely due to the fact that the deleted information pertains to chemicals or substances other than those for which reporting is required under the above rule. Union Carbide has included copies and lists of studies for only those chemical substances that are members of categories which it has manufactured or processed since 1972. Union Carbide Corporation believes that the enclosed copies of studies and lists of studies represent all of the studies which Union Carbide's file search has identified to date as reportable under the above-identified rule. Should any reportable studies be discovered subsequently, they will be forwarded immediately.

The Environmental Protection Agency and other appropriate government agencies are free to use the enclosed information as necessary in the normal discharge of their mandated responsibilities. However, identified authors, whether employees of Union Carbide or elsewhere, or their organizations are the rightful owners of the publication rights to the contained information.

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U.S. Environmental Protection Agency
Page 2

If you have questions concerning the enclosed reports and lists of studies or wish to request further basic underlying data pertinent to the studies, please contact me or Dr. Donald L. Heywood (203-794-5224) of this Department.

Very truly yours,


Jackson B. Browning, Director
Health, Safety and Environmental
Affairs (203) 794-5227

JBB/DLH/cr
Enclosure

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severe lung hemorrhage, mottled livers and kidneys and irritation of the stomach and intestines. This acute oral toxicity is roughly equivalent to that of 76% ethylene diamine and somewhat greater than that of triethylene tetramine LD₅₀ 4.34 gm./kg., and tetraethylene pentamine LD₅₀ 3.99 gm./kg.

Previous range finding results with a 10 or 50% aqueous dilution fed to rats placed the LD₅₀ at 1.8 gm./kg. It is not unreasonable to presume that current value of 2.33 gm./kg. reflects increased accuracy in the estimation of the LD₅₀ and is not necessarily indicative of a decrease in toxicity of the current sample.

Skin Penetration

The application of undiluted diethylene triamine to the clipped skin of the rabbit trunk resulted in an LD₅₀ of 1.09 (0.95 to 1.24) ml./kg. calculated by the method of Thompson. Immediate erythema, followed by nerosis and finally a leathery scab resulted from the direct action of the material on the skin. In animals that died, the lungs were often congested and sometimes hemorrhagic; the kidneys showed considerable gross damage including a pitted appearance of the surface and subcapsular hemorrhage. The stools of survivors were watery and sometimes blood-tinged. With a 10% aqueous dilution the corrosive action on the skin was eliminated and none of 6 rabbits succumbed to a dosage of 2.0 gm./kg. (20 ml./kg. of the 10% dilution). One of 6 rabbits died an unexplained death at 1.0 gm./kg. All lost weight save one, which gained only 4 grams. This demonstrates that the toxicity by skin absorption is enhanced by the corrosive action of the undiluted compound as the LD₅₀ for a 10% dilution would be above 4 gm./kg.

Previous tests with guinea pigs, on the 1940 sample, resulted in an LD₅₀ of 0.5 ml./kg. for guinea pigs poulticed with undiluted material for 4 days. Species difference can account for the variance between guinea pig and rabbit results, plus the longer contact period used in the guinea pig test.

Inhalation

Substantially saturated vapor produced at room temperature was not lethal to rats in an 8-hour exposure. Similar results were obtained with the 1940 sample.

A cooled mist produced by heating the compound to 170° C. while air was bubbled through it caused the death of 4 of 6 rats in an 8-hour exposure.

Exposures to accurately prepared concentrations were not made because of the very low concentration, circa 300 ppm., for saturated vapor at room temperature, which proved not to be lethal to rats in 8 hours.

Irritation

Undiluted diethylene triamine produced necrosis of the skin of the rabbit belly in the vesicant test. A 10% dilution in acetone was not irritating to 3 of 5 rabbits tested and only minimum reaction was noted on the others. This reactivity is comparable to that produced by the other members of this series.

Severe corneal damage is caused by a 15% dilution in propylene glycol, whereas a 5% dilution causes quite minor effects. This relegates the compound to the same Grade (8) in which 76% ethylene diamine and formaldehyde are placed and indicates the hazards of handling the compound as far as the eyes are concerned.

These data agree with the results obtained with the 1940 sample.

Charles P. Carpenter

SENIOR INDUSTRIAL FELLOW

Charles P. Carpenter

Typed: April 27, 1948 - met

Table 11-105

Diethylene Triamine

Single Doses to Male Albino Rats by Mouth

Fed by Stomach Tube as a Dilution in Water, 1 ml. = 0.10 gm.

Rat Number	1948 Date Dosed	Grams Wt.	Weight Change in 14 Days	Dosage; Grams per Kilo	Dose in Grams	Dose in ml. of Dilution	Days to Death
68060	2-5	97	-	3.16	0.305	3.1	1
68061	"	90	-	3.16	0.284	2.8	4
67833	"	90	-	3.16	0.284	2.8	2
67811	"	90	-	3.16	0.284	2.8	1
67814	"	90	-	3.16	0.284	2.8	3
67111	2-10	92	-	3.16	0.291	2.9	1
67117	"	98	-	3.16	0.310	3.1	3
67124	"	91	-	3.16	0.388	2.9	2
67125	"	90	-	3.16	0.284	2.8	2
67058	"	90	-	3.16	0.284	2.8	2
67932	1-29	95	-	2.52	0.239	2.4	3
67606	"	91	-	2.52	0.229	2.3	2
67620	"	97	-	2.52	0.244	2.4	2
67608	"	102	-	2.52	0.257	2.6	0
67916	"	102	+ 28	2.52	0.257	2.6	-
67928	"	92	+ 38	2.52	0.232	2.3	-
67927	"	98	+ 39	2.52	0.247	2.5	-
67921	"	95	+ 47	2.52	0.239	2.4	-
67918	"	98	+ 34	2.52	0.247	2.5	-
67913	"	102	+ 27	2.52	0.257	2.6	-
67188A	1-15	90	-	2.00	0.180	1.8	2
67191	"	90	-	2.00	0.180	1.8	1
67519	"	98	-	2.00	0.186	1.9	6
67192	"	91	+ 14	2.00	0.182	1.8	-
67501	"	92	+ 10	2.00	0.184	1.8	-
67550	1-27	97	+ 51	2.00	0.194	1.9	-
67549	"	90	+ 52	2.00	0.180	1.8	-
67548	"	90	+ 38	2.00	0.180	1.8	-
67672	"	93	+ 49	2.00	0.186	1.9	-
67929	1-29	91	+ 53	2.00	0.182	1.8	-
67557	1-29	91	-	1.58	0.144	1.4	12
67675	"	93	+ 53	1.58	0.147	1.5	-
67672	"	90	+ 50	1.58	0.142	1.4	-
67526	"	100	+ 72	1.58	0.158	1.6	-
67525	"	111	+ 71	1.58	0.175	1.8	-
67530	"	97	+ 73	1.58	0.153	1.5	-
67531	"	102	+ 74	1.58	0.161	1.6	-
67529	"	102	+ 82	1.58	0.161	1.6	-
67558	"	90	+ 20	1.58	0.142	1.4	-
67570	"	92	+ 47	1.58	0.147	1.5	-

Table 11-106

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Diethylene Triamine

Single Doses to Male Albino Rabbits by Skin Absorption

Administered undiluted under "Vinylite" dam for 24 hours

Rabbit Number	1948 Date Clipped	1948 Date Applied	Grams Wt.	Weight Change in 14 Days	Dosage; Ml. per Kilo	Dose in ml. of Material	Days to Death
70839	4-12	4-12	2200	-	1.26	2.8	1
70840	"	"	2178	-	1.26	2.7	8
70847	"	"	2266	-	1.26	2.8	4
70848	"	"	2114	-	1.26	2.7	2
70849	"	"	2526	-	1.26	3.2	2
70865	"	"	2124	-	1.26	2.7	4
70895	"	"	2388	-	1.26	3.0	3
70897	"	"	2772	-	1.26	3.5	1
70898	"	"	2922	- 470	1.26	3.7	-
70866	"	"	1958	- 244	1.26	2.5	-
70601	3-29	3-29	2406	-	1.12	2.7	6
70687	"	"	2520	-	1.12	2.8	4
70753	"	"	3040	-	1.12	3.4	6
70805	"	"	2190	-	1.12	2.4	2
70599	"	"	2590	- 282	1.12	2.9	-
70618	"	"	2480	- 124	1.12	2.8	-
70620	"	"	2340	- 58	1.12	2.6	-
60636	"	"	2272	+ 468	1.12	3.1	-
70652	"	"	2100	- 288	1.12	2.4	-
70752	"	"	2630	- 256	1.12	2.9	-
70590	2-27	2-27	2360	-	1.00	2.4	3
70593	"	"	2596	-	1.00	2.6	6
70595	"	"	2712	-	1.00	2.7	2
70596	"	"	2364	-	1.00	2.4	8
70594	"	"	2132	- 168	1.00	2.1	-
70730	3-18	3-18	2804	- 874	1.00	2.8	-
70731	"	"	2414	- 210	1.00	2.4	-
70735	"	"	2408	- 476	1.00	2.4	-
70738	"	"	2418	- 154	1.00	2.4	-
70740	"	"	2080	- 206	1.00	2.1	-
70748	3-18	3-18	2444	-	0.89	2.2	4
70754	"	"	2400	-	0.89	2.1	1
70727	3-22	3-23	2222	-	0.89	1.8	10
70732	"	"	2560	-	0.89	2.0	1
70744	3-18	3-18	2296	- 258	0.89	2.0	-
70806	3-29	3-29	2200	- 242	0.89	1.9	-
70719	3-22	3-23	2558	- 736	0.89	2.0	-
70726	"	"	2304	- 140	0.89	1.8	-
70728	"	"	2156	- 270	0.89	1.7	-
70729	"	"	2732	- 460	0.89	2.2	-

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OFFICE OF TOXIC SUBSTANCES
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	DIETHYLENETRIAMINE (1974-75 RESULTS)		
	RANGE FINDING TOXICITY AND 7-DAY DIETARY		
	INCLUSION STUDIES		
Chemical Name (300 per name)	25	CAS No. (10)	24
DIE THYLENETRIAMINE		111-4φ-φ	

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Diethylenetriamine
l.b.

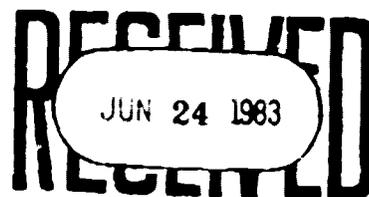
Project Report 40-45
9 Pages
April 7, 1977
Tel: (412) 327-1020

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878213670

CHEMICAL HYGIENE FELLOWSHIP
Carnegie-Mellon Institute of Research
Carnegie-Mellon University
4400 Fifth Avenue
Pittsburgh, Pa. 15213

Diethylenetriamine
(1974-75 Results)



Range Finding Toxicity and 7-Day Dietary Inclusion Studies

Sponsor: *Union Carbide Corporation*

Summary

	<u>1974-75 Results</u>	<u>Previous Results</u> ¹
Stomach Intubation, rat LD50	1.62 ml/kg; undiluted	1.8 to 2.33 gm/kg; 1 ml = 0.10 gm in water (1941,1948)
Intraperitoneal Injection, rat LD50	-	0.28 to 0.93 ml/kg; undiluted (1962, 1964)
Subcutaneous Injection, rat LD50	-	1.78 to 3.00 ml/kg; undiluted (1962, 1964)
Skin Penetration, rabbit LD50	0.707 ml/kg; undiluted	1.09 ml/kg; undiluted (1948)
Skin Penetration, guinea pig LD50	-	0.17 gm/kg; undiluted (1941)
Inhalation, rat Substantially saturated vapor	8 hr killed 0 of 6	8 hr killed 0 of 6 (1948,1955)
Mist from saturation at 170°C	-	8 hr killed 4 of 6 (1948)
Uncovered Skin Irritation, rabbit	Moderate, Grade 6	Moderate, Grade 6 (1948)
Eye Injury, rabbit	Severe, Grade 8	Severe, Grade 8 (1948)
Seven-Day Dietary Inclusion, rat	Minimum effect at 0.61 gm/kg/day; no significant ill-effect at 0.24 gm/kg/day	-

¹ Smyth, Henry F., Jr. et al, "Range Finding Toxicity Data List III"
Journal of Industrial Hygiene and Toxicology, Vol. 31, No. 1, 60-62
(January 1949) and Chemical Hygiene Fellowship (CHF) Reports 5-23 (1942),
11-61 (1948) and 26-6 (1963).

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Interpretation

Diethylenetriamine was moderately toxic following single stomach intubation and covered dermal application routes of administration. Application of the undiluted material to uncovered rabbit skin resulted in necrosis in the current and previously-reported tests. Therefore, it is rated by the Chemical Hygiene Fellowship as a Department of Transportation (D.O.T.) "corrosive" although the actual 4-hr D.O.T. covered skin test was not performed. Application of a 10% dilution in distilled water resulted in trace irritation to rabbit skin. Severe corneal injury, with iritis, resulted from instillation of the material in rabbit eyes. No hazard is anticipated from the infrequent inhalation of substantially saturated vapor evolved at room temperature under normal handling conditions. Rats that received diethylenetriamine in their diets for 90 days were affected by 0.61 gm/kg/day (average); this is the minimum effective dose (MIE). No significant effect, in the criteria examined, resulted at 0.15 gm/kg/day. The ratio of single peroral LD50/MIE was 2.66, probably indicating a low degree of chronicity.

The results of these latest range finding tests were similar to those done previously. Most of the previous results appear in report 11-61 (1948).

Included in the literature on diethylenetriamine is a study in which rabbits and guinea pigs received 1.0 and 0.6 mg/kg, respectively, for 6 months without effect (Trubko, E. I., Teplyakova, E. V., "Diethylenetriamine Studied in Connection with Determining its Hygienic Standard Levels in Reservoir Water". Gig. Sanit. 1972, 37 (7), 103-4 (Russ). One inhalation study (Brit. J. Industr. Med., 1970, 27, 1-18) consisted of 15 exposures, 6 hr per exposure, to 130 ppm without toxic effect among rats. During a study in Japan, subcutaneous injections (10 to 25 mg/kg/day) and daily topical applications (0.4 ml of 10% aqueous) reduced the life span of rats and affected kidneys, livers, spleens and adrenals (Fujino, Mitsuo, "Chronic Toxicity of Diethylenetriamine in Rats", Igaku Kenkyu 1970, 40 (2), 139-6).

Sample

Quantity: 2 quarts

Date Received: 7-26-74

CHF Sample No.: 37-417

Submitted By: R. V. Berthold

Division: Chemicals and Plastics
South Charleston, WV

Charge No.: 01067

000003

Peroral, Single Dose to Rats

LD50 - 1.62 (1.18 to 2.24) ml/kg; undiluted.

Conditions - Standard.

Dosage; ml/kg	Dead Dosed	Days to Death	Weight Change	Signs and/or Symptoms
4.0	5/5	0,0,1,1,1	-	Sluggish 2 min; prostrate 3 hr; death of 2 at 4 hr.
2.0	4/5	2,3,7,8	98 gm	Sluggish 10 min.
1.0	0/5	-	96 to 110 gm	Sluggish 10 min.

Gross Pathology - In victims, petechial hemorrhages of the lungs; stomachs liquid-filled, hemorrhaged; intestines liquid-filled, opaque, hemorrhaged, slightly yellow; kidneys and adrenals slightly congested; kidneys speckled; livers and spleens mottled. Nothing remarkable in survivors.

Conclusions - Moderately toxic following acute peroral intubation.

Skin Penetration, Single Dose to Rabbits

LD50 - 0.707 (0.324 to 1.54) ml/kg; undiluted.

Conditions - Standard. Dosed under polyethylene sheeting.

Dosage; ml/kg	Dead Dosed	Days to Death	Weight Change	Skin Irritation	Signs and/or Symptoms
1.0	3/4	2,5,6	-157 gm	necrosis	-
0.5	1/4	8	-181,-15, 212 gm	necrosis	-

Gross Pathology - In victims, congestion of lungs, livers, spleens and kidneys. Nothing remarkable in survivors.

Conclusions - Moderately toxic following acute covered dermal application.

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Inhalation, Single, by Rats

Conditions - Static exposure at 23°C. Procedure B of standard test procedures.

Procedure	Time	Concentration	Dead / Dosed	Death	Weight Change	Signs and/or Symptoms
B	8 hr	Substantially saturated vapor	0/6	-	66 to 81 gm	-

Gross Pathology - Nothing remarkable.

Conclusions - No hazard is anticipated from the infrequent inhalation of substantially saturated vapor evolved at room temperature under normal handling conditions.

Skin Irritation, Rabbit, Uncovered

Conditions - Standard. Applied undiluted or in distilled water.

Conclusions - Necrosis on 2 of 2 rabbits from the undiluted material; no irritation on 2 rabbits, moderate capillary injection on 3 from a 10% dilution in distilled water. Grade 6.

Eye Irritation, Rabbit

Conditions - Standard. Instilled undiluted or in distilled water.

Conclusions - Severe corneal injury, with iritis, from 0.005 ml undiluted per eye; moderate corneal injury from 0.5 ml per eye of a 15% dilution in distilled water; trace corneal injury on one of 5 eyes from 5% in distilled water. Grade 8.

Seven-Day Dietary Inclusion, RatsProcedure

Diethylenetriamine was added to ground PURINA Chow and fed in the diet for 7 days. Groups of 5 male and 5 female Harlan-Wistar albino rats, 30 days of age at the start of the study, were randomly assigned to each dosage level and to each of 2 control levels.

Results

The results are summarized in Table 40-1 and a synopsis of pathology is given in Table 40-2.

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Inclusion of diethylenetriamine in the diet for 7 days resulted in moderate to severe body weight depression at attained dosages of 1.35 gm/kg/day for the males and 1.58 gm/kg/day for the females. These were the highest dosage levels. Liver weights of the male rats at their highest level were slightly depressed compared to those of one of the control groups. There were no other organ weight effects noted at any of the dosage levels for males or females. Body weights of female rats at 0.62 gm/kg/day were moderately depressed while the weights of males at the middle level, 0.60 gm/kg/day, were slightly depressed after 5 days compared to one of the control groups. No body weight or organ weight effects were observed at the lowest dosage level, 0.24 gm/kg/day.

On micropathological examination, the only remarkable lesions were instances of bile duct proliferation in livers from females at the middle level, hydronephrosis among a few males at the higher level and chronic tracheitis among females at the higher level. These findings were not strictly dosage-related or present in significantly large numbers. Therefore, our pathologist considered them as sporadic, common lesions not related to treatment.

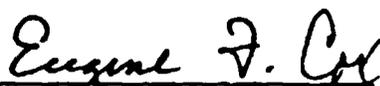
Conclusions

The maximum no significant ill-effect level was 0.24 gm/kg/day based on body weight, liver and kidney weight, and micropathology. The ratio between the single peroral LD50 and the minimum effect level (MIE) for the 7-day feeding study was $1.62 \text{ ml/kg} \pm 0.61 \text{ gm/kg}$ (average) or 2.66, probably indicating a low degree of chronicity. The medium predicted minimum effect level for 90-day rat feeding is 0.20 gm/kg; that for two years is 0.11 gm/kg (Weil, *et al*, "Toxicology and Applied Pharmacology" 14, 426-431, 1969).


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Charles P. Carpenter, Ph.D.
Advisory Fellow

Approved:


Eugene F. Cox, Ph.D.
Director

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Date: April 19, 1977

Typed: *icm*

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Standard Test Procedures

In all tests, the nonfasted animals are maintained on appropriate Wayne diets and water *ad lib* except during period of manipulation or confinement. Dosage levels differ by a factor of 2 in a geometric series. LD50s or LC50s are calculated by the moving average method based on a 14-day observation period.

Toxicity Terminology for Peroral and 24-Hr Dermal LD50s (A)/Inhalation 4-Hr LC50 (B)

	<u>A, gm/kg</u>	<u>B, ppm</u>		<u>A, gm/kg</u>	<u>B, ppm</u>
Extremely low order	> 15	> 100M	Highly	0.05-0.5	100-1M
Slightly	5-15	10M-100M	Seriously	0.01-0.05	10-100
Moderately	0.5-5	1M-10M	Dangerously	< 0.001	< 10

Peroral. Compounds administered by stomach intubation to Wistar derived male rats, 90-120 grams in weight and 3 to 4 weeks of age, reared in our own colony.

Skin Penetration. Male albino rabbits, 3 to 5 months of age, are immobilized during the 24-hour contact period with the compound retained under impervious sheeting on the clipped intact skin of the trunk. Thereafter, excess fluid is removed to prevent ingestion. Maximum dosage that can be retained is 16 to 20 ml/kg.

Inhalation. Procedure A. Concentrated vapor is generated in a gas washing bottle by passing dried air at 2.5 liters/min through a fritted glass disc immersed to a depth of at least 1-1/2 inches in the chemical which is delivered to rats in a 9-liter glass exposure chamber. Mean vapor concentration is calculated from the loss in weight of the liquid or estimated from the vapor pressure at the actual temperature of the chemical during aeration.

Procedure B. Substantially saturated vapor is prepared by spreading 50 grams of chemical over 200 cm² area on shallow tray placed near the top of a 120-liter glass chamber which is then sealed for at least 16 hours while an intermittently operated fan agitates the internal chamber atmosphere. Rats are then introduced in a gasketed drawer-type cage designed and operated to minimize vapor loss.

Procedure C. Mist, vapor and any oxidation or decomposition products of the chemical held at 170°C are generated and delivered as in A.

Procedure D. Vapor at metered concentration, not checked analytically, is generated by feeding the liquid at a constant rate down the inside of a spirally corrugated surface of a minimally heated one-inch Pyrex tube, through which metered air is passed. Resultant vapor is delivered as in A.

Procedure E. Spray - Solutions or suspensions are atomized in a glass VAPONEFRIN nebulizer using dried compressed air at 9 liters/min (corrected) and 22 psi. The resultant aerosol of droplets averaging 2 microns in diameter is conducted directly into a 60-liter cubic glass chamber containing rats. Mean aerosol concentration is calculated from the amount of material atomized.

Procedure F. Dust - Dust clouds are generated by a baffled Wright Dust Feed through which air is passed at 14 liters/min (uncorrected) at 5 psi. The dust is delivered directly to a 120-liter plexiglas chamber containing rats. Airborne dust concentrations are measured gravimetrically every half hour.

Skin Irritation. Chemical is applied in 0.01 ml amounts to clipped, uncovered intact skin of 5 rabbit bellies either undiluted or in progressive dilutions of 10, 1, 0.1, and 0.01% in solvent. Ten grades are recognized based on appearance of moderate or marked capillary injection, erythema, edema or necrosis within 24 hours. No injury from undiluted = Grade 1.

Eye Irritation. Eyes not staining with 5% fluorescein in 20 seconds contact are accepted. Single instillation of 0.005, 0.02, 0.10 or 0.5 ml undiluted or of 0.5 ml of 40, 15, 5 and 1% dilutions are made into conjunctival sac of 5 rabbits. Read immediately unstained and after fluorescein at 24 hours, with ten grades recognized. Trace or no injury from 0.5 ml undiluted = Grade 1.

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Table 40-1

Summary of Results of 7 Days of Inclusion of Diethylenetriamine
in the Diet of Rats

	Male Rats				
				A	B
Dosage goal, gm/kg	1.65	0.65	0.25	0.0	0.0
Concentration in diet, %	1.27	0.53	0.21	0.0	0.0
Dosage attained, gm/kg/day	1.35	0.60	0.24	0.0	0.0
Diet consumed, gm/rat/day	14.4	16.3	17.6	18.4	16.7
<u>Body weight change, gm</u>					
1 day of doses	-0.4 ^{b, b^x}	3.2	6.4	4.4	5.2
5 days of doses	17.0 ^{c, b}	23.6 ^{a, -}	27.4	30.2	27.2
7 days of doses	34.6 ^{b, a}	43.6	49.0	53.2	47.0
Liver weight, gm	6.78 ^{a, -}	7.49	8.42	8.62	7.46
Liver wt as % of body wt	4.42	4.46	4.85	4.66	4.42
Kidney weight, gm	1.51	1.56	1.62	1.69	1.54
Kidney wt as % of body wt	0.99	0.93	0.93	0.92	0.92
Mortality	0	0	0	0	0
	Female Rats				
Dosage goal, gm/kg	1.65	0.65	0.25	0.0	0.0
Concentration in diet, %	1.43	0.57	0.21	0.0	0.0
Dosage attained, gm/kg/day	1.58	0.62	0.24	0.0	0.0
Diet consumed, gm/rat/day	13.6	14.0	14.3	14.1	15.7
<u>Body weight change, gm</u>					
1 day of doses	-3.4 ^{c, c}	2.2 ^{-, a}	3.4	3.6	6.2
5 days of doses	9.2 ^{c, c}	15.4 ^{a, b}	21.6	21.0	25.2
7 days of doses	21.0 ^{c, c}	28.6 ^{-, b}	36.2	35.2	39.2
Liver weight, gm	5.83	6.48	6.81	6.70	7.2
Liver wt as % of body wt	4.39	4.55	4.66	4.88	4.6
Kidney weight, gm	1.37	1.35	1.39	1.34	1.3
Kidney wt as % of body wt	0.96	0.95	0.96	0.98	0.9
Mortality	0	0	0	0	0
^a 0.05 > P > 0.01	^b 0.01 > P > 0.001			^c P < 0.001	
^x 1st letter of superscript denotes degree of significance versus control group A; 2nd letter denotes degree of significance versus control group B.					

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Table 40-2

Synopsis of Pathology of Rats that Received Diethylenetriamine in their Diets for 7 Days

		Males				Females			
		gm/kg in Diet; Diethylenetriamine							
		1.65	0.65	0.25	0.00	1.65	0.65	0.25	0.00
<u>Total Number Examined Grossly:</u>		5	5	5	5	5	5	5	5
<u>LIVER: Number Examined</u>	(M)	5	5	5	5	5	5	5	5
Bile duct proliferation	(M)	2	0	0	2	0	3	2	0
Round cell foci	(M)	0	2	1	1	0	1	3	1
<u>KIDNEY: Number Examined</u>	(M)	5	5	5	5	5	5	5	5
Hydronephrosis	(G)	2	1	0	0	1	0	2	1
Hydronephrosis	(M)	2	1	0	0	1	0	1	0
Round cell foci	(M)	0	0	1	0	0	0	0	0
Tubular regeneration	(M)	0	0	0	0	0	0	1	0
<u>LUNG: Number Examined</u>	(M)	5	5	5	5	5	5	5	5
Petechiae	(G)	0	0	1	1	0	0	0	0
Inhaled blood due to kill	(G)	0	0	1	0	0	0	0	0
Inhaled blood	(M)	0	0	1	0	0	0	0	0
<u>TRACHEA: Number Examined</u>	(M)	5	5	5	5	5	5	5	5
Dilated tracheal glands	(M)	0	0	0	0	0	0	0	1
Purulent tracheitis	(M)	0	0	1	0	0	0	0	0
Chronic tracheitis	(M)	1	2	2	2	4	5	3	1
<u>UTERUS: Number Examined</u>	(M)	-	-	-	-	5	0	0	5
Dilated	(G)	-	-	-	-	0	3	1	1
<u>COLON: Number Examined</u>	(M)	5	0	0	5	5	0	0	5
Section parasites	(M)	0	-	-	1	0	-	-	0
<u>BRAIN: Number Examined</u>	(M)	5	5	5	5	5	5	5	5
Granulomas, parasitic	(M)	0	0	1	0	0	0	0	0

G = Gross

M = Microscopic

The following tissues were examined microscopically on the 1.65 and 0.0 gm/kg levels: lung, liver, kidneys, heart, spleen, adrenal, thyroids, parathyroids, trachea, esophagus, stomach, duodenum, pancreas, colon, urinary bladder, pituitary, brain and prostate, testicle, epididymis or uterus and ovary. On the 0.65 and 0.25 gm/kg levels the lung, liver, kidney, heart, spleen, adrenal, thyroids, parathyroids, trachea, esophagus and brain were examined microscopically.

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DIETHYLENETRIAMINE			111-40-0	

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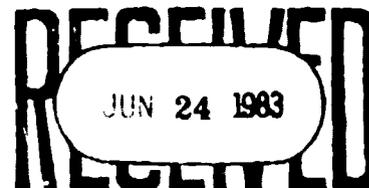
Diethylenetriamine - High Purity (DETA-HP)

In Vitro Mutagenesis Studies: 3-Test Battery

Authors: R. S. Slesinski, M. W. Gaunt, P. J. Guzzie, W. C. Hengler

Sponsor: Union Carbide Corporation

SUMMARY



Diethylenetriamine - High Purity (DETA-HP) was evaluated for potential mutagenic activity with a battery of three in vitro tests, which were: the Chinese Hamster Ovary (CHO) Mutation test, the Sister Chromatid Exchange (SCE) test and an assay for induction of Unscheduled DNA Synthesis (UDS) in rat liver cells. The pattern of negative responses obtained in the 3-test battery of mutagenicity assays indicated that DETA-HP did not produce a mutagenic effect typical of identified chemical mutagens and appeared to lack significant mutagenic potential in the three in vitro tests performed.

INTERPRETATION

In the CHO Mutation test, an indicator of agents which induce gene (point) mutations, DETA-HP was not active in stimulating a dose-related increase of mutant cells when tested either with or without the presence of an S9 metabolic activation system. The tests were apparently performed at sufficiently high doses of the test agent, because the highest dose level in the test with and without S9 metabolic activation ($40 \times 10^{-4}\%$) was completely cytotoxic. The results of the tests with or without S9 metabolic activation provided no indication of a significant mutagenic effect of the test agent. DETA-HP was considered inactive as a mutagenic agent for CHO cells in culture. The positive control agents EMS (ethylmethanesulfonate) and DMN (dimethylnitrosamine) induced mutation values that were highly statistically significant from the concurrent solvent controls and these values were within the historical range of acceptable variations for identical treatments in previous tests.

In the SCE test, an indicator of agents which induce DNA damage observable at the chromosome level of organization, DETA-HP was consistently not active in significantly stimulating the induction of SCE in vitro. The lack of any suggestion of a dose-effect relationship in the tests either with or without S9 metabolic activation indicated that DETA-HP was inactive in stimulating SCE in CHO cells. Highly statistically significant values of SCE, in comparisons to the concurrent solvent control, were obtained for tests of both positive control agents (EMS and DMN) and these data indicated that the test system had appropriate sensitivity for mutagenic detection.

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In the UDS-test, an indicator of agents which cause damage to the DNA molecule and stimulate "unscheduled" incorporation of radioactive thymidine detectable in nuclei and DNA of hepatocytes, DETA-HP failed to induce dose-related increases in the UDS detected with either nuclei or DNA. In evaluations over a relatively wide range of concentrations, DETA-HP produced marginally statistically significant levels of UDS activity in measurements of incorporation into nuclei at only one concentration and two concentrations in measurements of radioactive incorporation into DNA. Although these values were statistically significant, no consistent pattern or dose-effect relationship was observed in the response to DETA-HP. These isolated responses at single dose levels were not considered to be biologically significant. Although not all dose levels of the positive control agents NQO (4-nitroquinoline oxide) and DMN produced statistically significant responses in comparison to the concurrent solvent control, all doses produced numerical increases in UDS characteristic of a positive test. No evidence for a dose-related activity for DETA-HP was observed and DETA-HP was classified as inactive in the induction of UDS in the present test with the hepatocyte test system.

One literature reference reported that DETA was positive for inducing mutations in the Salmonella typhimurium (Ames) test (Hedenstedt, 1978). However, an alkylating contaminant was found in the DETA sample used in that test and no conclusion on the potential activity of pure DETA was possible.

The interpretation of the overall pattern of responses from the present 3-test battery of mutagenicity assays indicated that DETA-HP was not active as a mutagenic agent in the three in vitro tests employed.

SAMPLE

Quantity: 4 oz

CHF Sample No.: 42-102

Submitted by: D. C. Best

Date Received: 2/23/79

Division: Chemicals and Plastics
South Charleston, WV

Identification: Diethylenetriamine
511-01-0868
Clear Liquid

Ref.: 99CAG-37B

CAS#: 111-40-0

**Diethylenetriamine - High Purity
(DETA-HP)**

In Vitro Mutagenesis Studies: 3-Test Battery

Objective

The purpose of this study was to evaluate the potential of DETA-HP to induce genetic damage in mammalian cells at the gene, chromosome and/or DNA (deoxyribonucleic acid) level of molecular organization. A battery of three in vitro, short-term tests which detect each of these genetic end points was employed to evaluate DETA-HP for potential mutagenic activity. The classification of the test chemical as an active or inactive agent in these tests was evaluated in context with the overall pattern of responses observed in the complete battery of tests.

Sample Characteristics

A typical, commercial sample of DETA-HP was received for testing on 2/23/79. The available information from the Toxicology Data Bank or from "Material Safety Data Sheets" for this product specify the following chemical and physical characteristics:

Chemical Name:	Diethylenetriamine
Trade Name and/or Synonyms:	Bis-(2-Aminoethyl)Amine
Molecular Weight:	103.17
Formula:	$H_2NCH_2CH_2NHCH_2CH_2NH_2$
Specific Gravity (@ 20°C):	0.9542
Boiling Point:	206.9°C (760 mm Hg)
Solubility in H₂O (% by wt):	Complete at 20°C
Purity:	98.5%, minimum (see comments in additional analytical sections)
Vapor Pressure (@ 20°C):	< 1 mm Hg
pH:	Not available
Flash Point:	210°F, tag closed cup
Stability:	Stable
Incompatibility:	Avoid acids
Appearance and Odor:	Water-white liquid; mildly ammoniacal odor
Disposal:	Incineration

Protective Measures: Do not get on skin, eyes or clothing. Use rubber gloves, mechanical ventilation or local exhaust when handling. Avoid prolonged breathing of vapor or contact with skin. Safety goggles, organic vapor mask and rubber apron are recommended. Small spills should be flushed with large quantities of water.

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Health Hazard: Harmful if inhaled; skin and eye contact cause burns. DETA-HP is a sensitizer which may cause skin rash. Prolonged and repeated breathing will be irritating and may cause an asthma-like condition. In case of contact flush with large volumes of water for 15 minutes followed by soap and water. Remove contaminated clothing and wash before wearing again.

Additional analytical data on the sample received for testing was obtained from P. R. Umberger, UCC, S. Charleston, WV (Appendix IV - pertinent data attached to the complete report). Two different analytical methods were used: with a derivative method, the percentage by weight of DETA was 96.77%, with 0.49% ethylenediamine (EDA), 1.78% N-(2-aminoethyl)piperazine (AEP), 0.70% diethanolamine (DEA) and 0.25% triethanolamine (TEA); with a column of 10% CARBOWAX 30 M on 750 chromosorb 60/80 mesh, the area % of DETA was 99.34% and 0.76% AEP (Data in Table V, Appendix IV).

Methods

A description of the technical procedures used in the CHO test, the SCE test and the UDS assay are presented in greater detail in Appendices I, II and III, respectively (attached to the complete report). Testing was performed in compliance with Standard Operating Procedures used for these tests at the Bushy Run Research Center and deviations from procedures are noted in the individual test results. Diethylenetriamine - high purity is abbreviated DETA-HP in this report consistent with standard UCC Terminology for this product.

1. CHO Test (Detailed procedures in Appendix I): SOP #7.2.4A, 7.2.5, 7.2.11
- A. Cytotoxicity - DETA-HP was tested for cytotoxicity to CHO cells at six concentrations from 0.1% to $0.3 \times 10^{-3}\%$ (by volume) both in the presence and absence of a liver, S9 metabolic activation system. Selection of a maximum concentration for testing depended upon an estimate of dose levels which would permit survival of at least 10% of the treated cells. Glass-distilled water was used as the solvent and solvent control; glass-distilled dimethylsulfoxide (DMSO) was used as the negative control.

To simplify tables and to allow comparisons between different tests, concentrations of DETA-HP in the following sections of the report are given in terms of volume percentages $\times 10^{-2}$ to eliminate zeros in the lower concentration values (eg. 0.0001% = $0.01 \times 10^{-2}\%$).

- B. Mutation - CHO cells were exposed for 5 hours to five concentrations of DETA-HP from $40 \times 10^{-2}\%$ to $1.25 \times 10^{-2}\%$ (by volume) without the addition of an S9 metabolic activation system and to an identical range of concentrations with S9 activation. Dilutions of DETA-HP for testing were prepared by either direct addition of the test agent into the cell culture media or by making sequential one-half dilutions in sterile, glass-distilled H₂O. The surviving fraction was determined at 20 to 24 hours after treatment and the mutant fraction was determined after a 7 to 9-day period to allow "expression" of the mutant phenotype. Only the top five concentrations which allowed sufficient cell survival were assessed for survival and induction of mutants. The percentage of cells surviving the treatment, the frequencies of mutant colonies and the number of mutants/ 10^6 viable cells are presented in tabular form.

2. SCE Test (Detailed procedures in Appendix II): SOP #7.2.12A

Induction of SCE's by exposure to various concentrations of DETA-HP was studied in CHO cells both with and without the incorporation of an S9 metabolic activation system. Selection of dose levels which would permit survival of at least 50% of the treated cells was based on the prescreening test for cytotoxicity performed as part of the CHO Mutation test. Dilutions of DETA-HP for testing, ranging from $20 \times 10^{-2}\%$ to $1.25 \times 10^{-2}\%$ (by volume), were prepared either by direct addition into the culture medium or by making sequential one-half dilutions of the maximum dose level in H₂O. For determination of direct mutagenic action, CHO cells were exposed to DETA-HP and appropriate controls for 5 hours without S9 activation. Indirect mutagenic action, requiring metabolic activation by liver S9 homogenate, was studied with a 2-hour exposure period. Bromodeoxyuridine (BrdU) required to differentiate between the individual "sister" chromatids by SCE staining, was present at a concentration of 3 ug/ml in the growth medium during treatment and during the culture period following exposure. A total of 15 cells/dose level and 5 dose levels, with or without metabolic activation were examined. The number of SCE/cell, mean # of SCE/chromosome and the level of statistical significance of the increases above concurrent solvent control values are presented in tabular form.

3. UDS Test (Detailed procedures in Appendix III): SOP #7.2.6; 7.2.7; 7.2.8A; 7.2.9A

Induction of DNA damage in rat liver cells (hepatocytes), resulting in stimulation of Unscheduled DNA Synthesis (UDS), was studied at a minimum of six dose levels which spanned a 1000-fold range of concentrations. Cells were treated with DETA-HP for 2 hours in culture medium containing ³H-thymidine, hydroxyurea and appropriate dilutions of DETA-HP prepared in DMSO. UDS activity was determined by analyses of the amount of radioactive incorporation into isolated hepatocyte nuclei or in DNA (precipitated from aliquots of the isolated nuclei) using a Searle Analytic Model 81 or Packard Model 2650 scintillation spectrometer. Data are presented in tabular form with an indication of the level of statistical significance above the concurrent solvent control values.

4. Controls - appropriate dose levels of positive, negative and solvent controls were used for each test. In the CHO and SCE assays, dimethylnitrosamine (DMN) and ethylmethanesulfonate (EMS) were used as positive control agents with and without metabolic activation, respectively. Deionized, glass-distilled water, sterilized by membrane filtration or glass-distilled dimethylsulfoxide (DMSO) were used as the solvent and negative controls, respectively.

In the UDS assay, DMN and 4-nitroquinoline oxide (4-NQO) were used as positive controls for indirect- or direct-acting mutagens, respectively. Glass-distilled DMSO was used as the solvent and the solvent control.

5. Metabolic Activation - S9 liver homogenate, prepared from Arochlor 1254-induced, Sprague-Dawley male rats, was purchased from Litton Bionetics. The S9 preparation used for the CHO test contained 27 mg/ml protein and had a benzo(a)pyrene hydroxylase activity of 9.97 nmol hydroxybenzpyrene/20 min/mg protein, (assayed by Litton). A concentration of 1500 ug of S9 protein was added to 5 ml of culture media.

For the SCE test, a serial lot of S9 homogenate, containing 40 mg/ml protein and a benzo(a)pyrene hydroxylase activity of 15.0 nmol hydroxybenzpyrene/20 min/mg protein (assayed by Litton), was used at a concentration of 600 ug of S9 protein/5 ml culture media.

6. Statistical Analyses - Data from the SCE and UDS tests were analyzed by appropriate parametric tests following Standard Operating Procedures for statistical analyses at the Busby Run Research Center. Data from the CHO test do not follow a normal distribution according to experience with historical controls, thus, standard parametric tests were applied only after suitable transformation of the mutation frequencies (MF): specifically $(MF + 1)^{0.15}$ (Irr, J. D. and R. Snee, Proceedings of the Cold Spring Harbor-Banbury Conference, II (1979), 263-274). Rounding of data to either two decimal places or to the appropriate number of significant figures was performed for presentation on tables. Although statistically significant decreases in mutation indices can occur because of cytotoxic responses, only statistically significant increases in responses above control values are indicated on Tables for simplicity. The degree of statistical significance is denoted by: a: $0.05 > p > 0.01$, b: $0.01 > p > 0.001$, or c: $p < 0.001$. No superscript (or NS) indicates $p > 0.05$.
7. Raw Data Storage - Copies of the final report, statistical analyses, available analytical data and data used to prepare the final report are stored in the BRRC Archives. Slides are stored in the Genetic Toxicology slide storage area.

RESULTS:

SECTION I - CHO MUTATION TEST - DIETHYLENTRIAMINE - HIGH PURITY (DETA-HP)

A. Test Dates - Initiated: January 15, 1980
Completed: June 24, 1980

B. Selection of Test Concentration (Data not shown in tables)

CHO Cells were exposed for five hours to six concentrations of DETA-HP which spanned a concentration range from $10 \times 10^{-2}\%$ to $0.03 \times 10^{-2}\%$ by volume. The percentage of cells which survived the exposure, either in the presence or absence of an S9 metabolic activation system, was determined by counting the number of colonies produced by the survivors after a 5 to 7 day incubation period. Because there was no apparent cytotoxicity at the highest dose tested, two higher doses were tested in the mutation induction experiments and a concentration of $40 \times 10^{-2}\%$ was selected as the maximum concentration for testing with and without S9 activation.

C. Determination of Mutation Induction

1. Survival (Cytotoxicity)

Table 1 presents the cytotoxicity data for CHO cells treated with DETA-HP in the presence or absence of a liver S9 metabolic activation system. A steep dose-response effect with the test agent was suggested from the complete cytotoxicity observed for the top concentration $40 \times 10^{-2}\%$, with or without S9 activation, in comparison to the markedly lower cytotoxicity obtained at only one-half the top dose-level. The cytotoxicity of DETA-HP was similar in the presence or absence of S9 activation, particularly apparent in the similar numbers of colonies produced by each respective treatment before expression of the data as a percentage of the control. The H₂O solvent control in the test with S9 was atypically lower than generally observed in similar experiments.

2. Mutation

Table 2 presents the data for induction of mutants by DETA-HP and control agents. No evidence was obtained to indicate the presence of a dose-related increase in the frequency of mutants/ 10^6 viable cells over the 32-fold range of concentrations tested for potential mutagenic action either with or without the presence of an S9 metabolic activation system. No concentration of DETA-HP produced an increase in the mutation frequency which was statistically significant from the concurrent solvent control in the test without S9 activation. Mutation data from cells treated with DETA-HP did not indicate the presence of a dose-response relationship and the test agent was considered not mutagenic to CHO cells in the present test.

Mutation frequencies for the solvent controls for tests both with and without S9 activation were in an acceptable and low range based upon experience with historical control values.

Highly statistically significant mutation frequencies were obtained for the DMN and EMS positive controls for both experiments in comparison to the concurrent solvent control. The values for the positive control agents were similar to values encountered in previous tests with these chemicals and indicated that these data represented a valid, acceptable test. Three concentrations of DMN were used in this test to show the effectiveness of the S9 activation system. A dose-related effect to increasing DMN concentration is apparent from the results.

D. Deviations from Standard Procedures

Three concentrations of DMN were tested to assure the activity of the metabolic activation system.

E. Conclusions

DETA-HP was consistently inactive as a mutagenic agent for CHO cells when tested with or without an S9 metabolic activation system over a 32-fold range of concentrations. None of the mutation values were statistically significant from the concurrent solvent control and values were within the expected variation in mutant frequencies observed in historical control data.

RESULTS:**SECTION II - SCE TEST - DIETHYLENTRIAMINE - HIGH PURITY (DETA-HP)**

A. Test Dates - Initiated: February 20, 1980
Completed: June 16, 1980

B. Selection of Test Concentrations

A maximum concentration of $20 \times 10^{-2}\%$ by volume of DETA-HP was chosen as the top dose levels for testing with or without S9 activation based on cytotoxicity data from the CHO mutation test. Higher concentrations were expected to produce delays in the mitotic cycle and to decrease the number of cells with SCE staining. A 16-fold range of concentrations from $20 \times 10^{-2}\%$ to $1.25 \times 10^{-3}\%$ was examined in SCE experiment #1 without S9 activation. A 32-fold range of concentrations of $20 \times 10^{-2}\%$ to $0.625 \times 10^{-2}\%$ was examined in SCE experiment #2 with S9 activation. A wider concentration range of DETA-HP was employed in the test with S9, in comparison to the test without S9, because a greater degree of cytotoxicity and delays in cell division were observed in the test with a metabolic activation system.

C. Determinations of SCE Induction

The data for SCE induction in CHO cells treated with various dose levels of DETA-HP or with appropriate positive, negative or solvent control agents are summarized in Tables 3 and 4. A single statistically significant increase in the SCE frequency was produced by the highest dose-level of $20 \times 10^{-2}\%$ DETA-HP tested for direct action in the absence of a metabolic activation system (Table 3). A statistically significant result at the highest dose-level should be regarded with suspicion. However, we observed no suggestion of a dose-response effect and the dose-level in question was identical to that which produced complete cytotoxicity with S9. The test without S9 activation was considered a negative indication of potential direct mutagenic action of DETA-HP. Although the possibility of a very narrow range of activity at slightly higher, moderately toxic dose-levels cannot be excluded using the present data, this possibility is considered extremely remote. Induction of SCE by the concurrent EMS positive control was highly statistically significant from the concurrent solvent control and these data indicated an appropriate sensitivity of the test system comparable to our historical positive control data. The numbers of SCE obtained with the H₂O solvent and DMSO controls were also in an acceptable range of values within or slightly below values encountered in our historical control data for this test.

SCE values obtained from treatments with DETA-HP of CHO cells in the presence of an S9 metabolic activation system are presented in Table 4. No statistically significant increase in the SCE values was observed with any of the tested concentrations of DETA-HP. Also, there was a lack of any suggestion of a dose-response effect of treatment with DETA-HP which indicated that the test results were negative and consistent with the findings in the test without addition of S9 (Table 3). DETA-HP was considered inactive as a mutagenic agent in the induction of SCE in vitro.

The negative (DMSO) and solvent controls in the test with S9 activation were higher than values for those agents tested without S9, a finding consistent with the observation of a weak cytotoxic/mutagenic potential for some liver homogenate preparations. A numerical increase and highly statistically significant numbers of SCE were produced by the two different concentrations of the DMN positive control. This result again indicated that the metabolic activation system was functioning in an appropriate manner for an acceptable test.

D. Deviations from Standard Procedures

Because of the cytotoxicity of the liver S9 homogenate, cells treated with the test agent and S9 were incubated for a total of 38 to 42 hours before harvesting cells and preparation of chromosomes for SCE slides. This extended growth period allowed for the cell division delay induced by the liver homogenate.

Due to the alkalinity of the test chemical, the flasks were equilibrated with 10% CO₂ and sealed to help neutralize the pH of the media. Colcemid® at a concentration of 0.1 ug/ml was used rather than colchicine to arrest cells in mitosis. Media purchased from K. C. Biological Co. rather than from Grand Island Biological Co. was used. A concentration of 0.075% trypsin, rather than 0.05% as stated in the SOP was used to improve cell dissociation.

E. Conclusions

Treatments of CHO cells with DETA-HP over a 16 to 32-fold range of concentrations failed to indicate a potential mutagenic activity in tests either with or without addition of an active S9, metabolic activation system. No evidence of a dose-related effect of exposure to DETA-HP on the SCE frequency was evident in tests with or without S9 metabolic activation and the test agent was considered to be inactive in the present in vitro assay.

RESULTS:

SECTION III - UDS TEST - DIETHYLENTRIAMINE - HIGH PURITY (DETA-HP)

A. Test Dates - Initiated: April 4, 1980
Completed: May 12, 1980

B. Selection of Test Concentrations

Standard procedures were followed and DETA-HP was tested over a 3-log range of concentrations from $10 \times 10^{-2}\%$ to $0.01 \times 10^{-2}\%$ by volume. The maximum dose-level was selected according to cytotoxicity data obtained in the CHO Mutation test which indicated that higher values would result in excessive cell killing. These dose-levels were considered to be appropriate for testing because uptake and incorporation of ^3H -thymidine into hepatocytes are generally even more sensitive to chemical effects than survival measurements with CHO cells (in which cytotoxicity is measured after a 24 hour recovery period following treatment).

C. Determination of UDS Induction

1. Nuclear-Bound Radioactive Label (Data in Table 5)

Induction of "unscheduled" incorporation of radioactive thymidine into nuclei of hepatocytes exposed to DETA-HP or to appropriate positive and negative controls is presented in Table 5. Both of the positive control agents, NQO and DMN, induced numerically elevated increases in UDS over values obtained with the solvent control. With both positive controls a moderate increase in the amount of UDS in relation to the exposure concentration indicated the responsiveness of the test system with measurements using nuclei.

Although only the highest dose levels of DMN or NQO produced statistically significant increases above the concurrent solvent control, all concentrations produced numerical increases above the solvent control indicative of an acceptable test. In hepatocytes treated with DETA-HP, only the lowest concentration, $0.01 \times 10^{-2}\%$, tested for potential activity induced a statistically significant increase in the amount of ^3H -thymidine incorporation. A gradual decrease in the amounts of radioactive incorporation, particularly at the high dose level of $10 \times 10^{-2}\%$, was considered an indication of the cytotoxicity of the test agent, DETA-HP. The lack of a clear indication of statistically significant results or of a possible dose-response relationship resulting from DETA-HP exposure indicated that the test results were a negative indication of potential activity. DETA-HP was considered inactive in the present test.

2. DNA-Bound Radioactive Label (Data in Table 6)

Analyses of DNA, from aliquots of hepatocyte nuclei used for the UDS studies presented on Table 5, were performed as a second assessment of "unscheduled" incorporation of radioactive thymidine. Values for radioactivity incorporated into the DNA of these hepatocyte nuclei are presented in Table 6.

Two concentrations of DETA-HP, $0.3 \times 10^{-2}\%$ and $0.01 \times 10^{-2}\%$, induced levels of UDS significantly above that of the concurrent control. By comparing the nuclear-bound values with the DNA values, a similar pattern of quantitative response was seen. However, the $0.3 \times 10^{-2}\%$ dose-level was also statistically significant using the DNA method. The numerically-increased values in the DNA experiment for the dose-levels 0.3, 0.1 and $0.01 \times 10^{-2}\%$ are all outside the 95% confidence interval for historical controls for the DNA-bound label method (2523 to 3816 DPM/ 10^6 cells). Although these data may suggest the presence of an extremely weak activity in the test sample, statistical analyses with respect to the concurrent control indicated that DETA-HP was most likely an inactive agent in the present test on induction of UDS. The lack of significant or dose-related effects at any of the higher dose-levels (which showed no apparent cytotoxicity in the UDS or the CEO tests) indicated that a negative classification was justified and correct.

In measuring UDS by either the nuclear-bound or DNA-bound label method, the positive control agents, NQO and DMN, both induced numerically increased and statistically significant levels of UDS. The lack of significant values at the two lower concentrations of NQO and DMN may have been due to cytotoxic effects at the lower doses or simply insufficient dose for induction of UDS. The test was considered valid based on the distinct positive responses with the highest dose levels of the positive control agents.

D. Deviations from Standard Procedures

None.

E. Conclusions

DETA-HP failed to stimulate consistently a dose-related incorporation of radioactive thymidine in cells treated over a 1000-fold range of test concentrations. Although values for the positive controls were low, in the assessment of UDS with nuclei, measurements of radioactive incorporation into the precipitated DNA from those nuclei verified the activity of the positive control agents and the inactivity of the DETA-HP sample. DETA-HP was considered inactive in the tests with hepatocytes.

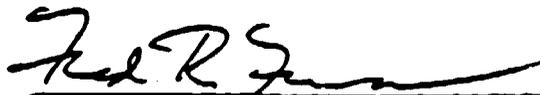
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Table 1
 Chinese Hamster Ovary (CHO) Mutation Assay:
 Determination of Toxic Effects of Chemical Treatment During 5 Hr Mutation Induction Period

Test Chemicals	Total # Colonies	Total # Cells Plated	% Survival	% of Solvent Control
Without S9 Activation				
[Diethylenetriamine - High Purity] (X, v/v)				
40.0 x 10 ⁻²	-	TOXIC	-	-
20.0 x 10 ⁻²	363	400	90.8	92.4
10.0 x 10 ⁻²	381	400	95.2	96.9
50.0 x 10 ⁻²	393	400	98.2	100.0
2.5 x 10 ⁻²	327	400	81.8	83.2
1.25 x 10 ⁻²	405	400	101.2	103.1
Controls				
H ₂ O - Solvent (20 ul/ml)	393	400	98.2	-
DMSO - (20 ul/ml)	412	400	103.0	104.8
EMS - (200 ug/ml)	343	400	85.8	87.3
With S9 Activation				
[Diethylenetriamine - High Purity] (X, v/v)				
40.0 x 10 ⁻²	-	TOXIC	-	-
20.0 x 10 ⁻²	395	400	98.8	134.4
10.0 x 10 ⁻²	395	400	98.8	134.4
5.0 x 10 ⁻²	306	400	76.5	104.1
2.5 x 10 ⁻²	357	400	89.2	121.4
1.25 x 10 ⁻²	378	400	94.5	128.6
Controls				
H ₂ O - Solvent (20 ul/ml)	294	400	73.5	-
DMSO - (20 ul/ml)	143	400	35.8	48.6
DMN - (3700 ug/ml)	61	400	15.2	20.7
DMN - (3000 ug/ml)	48	400	12.0	16.3
DMN - (2000 ug/ml)	83	400	20.8	28.2

Abbreviations: H₂O - water; S9 - liver homogenate; DMSO - dimethylsulfoxide
 EMS - ethylmethanesulfonate; DMN - dimethylnitrosamine

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Table 2
Chinese Hamster Ovary (CHO) Mutation Assay:
Results on Evaluation of Mutant Induction by Diethylenetriamine - High Purity

Test Chemicals	Plating Efficiency			Mutation Induction		
	Total # Colonies	Total # Cells Plated	Viable Fraction	Total # Mutant Colonies	Total # Cells Plated	Mutants ¹ 10 ⁶ Viable Cells
Without S9 Activation						
[Diethylenetriamine - High Purity] (% v/v)						
40.0 x 10 ⁻²	-	-	TOXIC	-	-	-
20.0 x 10 ⁻²	293	400	0.732	0	1 x 10 ⁶	0
10.0 x 10 ⁻²	277	400	0.692	0	1 x 10 ⁶	0
5.0 x 10 ⁻²	257	400	0.642	0	1 x 10 ⁶	0
2.5 x 10 ⁻²	325	400	0.812	0	1 x 10 ⁶	0
1.25 x 10 ⁻²	258	400	0.645	0	1 x 10 ⁶	0
Controls:						
H ₂ O - Solvent (20 ul/ml)	321	400	0.802	3	1 x 10 ⁶	3.7
DMSO - (20 ul/ml)	313	400	0.782	0	1 x 10 ⁶	0
EMS - (200 ug/ml)	350	400	0.875	83	1 x 10 ⁶	94.9 ^c
With S9 Activation						
[Diethylenetriamine - High Purity] (% v/v)						
40.0 x 10 ⁻²	+	-	TOXIC	-	-	-
20.0 x 10 ⁻²	208	400	0.520	0	1 x 10 ⁶	0
10.0 x 10 ⁻²	223	400	0.558	0	1 x 10 ⁶	0
5.0 x 10 ⁻²	181	400	0.452	0	1 x 10 ⁶	0
2.5 x 10 ⁻²	190	400	0.475	0	1 x 10 ⁶	0
1.25 x 10 ⁻²	187	400	0.468	1	1 x 10 ⁶	2.1
Controls:						
H ₂ O - Solvent (20 ul/ml)	165	400	0.412	1	1 x 10 ⁶	2.4
DMSO - (20 ul/ml)	274	400	0.685	4	1 x 10 ⁶	5.8
DMN - (3700 ug/ml)	98	400	0.245	62	1 x 10 ⁶	253.1 ^c
DMN - (3000 ug/ml)	131	400	0.328	5	1 x 10 ⁶	106.9 ^b
DMN - (2000 ug/ml)	114	400	0.285	11	1 x 10 ⁶	38.6 ^b

¹Total # mutant colonies per 10⁶ cells plated divided by viable fraction.
 Statistical significance above solvent control: b: 0.01 > p > 0.001; c: p < 0.001;
 no superscript indicates p > 0.05. Data analyzed by Student's t-test.

Abbreviations: H₂O - water; S₉ - liver homogenate; DMSO - dimethylsulfoxide; EMS - ethylmethanesulfonate;

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Table 3
Sister Chromatid Exchange (SCE) Assay:
Induction of SCE's by Diethylenetriamine High Purity Without S9 Metabolic Activation
5 Hour Treatment
Experiment #1

Test Chemicals	Total # of Chromosomes	Total # of SCE	SCE/Cell ¹	Mean Number SCE/Chromo- some ² + S.D.	Significance Above Solvent Control ³
[Diethylenetriamine - High Purity] (%, v/v)					
20.0 x 10 ⁻²	295	236	15.73	0.794 + 0.234	a
10.0 x 10 ⁻²	298	195	13.00	0.653 + 0.140	NS
5.0 x 10 ⁻²	286	184	12.27	0.640 + 0.170	NS
2.5 x 10 ⁻²	298	191	12.73	0.643 + 0.192	NS
1.25 x 10 ⁻²	301	198	13.20	0.660 + 0.194	NS
Controls					
H ₂ O - (5 ul/ml) - Solvent	298	177	11.80	0.593 + 0.169	-
DMSO - (5 ul/ml)	299	154	10.27	0.517 + 0.190	NS
EMS - (100 ug/ml)	300	427	28.47	1.415 + 0.390	c
¹ Fifteen cells examined per dose level. ² Mean value of SCE/chromosome determined from the values of the individual cells examined. ³ Statistical significance above solvent control: a: 0.05 > p > 0.01; c: p < 0.001 NS: p > 0.05. Data analyzed by Student's t-test.					

Abbreviations: H₂O - water; S9 - liver homogenate; DMSO - dimethylsulfoxide;
EMS - ethylmethanesulfonate; S.D. - standard deviation

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Table 4
Sister Chromatid Exchange (SCE) Assay:
Induction of SCE's by Diethylenetriamine High Purity With S9 Metabolic Activation
2 Hour Treatment
Experiment #2

Test Chemicals	Total # of Chromosomes	Total # of SCE	SCE/Cell ¹	Mean Number SCE/Chromo- some ² + S.D.	Significance Above Solvent Control ³
[Diethylenetriamine - High Purity] (X, v/v)					
20.0 x 10 ⁻²	-	-	TOXIC	-	-
10.0 x 10 ⁻²	285	175	11.67	0.626 + 0.256	NS
5.0 x 10 ⁻²	295	157	10.47	0.535 + 0.161	NS
2.5 x 10 ⁻²	285	129	8.60	0.455 + 0.150	NS
1.25 x 10 ⁻²	293	132	8.80	0.452 + 0.126	NS
0.625 x 10 ⁻²	296	153	10.20	0.517 + 0.155	NS
Controls					
H ₂ O - (5 ul/ml) - Solvent	292	178	11.87	0.614 + 0.212	-
DMSO - (5 ul/ml)	293	188	12.53	0.644 + 0.168	NS
DMN - (1000 ug/ml)	296	954	63.60	3.201 + 0.716	c
DMN - (800 ug/ml)	304	883	58.87	2.914 + 0.798	c

¹Fifteen cells examined per dose level.

²Mean value of SCE/chromosome determined from the values of the individual cells examined.

³Statistical significance above solvent control: c: p < 0.001;
NS: p > 0.05. Data analyzed by Student's t-test.

Abbreviations: H₂O - water; S9 - liver homogenate; DMSO - dimethylsulfoxide;
DMN - Dimethylnitrosamine; S.D. - standard deviation

Table 3
Unscheduled DNA Synthesis in Hepatocytes from Rat Liver

Nuclear-bound label: all DPM values are calculated from nuclei per 10^6 viable hepatocytes. Each average is calculated from duplicate samples, except for DMSO which was done in quadruplicate.

Test Chemical	Concentration	Radioactivity in Nuclei Avg. DPM \pm S.D.	% of Solvent Control \pm S.D.	Significance Above Solvent Control ¹
Solvent - DMSO	2.0%	3195 \pm 679	100.0% \pm 21.2%	-
Positive Controls:				
4 - NQO	3.0 ug/ml	7950 \pm 1662	248.8% \pm 52.0%	c
	1.0 ug/ml	3925 \pm 1291	122.8% \pm 40.4%	NS
	0.3 ug/ml	4369 \pm 1321	136.7% \pm 41.4%	NS
DMN	1000 ug/ml	5341 \pm 59	167.2% \pm 1.9%	a
	300 ug/ml	4066 \pm 317	127.3% \pm 9.9%	NS
	100 ug/ml	3850 \pm 617	120.5% \pm 19.3%	NS
Test Chemical:				
[(Diethylenetriamine - High Purity) (%, v/v)]	10 x 10 ⁻² %	1970 \pm 290	61.6% \pm 9.1%	NS
	3 x 10 ⁻² %	3588 \pm 330	112.3% \pm 10.3%	NS
	1 x 10 ⁻² %	3607 \pm 299	112.9% \pm 9.4%	NS
	0.3 x 10 ⁻² %	5057 \pm 615	158.3% \pm 19.3%	NS
	0.1 x 10 ⁻² %	4362 \pm 952	136.5% \pm 29.8%	NS
	0.01 x 10 ⁻² %	5260 \pm 1576	164.6% \pm 49.3%	a

¹Statistical significance above solvent control: a: $0.05 > p > 0.01$; c: $p < 0.001$;
NS: $p > 0.05$; Data analyzed by Duncan's Multiple Range Analysis.

Abbreviations: DMSO - dimethylsulfoxide; 4-NQO - 4-nitroquinoline oxide; DMN - dimethylnitrosamine;
DPM - disintegrations per minute; S.D. - standard deviation

Table 6
 Unscheduled DNA Synthesis in Hepatocytes from Rat Liver

DNA-bound label: all DPM values are calculated from DNA precipitated per 10^6 viable hepatocytes. Each average is calculated from duplicate samples, except for DMSO which was done in quadruplicate.

Test Chemical	Concentration	Radioactivity in DNA Avg. DPM \pm S.D.	% of Solvent Control \pm S.D.	Significance Above Solvent Control ¹
Solvent - DMSO	2.0%	2907 \pm 524	100.0% \pm 18.0%	-
Positive Controls:				
4 - NQO	3.0 ug/ml	9035 \pm 1461	310.8% \pm 50.2%	c
	1.0 ug/ml	4270 \pm 1440	146.9% \pm 49.5%	NS
	0.3 ug/ml	4424 \pm 930	152.2% \pm 32.0%	NS
DMN	1000 ug/ml	5225 \pm 389	179.7% \pm 13.4%	a
	300 ug/ml	4356 \pm 610	149.8% \pm 21.0%	NS
	100 ug/ml	3769 \pm 1047	129.6% \pm 36.0%	NS
Test Chemical:				
(Diethylenetriamine - High Purity) (X, v/v)	$10 \times 10^{-2}\%$	1736 \pm 174	59.7% \pm 6.0%	NS
	$3 \times 10^{-2}\%$	3468 \pm 304	119.3% \pm 10.5%	NS
	$1 \times 10^{-2}\%$	3638 \pm 163	125.1% \pm 5.6%	NS
	$0.3 \times 10^{-2}\%$	4923 \pm 185	169.3% \pm 6.4%	a
	$0.1 \times 10^{-2}\%$	4349 \pm 1006	149.6% \pm 34.6%	NS
	$0.01 \times 10^{-2}\%$	4800 \pm 1303	165.1% \pm 44.8%	a

¹Statistical significance above solvent control: a: $0.05 > p > 0.01$; c: $p < 0.001$;
 NS: $p > 0.05$; Data analyzed by Duncan's Multiple Range Analysis.

Abbreviations: DMSO - dimethylsulfoxide; 4-NQO - 4-nitroquinoline oxide; DMN - dimethylnitrosamine;
 DPM - disintegrations per minute; S.D. - standard deviation

APPENDIX I

Chinese Hamster Ovary (CHO) Mutation AssayTheoretical Basis

Mutation is a heritable alteration in a cell in which a gene specifying the genetic code for a specific protein is modified in structure and/or function. Mutations, induced by chemical or physical agents, of the HGPRT (hypoxanthine-guanine phosphoribosyltransferase) gene may be detected by the growth of colonies of "mutant" cells which are resistant to the purine analogs 6-thioguanine (TG) or 8-azaguanine. Normal cells contain a functional HGPRT enzyme which phosphorylates TG and allows its incorporation into DNA causing the cells to die. Mutant cells with a non-functional HGPRT enzyme are unable to phosphorylate or incorporate TG, thus survive and grow in its presence.

The CHO mutation test is an assay which detects "forward mutations" from TG-sensitivity to TG-resistance caused by a direct loss of the activity of the HGPRT enzyme ($HGPRT^+ \rightarrow HGPRT^-$). An assessment of the ability of several hundred agents to cause gene mutations in vitro indicates that the CHO mutation assay provides a reasonable estimate of the potential genetic activity of the test chemical.

Methods

Cell Culture Procedures: CHO cells used in these studies were obtained from Abraham Hsieh at Oak Ridge National Laboratory with the designation CHO-K1-BH4-D1 (or simply CHO for report purposes). Cells are maintained in active growth by subculturing 2 to 3 times/week in antibiotic-free, Ham's Modified F12 Medium supplemented with 10% (v/v) heat-inactivated, fetal bovine sera (F12-10), and lacking in hypoxanthine. For treatment of cells without metabolic activation, F12 medium with 50 units/ml of penicillin, 50 ug/ml streptomycin and 5% (v/v) of dialyzed bovine serum (F12-D5) is used. For treatments incorporating an S9 metabolic activation system, identical medium, but without serum, is employed. For determination of mutant frequencies, F12-D5 medium containing 2.0 ug/ml TG (6-thioguanine) is used as a "selective medium." Cell numbers are determined routinely with a Coulter Model F electronic cell counter which is standardized periodically with a pre-counted suspension of latex beads. Presence of Mycoplasma cell contaminants is determined by a microscopic fluorescence assay employing Hoechst 33258 dye. All culture procedures and treatments with test chemicals are performed under aseptic conditions in a laminar-flow, biohazard hood.

Positive and Negative Controls: Sterile, de-ionized, glass-distilled water or glass-distilled dimethylsulfoxide (DMSO) are the usual solvents for test chemicals and the respective solvent is tested as a control at the maximum concentration used to add the test agent. Dimethylnitrosamine (DMN) or ethylmethanesulfonate (EMS) are used as positive control mutagens for tests with or without an S9 metabolic activation system, respectively. Mutation frequencies obtained with concurrent positive and negative controls are used as the basis for monitoring the sensitivity and stability of the CHO mutation test system. Comparison of concurrent control values with historical controls is used to delineate the range of acceptable variations in the test system.

Metabolic Activation: Rat liver, S9 homogenate prepared from Arochlor-1254 induced, Sprague-Dawley, male rats is purchased from Litton Bionetics, Kensington, MD. Each lot of liver homogenate is prescreened for metabolic capability to activate DMN in our laboratory before use in the testing program. The complete S9 metabolic activation system contains the following: 8 umoles/ml $MgCl_2$, 33 umoles/ml KCl, 5 umoles/ml glucose-6-phosphate, 4 umoles/ml NADP-oxidized (nicotinamide adenine dinucleotide phosphate), 100 umoles/ml Na_2HPO_4 , and between 500 to 4000 ug/ml of S9 protein (depending on metabolic activity); a volume of 1.0 ml of the complete mixture of the above reagents is added to each 4.0 ml of culture medium.

Dose Selection: Toxicity of the test chemical is determined prior to assessment of mutagenic potential to select doses which produce a maximum of 80 to 90% cell killing. Cytotoxicity is determined by either of the following two methods:

- (1) **Clonal assay** - 200 to 400 CHO cells are exposed to a minimum of five dose levels of the test agent at concentrations from 0.1% to 3×10^{-4} % (by weight or volume, as appropriate) with and without the presence of a metabolic activation system. The number of cells which survive the treatment is determined by counting the number of colonies produced after a 7- to 8-day incubation period (37°C) in comparison with the colonies formed by cells treated only with appropriate concentrations of solvent (generally 20 ul/ml).
- (2) **Growth Inhibition** - 5×10^5 cells in 25 cm² culture flasks are treated for 5 hours with a minimum of five test concentrations both with and without S9 metabolic activation. Following treatment the cells are rinsed, fresh F12-DS medium is added and the flasks are incubated for an additional 18 to 24 hours. Cytotoxicity is determined by comparing the relative number of cells in control (untreated cells) and in cells treated with various concentrations of the test agent.

If no cytotoxicity is evident at the highest concentrations in the cytotoxicity tests, the test is either repeated at higher concentrations, or mutation testing is performed with a greater number of treatment flasks starting at higher dose levels. If marked toxicity is evident even at the lowest dose, the cytotoxicity test is repeated at a concentration range of 3×10^{-6} to 3×10^{-8} percent by volume.

Dose levels which are moderately toxic but permit survival of at least 10 to 20% of the cells, in comparison to the solvent control, are selected as the maximum dose, and at least four additional one-half dilutions are tested for induction of mutations. If cytotoxicity data are equivocal, a total of 5 to 8 one-half dilutions of the selected, maximum concentration are used to treat cells; but only the highest five concentrations which permit survival of a sufficient number of cells are assessed for mutation induction.

Chemical samples are sterilized by membrane filtration when microbiological tests indicate this is required to assure sterility. Liquid test agents are tested on a percentage by volume basis. Solid chemicals are dissolved in an appropriate solvent by making a 10 to 20% stock solution (by weight) and subsequent dilutions are made from this stock on a volume/volume basis.

Treatment with Test Chemicals: For tests of direct acting chemicals without incorporation of an S9 metabolic activation system, 5×10^5 cells are inoculated 20 to 24 hours prior to treatment into 25 cm² culture flasks containing F12-D5 medium and incubated at 37°C in a 5 to 6% CO₂ atmosphere. Appropriate concentrations of the test agent or control chemicals are added to the cells and cultures are treated for 5 hr at 37°C. The medium and test agents are removed by suction, cells are rinsed once or twice and fresh F12-D5 medium is added. The cells are allowed a period of 20 to 24 hours of recovery from treatment before survival is determined. Treatment of cells for testing of chemicals which require metabolic activation for mutagenic capacity is performed identically with the procedure above, with the exception that F12 medium without serum and containing 1.0 ml of S9 activation mixture per 4.0 ml of medium is employed.

Determination of Cytotoxicity: The relative survival of treated cells, in comparison to solvent controls, is determined one day after the exposure to the test agents. The level of cytotoxicity is often correlated with the mutation frequencies induced by known chemical mutagens. Thus, excessive cytotoxicity may kill both normal cells and mutants and may depress the actual mutation frequencies; insufficient cytotoxicity may indicate an insufficient concentration of the test agent was employed. The colony-forming potential of 100 to 200 treated cells is used as the measure of treatment-induced cytotoxicity.

Survival values which indicate the cytotoxic effects of the test agents are included in reports in tabular form. Statistical analyses are not performed on these data, since they are only useful as a frame-of-reference and are not used to calculate mutation frequencies.

Determination of Mutant Induction: On days 1, 3 and 6 (or alternatively 1, 4 and 6) after treatment with the various test agents, approximately 5×10^5 cells are subcultured in 100 mm tissue culture dishes in F12-D5 medium and incubated at 37°C in a 5 to 6% CO₂ atmosphere. After a total of 7 days to allow "expression" of the mutant phenotype, cells are dissociated with 0.05 to 0.075% trypsin, counted and plated at a concentration of 2.5×10^5 /dish in four culture dishes (1×10^6 total cells) which each contain 5 ml of F12-D5 (TG) selective medium. At this time, cells are diluted and 100 cells/dish are added to four culture plates containing F12-D5 medium (without TG) to assess viability (plating efficiency) of the treated cell population and to determine the surviving fraction. All cultures are then incubated for an additional 6 to 8 days to allow growth of cells; medium is then discarded and colonies are fixed and stained for counting. The number of colonies in selection plates and in the viability test are counted by electronic methods, checked by manual counts and data are recorded both as total mutants, mutants/ 10^6 total cells and mutants/ 10^6 viable cells.

Statistical Analyses: Uniform statistical procedures to evaluate in vitro mutation data have not been developed. The distribution of mutation frequencies from historical controls in at least two laboratories indicates that the frequency distribution and variances encountered do not justify the use of parametric analyses unless data is transformed before application of standard parametric tests. Analysis of mutation frequencies in the CHO test follow the procedure of Irr and Snee (Reference 4) which employs the Box-Cox Transformation (Reference 5) to transform data before parametric analyses. The mutation frequency for each plate is increased by 1.0 (to eliminate zeros) and raised to the 0.15 power. Experience with historical negative control data in our laboratory indicates that a normal probability distribution of the data suitable for parametric analyses is achieved by this transformation. Parametric analysis of mutation data following the ERRC Standard Operating Procedures for Statistical Analysis is performed on the transformed data. The degree of statistical significance for the mutation values are indicative of a difference from the concurrent solvent control, but these statistical indicators must be viewed conservatively until additional historical control data are available.

Interpretation of Data: The criteria for interpretation of the test results as a positive or negative response depend upon both the level of statistical significance from the concurrent control and the evidence of a dose-response following treatment. When a clear dose-response relationship is not evident but one or more marginally significant values are obtained, a careful examination of the data from the concurrent positive and negative controls and comparisons to historical control data are used to evaluate the possible significance of the responses. Historical control data indicate that a spontaneous mutation frequency in CHO cells of approximately 4 to 5 mutants/ 10^6 viable cells, with a range of 0 to 25 mutants/ 10^6 viable cells, can be obtained in the absence of mutagenic treatment. Statistical comparisons against unusually high or low spontaneous controls are subjectively scrutinized in respect to the above variability.

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Determination of Sister Chromatid Exchange (SCE) Frequencies
in Chinese Hamster Ovary (CHO) Cells In Vitro

Theoretical Basis

Exchanges of genetic material between the individual arms of a chromosome (i.e. sister chromatids) are thought to arise from breakage and physical interchanges in the DNA of a cell during cell division. An increase in the frequency of such interchanges between sister chromatids can be observed in cells treated with physical or chemical mutagenic agents, or in cells exposed to many suspect or proven human carcinogens. Thus, analysis of SCE frequencies in cells treated with a test agent has been suggested as a sensitive screening test for potential mutagenic/carcinogenic chemicals.

The method used in our study to visualize SCE's in CHO cells grown in culture is based on the procedure described by Ferry and Wolff (1974). A standard concentration of 3.0 ug/ml of bromodeoxyuridine (BrdU) was used in the growth medium to allow a visualization of SCE's after two cell divisions in the presence of BrdU. A concentration of 5.0 ug/ml of 33258-Hoechst fluorescent dye and Giemsa stain was used to differentiate chromosomes for SCE's.

Methods

Cell Culture Procedures: Chinese hamster ovary (CHO) cells were obtained from Abraham Hsieh at Oak Ridge National Laboratory with the designation CHO-K1-BH4-D1 (referred to simply as CHO for report purposes). CHO cells are maintained in active growth by 2 to 3 weekly subcultures into fresh antibiotic-free, Ham's F12 (modified) medium fortified with 10% (v/v) of heat-inactivated fetal bovine serum and lacking hypoxanthine and thymidine. Cell concentrations are determined routinely with a Coulter® Model-F electronic cell counter calibrated with a precounted suspension of latex beads. All cell culture procedures prior to final harvesting of cells for chromosome preparations are performed under aseptic conditions in a laminar flow, biohazard hood. Presence of Mycoplasma cell contaminants is determined using a fluorescent microscopic assay employing Hoechst 33258 dye.

For treatments with test chemicals without S9 metabolic activation, modified F12 medium is used with 50 units/ml of penicillin, 50 ug/ml streptomycin and 5% (v/v) of heat-inactivated, dialyzed fetal bovine serum (F12-D5). Identical medium but without serum is used for treatments incorporating an S9 metabolic activation system.

Positive and Negative Controls: Sterile, deionized, glass-distilled water or glass-distilled dimethyl sulfoxide (DMSO) are the usual solvents used for test chemicals and the respective solvent is tested as a control at the maximum concentration used to add the test agent. Dimethylnitrosamine (DMN) and ethylmethanesulfonate (EMS) are used as positive control mutagens for tests with or without the addition of an S9 metabolic activation system, respectively. Results from treatments with concurrent control agents are used as a basis of comparison and for demonstrating the sensitivity and stability of the SCE test system. Comparison of concurrent control values with historical controls is used to delineate the range of acceptable variations in the test system.

Metabolic Activation: Rat liver S9 homogenate (prepared from Arochlor 1254 induced, Sprague-Dawley, male rats) is purchased from Litton Bionetics, Kensington, MD. Each lot of liver homogenate is prescreened for activity in our laboratory before use in the testing program. The complete S9 metabolic activation system contains the following: 8 umoles/ml $MgCl_2$, 33 umoles/ml KCl , 5 umoles/ml KCl , 5 umoles/ml glucose-6-phosphate, 4 umoles/ml NADP-oxidized form (nicotinamide adenine dinucleotide phosphate), 100 umoles/ml Na_2HPO_4 and between 500 to 4000 ug/ml of S9 protein (depending on metabolic activity). A volume of 1.0 ml of the complete mixture of the above reagents is added to each 4.0 ml of culture medium.

Dose Selection: Toxicity of the test chemical is determined prior to assessment of mutagenic potential to select doses which produce a maximum of 80 to 90% cell killing. Cytotoxicity is determined by either of the following two methods:

- (1) **Clonal assay** - 200 to 400 CHO cells are exposed to a minimum of five dose levels of the test agent at concentrations from 0.1% to $3 \times 10^{-4}\%$ (by weight or volume, as appropriate) with and without the presence of a metabolic activation system. The number of cells which survive the treatment is determined by counting the number of colonies produced after a 7- to 8-day incubation period ($37^\circ C$) in comparison with the colonies formed by cells treated only with appropriate concentrations of solvent (generally 20 ul/ml).
- (2) **Growth Inhibition** - 5×10^5 cells in 25 cm^2 culture flasks are treated for 5 hours with a minimum of five test concentrations both with and without S9 metabolic activation. Following treatment the cells are rinsed, fresh F12-D5 medium is added and the flasks are incubated for an additional 18 to 24 hours. Cytotoxicity is determined by comparing the relative number of cells in control (untreated cells) and in cells treated with various concentrations of the test agent.

If no cytotoxicity is evident at the highest concentrations in the cytotoxicity tests, the test is either repeated at higher concentrations, or mutation testing is performed with a greater number of treatment flasks starting at higher dose levels. If marked toxicity is evident even at the lowest dose, the cytotoxicity test is repeated at a concentration range of 3×10^{-4} to 3×10^{-6} percent by volume.

Dose levels which are moderately toxic but permit survival of at least 40 to 50% of the cells, in comparison to the solvent control, are selected as the maximum dose, and at least four additional one-half dilutions are tested for induction of mutations. If cytotoxicity data are equivocal, a total of 5 to 8 one-half dilutions of the selected, maximum concentration are used to treat cells; but only the highest five concentrations which permit survival of a sufficient number of mitotic cells with SCE staining, are evaluated for SCE induction.

Chemical samples are sterilized by membrane filtration when microbiological tests indicate this is required to assure sterility. Liquid test agents are tested on a percentage by volume basis. Solid chemicals are dissolved in an appropriate solvent by making a 10 to 20% stock solution (by weight) and subsequent dilutions are made from this stock on a volume/volume basis.

Treatment With Test Chemicals: Testing of chemicals for direct mutagenic action (without S9 metabolic activation) is performed first. For chemicals with clearly positive mutagenic capabilities by direct action, testing with metabolic activation is generally not performed.

For testing direct acting chemicals for SCE induction, 2×10^6 cells are plated with 75 cm² culture flasks in F12-D5 medium 20 to 24 hrs prior to treatment and incubated at 37°C in a 5 to 6% CO₂ atmosphere. Appropriate concentrations of the test agent or control chemicals are added to the cells and 3 ug/ml BrdU is added to all flasks. Cells are treated with test agents for 5 hrs, media is then removed by suction, cells are rinsed with buffered, physiological salt solution and fresh medium containing 3 ug/ml BrdU is added for 24 to 30 hrs of additional incubation at 37°C. Cells are harvested and chromosomes are prepared for SCE staining.

Treatment of cells for testing of chemicals which require metabolic activation for mutagenic effectiveness is performed similarly as for treatments without activation, except for three modifications:

1. Before treatment with the test agents, F12-D5 medium is removed and F12 medium without serum is added.
2. S9 metabolic activation mixture is added to each flask (including solvent and positive controls) before addition of test agents.
3. Cells are treated for a total of 2 hrs (rather than 5 hrs) and then incubated for 36 to 42 additional hours before harvest for chromosome preparation.

Preparation of Chromosomes: Colcemid® (0.1 ug/ml) or Colchicine (0.5 ug/ml) is added to culture flasks 2 to 3 hrs prior to harvesting to arrest cells in mitosis. Cells are then removed from flasks, after a brief incubation with 0.01% DIFCO trypsin, suspended in 0.075M KCl (hypotonic) solution and incubated for 15 to 20 min at 37°C. Cells are centrifuged, fixed with 3 or 4 changes of Carnoy's fixative (3:1 methanol acetic acid) and chromosome spreads are prepared from cells suspended in a small volume of fixative. One slide/dose level is prepared, but fixed cells are saved if needed for preparation of additional slides.

Chromosomes are stained for SCE's by treatment with 5.0 ug/ml of Hoechst 33258 dye for 20 min, rinsed in distilled water, immersed in Sorenson's buffer and exposed to a high intensity sunlamp for 15 to 20 min. Irradiated chromosomes are stained in Gurr's giemsa (diluted 1:25 with water), rinsed in water and dried before application of coverslips.

Examination of SCE's: All slides are coded and read in a blind fashion without indication of the specific treatment or concentration of the test agent. The number of chromosomes and the number of SCE's in a minimum of 15 cells are recorded for each dose level. The mean number of SCE/cell and SCE/chromosome are calculated and recorded. Slides are decoded only after examination of all slides in the experiment has been completed.

Statistical Analyses: Data are analyzed by appropriate parametric statistical procedures which follow BRRC standard operating procedures for analyses of data. Significance values and the statistical test employed are shown for data summarized in tabular form.

Interpretation of Data: The criteria for evaluation of a positive or negative response depend both on the level of statistical significance and subjective analyses of concurrent and historical control data. The key determinant is whether a dose-dependent increase in SCE's is induced by the test agent. When no clear dose-response relationship is evident and when one or more responses of marginal statistical significance are obtained, a careful examination of the data in comparison to the concurrent controls and historical data base is necessary. Testing may be repeated to clarify unusual responses, if data for the concurrent positive or negative controls suggest a defect in the original experiment. Overall assessment will also rely on corroborating data from the other tests in the testing battery. Clearly positive responses will include any of the following: (i) Doubling in the SCE frequency at a minimum of two of the five concentrations tested; (ii) Statistically significant responses of $p < 0.05$ at three concentrations or at 2 concentrations if $p < 0.01$; (iii) Induction of a statistically significant, dose-related number of SCE.

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Unscheduled DNA Synthesis (UDS) in Hepatocytes from Rat LiverTheoretical Basis

Chemicals may interact with both the cellular components and the genetic material of a cell (e.g. DNA and RNA) because of their electrophilic nature or by conversion into reactive electrophiles by the metabolic enzymes of the cell. Damage to the DNA of a cell can result in cell death, mutation or, theoretically, carcinogenic transformation. Studies of agents which are capable of reacting and damaging the cellular DNA have suggested that such methods may be useful as a sensitive screening test for detecting potential mutagenic/carcinogenic chemical properties.

Detection of the relatively small amounts of DNA damage induced by chemical treatment requires a cellular system in which normal, semi-conservative DNA replication, which occurs during cell division, is inhibited. The system employed for the present study uses a suspension culture of primary hepatocyte cells isolated from rat liver according to the general methods of Seglen (1973) and Williams (1976). Hepatocytes do not normally divide in the minimal culture medium employed and stimulation of "unscheduled" incorporation of radioactive DNA precursors can be detected by scintillation spectrometry. The stimulation of incorporation of tritiated thymidine into both purified hepatocyte nuclei and DNA is used as the indicator of chemically induced DNA damage. The amount of unscheduled DNA synthesis (UDS) following treatment is compared with both concurrent positive and negative controls as well as with historical data for similar tests.

Methods

Preparation of Hepatocyte Suspensions: Hilltop-Wistar albino rats are anesthetized with Metafane(R). The abdominal cavity is surgically exposed and 1250 units of heparin is injected intravenously. A catheter is inserted into the portal vein and warm Hanks Balanced Salt Solution (HBSS) is pumped into the vein and through the liver. This first solution contains heparin and EGTA, [ethylene glycol-bis-(beta-aminoethyl-ether)N,N-tetracetic acid], which preferentially chelates calcium; the solution contains no magnesium or calcium. After the liver is blanched, a second solution of HBSS containing 60 units/ml collagenase is perfused. This solution is pumped through the liver until the liver is digested. The liver is then removed and the cells are freed in cold medium 199 by combing through the lobes with a sterile metal comb. The cell suspension is passed through two nylon meshes to remove cell clumps and the cells are washed once at low centrifugation speed. After resuspension in medium 199, equal volumes of cells and 0.4% trypan blue are mixed together and the cell viability and number of viable cells per ml is determined microscopically.

Preincubation of Hepatocytes: Approximately 2×10^6 viable hepatocytes are added to 5 ml medium 199 containing 10 mM hydroxyurea and 30 HEPES (N-2-hydroxyethyl piperazine-N-2 ethane sulfonic acid) buffer. After the cells are dispensed into the tubes, they are placed on a rocker platform and incubated at 37°C for 1 hour. Although hepatocytes do not normally divide in culture, medium 199 which lacks serum and contains hydroxyurea is used to further block semi-conservative DNA synthesis. Thus, any radioactive thymidine incorporated into the nuclei is expected to result from repair or unscheduled DNA synthesis.

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Selection of Doses of Test Chemical: Initially, the following concentrations $\times 10^{-5}\%$ (by volume) are tested: 100, 30, 10, 3, 1, and 0.1. If these concentrations prove to be cytotoxic, or if additional information is available from other in vitro tests as to the proper dose levels, then a lower series of concentrations is used over a 3-log range of concentrations.

Treatment of Hepatocytes: After preincubation, 25 microCuries of tritiated thymidine (20 Curies/millimole) is added to each tube. The test chemical and positive controls are diluted in an appropriate solvent and they are then added to each labeled tube. Generally, at least six concentrations of the test chemical over a 3-log range of concentrations are tested and each concentration is run in duplicate. The tubes are returned to the rocker platform for a 2-hour exposure at 37°C.

Positive and Negative Controls: 4-nitroquinoline oxide (NQO), a direct-acting mutagen, which induces UV-type DNA repair, is used at dose levels of 3, 1, and 0.3 $\mu\text{g/ml}$, run in duplicate. Dimethylnitrosamine (DMN), which requires metabolic activation by microsomal enzymes for activity, is used at dose levels of 100, 30, and 10 $\mu\text{g/ml}$, run in duplicate. In recent experiments, the concentration of DMN may be tested at 1000, 300 and 100 $\mu\text{g/ml}$ to assure detection. The solvent control is run in quadruplicate and consists of 100 to 150 microliters (concentration specified in individual reports) of the solvent used to dilute the sample. Dimethylsulfoxide (DMSO) or water are the usual solvents and negative control agents.

Harvest: At the end of incubation with the test agent, the cells are centrifuged from the medium at 200 $\times g$ at 5°C. The cells are rinsed once in 5 ml of cold medium 199 and are resuspended in 0.25% Triton X-100, 5% citric acid and 3 mM MgCl_2 , a lysing solution which liberates the nuclei. The nuclei are rinsed once in this solution and resuspended in 0.25 M sucrose, 2.5% citric acid and 3 mM MgCl_2 . The nuclei are then centrifuged at 600 $\times g$ for 10 min at 5°C and resuspended in 2 ml of the lysing solution.

Determination of Nuclear-Bound Label: To measure the amount of radioactive thymidine incorporated into the nuclei, 0.25 ml of the nuclear suspension is mixed with 1.0 mg of NCS tissue solubilizer in a scintillation vial. Ten ml of Dimilume® scintillation cocktail is added and the radioactive disintegrations per minute (DPM) are determined by counting twice in a scintillation counter for ten minutes. The measured DPM are then used to calculate the DPM/ 10^6 viable hepatocytes presented on tables.

Determination of DNA-Bound Label: The amount of radioactive thymidine incorporated into DNA is quantitated in DNA isolated and precipitated from 1.00 to 1.25 $\times 10^5$ viable hepatocytes. To 1.25 ml of the nuclear suspension, 2.75 ml of 1% sodium dodecyl sulfate (SDS) and 5 mM Ethylenediaminetetraacetic Acid (EDTA) is added to lyse the nuclei. The DNA is precipitated from this solution with 4 ml of ice-cold 10% trichloroacetic acid (TCA) and the sample tubes are incubated at 0°C for at least 30 minutes. The solution is then poured onto Whatman glass fiber filters under vacuum and the tubes and filters are washed twice with cold 5% TCA. Finally, each filter is rinsed once with methanol, dried and placed in a scintillation vial. The filters are incubated at 50°C for 1 hour with 1 ml of a diluted solution of NCS tissue solubilizer; prepared by adding 1 part solubilizer to 2 parts of Dimilume® cocktail. Dimilume® is then added to each vial and the vials are counted twice in a scintillation counter for ten minutes.

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Statistical Analysis: The average DPM is calculated for each dose level and the controls and final results are expressed as DPM/10⁶ viable hepatocytes. Data are also expressed as a percent of the solvent control for purposes of comparison. The original data are statistically analyzed by the appropriate parametric test, following the BRRC standard procedures for statistical analyses and the test(s) employed is indicated on the respective tables. Comparison between the mean for each dose level with the 95% confidence limits of the historical solvent control may also be used in some cases to assess the potential biological significance of the data. Testing may be repeated to clarify unusual responses, if data with the concurrent controls suggest a defect in the original experiment.

Interpretation of Results: The classification of a chemical as a positive, active agent depends upon the production of a statistically significant, dose-related amount of UDS activity. If a clear dose-response relationship is not evident, or when a few increases with marginal statistical significance are obtained, comparison of the responses to historical control data provides a meaningful assessment of the possibility for random variations which may be statistically significant only in relation to the concurrent control. A key determinant of the reliability of the UDS data is the detection of a similar response with both DNA and isolated nuclei determined at two or three consecutive concentrations.

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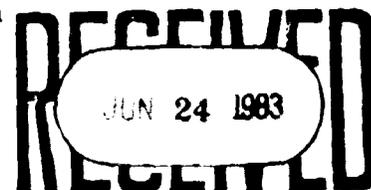
Project Report 43-113
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Diethylenetriamine - Commercial Grade (DETA-Comm.)

In Vitro Mutagenesis Studies: 3-Test Battery

Authors: R. S. Slesinski, M. W. Gaunt, P. J. Guzzie, W. C. Hengler

Sponsor: Union Carbide Corporation



SUMMARY

Diethylenetriamine - Commercial grade (DETA-Comm.) was evaluated for potential mutagenic activity with a battery of three in vitro tests, which were: the Chinese Hamster Ovary (CHO) Mutation test, the Sister Chromatid Exchange (SCE) test and an assay for induction of Unscheduled DNA Synthesis (UDS) in rat liver cells. The results indicated that DETA-Comm. did not produce a statistically significant mutagenic effect in any of the three in vitro tests performed.

RESULTS AND INTERPRETATION

Selection of Test Concentrations - Preliminary experiments were performed to select an appropriate range of test concentrations which at the maximum concentration would allow survival of approximately 10% of the treated cells. A maximum concentration of $40 \times 10^{-2}\%$ (by volume) was chosen for the highest dose-level and a total of six concentrations of DETA-Comm. were tested because the prescreening data suggested a steep dose-response effect.

CHO Mutation Test - DETA-Comm. did not produce a statistically significant increase in the frequency of mutations of CHO cells at any concentration between $40 \times 10^{-2}\%$ to $1.25 \times 10^{-2}\%$ (by volume) in tests with and without the incorporation of a liver S9 metabolic activation system. The lack of a dose-related effect on the mutation frequency indicated that DETA-Comm. was not active in producing gene mutations in CHO cells. A repeat test to assure the activity of the S9 metabolic activation system and the repeatability of the negative results of the first test was negative and these data verified the lack of mutagenic effect upon CHO cells in vitro.

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SCE Test - DETA-Comm. did not produce a dose-related increase in the frequency of SCE in CHO cells in tests both with and without the incorporation of an S9 metabolic activation system. An overall range of concentrations between $20 \times 10^{-2}\%$ to $0.625 \times 10^{-2}\%$ (by volume) was used. A single statistically significant increase in the frequency of SCE was obtained at the highest test concentration ($20 \times 10^{-2}\%$) in the test without metabolic activation. The possibility for an extremely narrow range of active concentrations cannot be excluded with the present data, but this possibility would be unlikely based on previous experience with known mutagenic agents. The highest concentration of DETA-Comm. ($20 \times 10^{-2}\%$) was cytotoxic to CHO cells in tests with S9 metabolic activation and one additional lower dose was employed for those studies.

UDS Test - DETA-Comm. did not produce statistically significant or dose-related increases in the amount of UDS activity in evaluations of concentrations between $10 \times 10^{-2}\%$ to $0.01 \times 10^{-2}\%$ (by volume). DETA-Comm. was considered to be inactive in the present test with the hepatocyte test system.

Comparative Mutagenicity - The pattern of negative responses produced by DETA-Comm. in the 3-test battery to determine potential mutagenicity indicated that DETA-Comm. was inactive in comparison to known mutagenic agents, such as the positive controls tested concurrently with the test chemical.

One literature reference reported that DETA was positive for inducing mutations in the Salmonella typhimurium (Ames) test (Hedenstedt, 1978). However, an alkylating contaminant was found in the DETA sample used in that test and no conclusion on the potential activity of pure DETA was possible.

SAMPLE

Quantity: 1 quart

GHF Sample No.: 42-103

Submitted by: D. C. Best

Date Received: February 23, 1979

Division: Specialty Chemicals and Plastics
Union Carbide Corporation
South Charleston, WV

Identification: 511-01-0875;
amber liquid

Ref.: 1PRU-50
(analytical data)

CAS#: 111-40-0

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Diethylenetriamine - Commercial Grade (DETA-Comm.)

In Vitro Mutagenesis Studies: 3-Test Battery

Sponsor: Union Carbide Corporation

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OBJECTIVE

The purpose of this study was to evaluate the potential of DETA-Comm. to induce genetic damage in mammalian cells at the gene, chromosome and/or DNA (deoxyribonucleic acid) level of molecular organization. A battery of three in vitro, short-term tests which detect each of these genetic end points was employed to evaluate DETA-Comm. for potential mutagenic activity. A general description of the theoretical basis of these three tests is presented in Appendices I, II and III attached to the complete report.

SAMPLE CHARACTERISTICS

A typical, commercial sample of DETA was received for testing on February, 23, 1979. The physical and chemical information available from the Toxicology Data Bank or from "Material Safety Data Sheets" for this product are attached to this report as Appendix IV.

Additional gas chromatographic analyses of the sample received for testing were performed by P. R. Umberger, UCC, South Charleston, WV. Copies of the pertinent sections of the analytical study are attached as Appendix V of this report. Briefly, gas chromatographic analyses on a 10% CARBOWAX 30M-750 Chromosorb 60/50 mesh column indicated a composition of 92.74% DETA and 7.26% N-(2-aminoethyl)piperazine (AEP). With a second derivative method, the composition was 90.75% DETA, 8.91% AEP and 0.34% ethylenediamine (EDA) (Data in Table 2, Appendix V).

METHODS

A description of the technical procedures used in the CHO test, the SCE test and the UDS assay are presented in greater detail in Appendices I, II and III, respectively (attached to the complete report). Testing was performed in compliance with Standard Operating Procedures used for these tests at the Bushy Run Research Center and deviations from procedures are noted in the individual test results. Diethylenetriamine is abbreviated as DETA-Comm. in this report.

1. CHO Test (Detailed procedures in Appendix I): SOP #7.2.4A; 7.2.5, 7.2.11.
- A. Dose Selection - Appropriate concentrations of DETA-Comm. for testing were determined by measurements of cytotoxicity to CHO cells of six concentrations tested both in the presence and absence of a liver S9 metabolic activation system. Selection of a maximum concentration for testing depended upon an estimate of a dose level which would permit survival of at least 10% of the treated cells. Sterile water (H₂O) was used as the solvent and solvent control.

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To simplify tables and to allow comparisons between different tests, concentrations of DETA-Comm. in the following sections of the report are given in terms of volume percentages $\times 10^{-2}$ to eliminate zeros in the lower concentration values (eg. 0.0001% = $0.01 \times 10^{-2}\%$).

B. Mutation - CHO cells were exposed for 5 hours to a minimum of five concentrations of DETA-Comm. both with and without the addition of an S9 metabolic activation system. A second repeat experiment of the treatments incorporating an S9 metabolic activation system was performed to assure the reproducibility of the data from the first test with S9. Dilutions of DETA-Comm. for testing were prepared by either direct addition of various aliquots of the test agent into the cell culture media or by making sequential one-half dilutions of the stock solution for the maximum concentrations using sterile H₂O. The surviving fraction was determined at 20 to 24 hours after treatment and the mutant fraction was determined after a 7-day period to allow "expression" of the mutant phenotype. Only the data from the top five concentrations which allowed sufficient cell survival for assessment of survival and induction of mutants are usually presented in the tables. The percentage of cells surviving the treatment, the frequencies of mutant colonies and the number of mutants/10⁶ viable cells are presented in tabular form.

2. SCE Test (Detailed procedures in Appendix II):

Production of SCE's following exposure to various concentrations of DETA-Comm. was studied in CHO cells both with and without the incorporation of an S9 metabolic activation system. Selection of a maximum dose level which would permit survival of at least 50% of the treated cells was based on the prescreening test for cytotoxicity performed as part of the CHO Mutation test. Dilutions of DETA-Comm. for testing, were prepared either by direct addition of various aliquots into the culture medium or by making sequential one-half dilutions of the stock solution for the maximum dose level using sterile H₂O. For determination of direct mutagenic action, CHO cells were exposed to DETA-Comm. and appropriate controls for 5 hours without S9 activation. Indirect mutagenic action, requiring metabolic activation by liver S9 homogenate, was studied with a 2-hour exposure period. Bromodeoxyuridine (BrdU) required to differentiate between the individual "sister" chromatids by SCE staining, was present at a concentration of 3 ug/ml in the growth medium during treatment and during the culture period following exposure. A total of 15 cells/dose level and 5 dose levels, tested either with or without metabolic activation, were examined. The number of SCE/cell, mean number of SCE/chromosome and the level of statistical significance of the increases above the concurrent solvent control values are presented in tabular form.

3. UDS Test (Detailed procedures in Appendix III):

Induction of primary DNA damage in rat liver cells (hepatocytes), was studied at a minimum of six dose levels which spanned a 1000-fold range of concentrations. Cells were treated with DETA-Comm. for 2 hours in

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culture medium containing ^3H -thymidine, hydroxyurea and appropriate dilutions of DETA-Comm. prepared in DMSO. Determination of UDS activity was performed by analyses of incorporation of ^3H -thymidine into isolated hepatocyte nuclei or in DNA (precipitated from aliquots of the isolated nuclei) using a Searle Analytic Model 81 or Packard Model 2650 scintillation spectrometer. Data are presented in tabular form with an indication of the level of statistical significance above the concurrent solvent control values.

4. Controls - Positive, negative and solvent controls were tested concurrently with the test sample to assure the sensitivity of the test system and the concurrence of the results to previous test performance. For the CHO and SCE assays, dimethylnitrosamine (DMN) and ethylmethanesulfonate (EMS) were used as positive control agents to assure the sensitivity of the test system for detecting indirect and direct-acting mutagens, respectively. Deionized water, sterilized by membrane filtration and glass-distilled dimethylsulfide (DMSO) were used as the solvent and negative controls, respectively.

In the UDS assay, DMN and 4-nitroquinoline oxide (4-NQO) were used as positive controls representing indirect- or direct-acting mutagens, respectively. DMSO was used as the solvent and the solvent control.

5. Metabolic Activation - S9 liver homogenate, prepared from Arochlor 1254-induced, Sprague-Dawley male rats, was purchased from Litton Bionetics. The S9 preparation used for the CHO test in experiment #1 contained 27 mg/ml protein and had a benzo(a)pyrene hydroxylase (BPH) activity of 9.97 nmol hydroxybenzpyrene/20 min/mg protein, (assayed by Litton). A concentration of 1200 ug of S9 protein was added to 5 ml of culture media. In experiment #2, the S9 preparation contained 40 mg/ml protein and a BPH activity of 13.93 nmol hydroxybenzpyrene/20 min/mg protein; 3600 ug of S9 protein was added to 5 ml of culture media.

For the SCE test, another lot of S9 homogenate, containing 40 mg/ml protein and a BPH of 15 nmol hydroxybenzpyrene/20 min/mg protein (assayed by Litton); a concentration of 600 ug of S9 protein was added per 5 ml culture media.

6. Statistical Analyses - Data from the SCE and UDS tests were analyzed by appropriate parametric tests following Standard Operating Procedures for statistical analyses at the Bushy Run Research Center. Data from the CHO test do not follow a normal distribution according to experience with historical controls. Thus, the Student's t-test was used after transformation of the mutation frequencies (MF) according to the method of Irr and Snee (MF + 1)^{0.15} (Irr, J. D. and R. Snee, Proceedings of the Cold Spring Harbor-Banbury Conference, II (1979), 263-274).

Rounding of data to either two decimal places or to the appropriate number of significant figures was performed for presentation on tables. Although statistically significant decreases in mutation indices can occur because of cytotoxic responses, only statistically significant increases in responses above control values are indicated on Tables for simplicity. The degree of statistical significance is denoted by: a: $0.05 > p > 0.01$, b: $0.01 > p > 0.001$, or c: $p < 0.001$. No superscript (or NS) indicates $p > 0.05$.

7. Raw Data Storage - Copies of the final report, statistical analyses, analytical data and data used to prepare the final report are stored in the ERRC Archives. Slides are stored in the Genetic Toxicology slide storage area.

RESULTS

SECTION I - CHO MUTATION TEST - Diethylenetriamine - Commercial Grade (DETA-COMM.)

- A. Test Dates - Initiated: January 8, 1980
Completed: August 8, 1980
- B. Selection of Test Concentration (Data not shown in tables)

CHO cells were exposed for five hours to concentrations of DETA-Comm. which spanned a concentration range from $10 \times 10^{-2}\%$ to $0.03 \times 10^{-2}\%$ by volume. The percentage of cells which survived the exposure, both in the presence and absence of an S9 metabolic activation system, was determined by counting the number of colonies produced by the survivors after a 5 to 7 day incubation period. A concentration of $40 \times 10^{-2}\%$ was selected as the maximum concentration for testing with and without S9 activation. A total of six concentrations were tested in the later mutation tests because the prescreening results suggested the existence of a probable steep dose-response effect.

- C. Determination of Mutation Induction

1. Survival (Cytotoxicity)

Table 1 presents the cytotoxicity data for CHO cells treated with DETA-Comm. in the presence and absence of a liver S9 metabolic activation system. A steep dose-response effect with the test agent was observed from the $> 99\%$ cell killing obtained with the top concentration ($40 \times 10^2\%$) of DETA-Comm., in tests with or without S9 activation, in comparison to the markedly lower cytotoxicity obtained at only one-half the top dose-level. Cytotoxicity with this test agent was inconsistent in the prescreening test and the survival test; a finding which is probably attributable to the inconsistent buffering of this alkaline chemical by the cell culture medium. We attempted to moderate this pH effect in these tests by equilibrating the cell culture medium with $10\% \text{ CO}_2$ in air.

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2. Mutation

Table 2 presents the data for induction of mutants by DETA-Comm. and control agents. Deta-Comm. did not produce a dose-related increase in the frequency of mutants/10⁶ viable cells over the 32-fold range of concentrations in tests either with or without the presence of an S9 metabolic activation system. Also, no concentration of DETA-Comm. produced an increase in the mutation frequency which was statistically significant from the concurrent solvent control. Small numerical increases in the mutant frequency obtained at some dose levels were not considered to be biologically significant because occasional increases of similar magnitude have been obtained in previous tests with solvent or negative controls.

To be certain that the negative responses observed with the test incorporating a metabolic activation system were reproducible, a second experiment presented on Tables 3 and 4 was performed. The data from the repeat test were consistent with the first experiment and no dose level of DETA-Comm. produced a statistically significant increase in the frequency of mutants. The numerical increase in mutants obtained at the 20 x 10⁻²% concentration of DETA is a finding which prompted some suspicion of weak activity but this finding was not statistically significant from the concurrent controls and it was not reproducible in experiments #1 and #2.

Mutation frequencies for the solvent controls for tests both with and without S9 activation in experiments #1 and #2 were in an acceptable and low range based upon experience with historical control values. Statistically significant increases in the mutation frequencies were obtained for the DMN and EMS positive controls for both experiments and these values were within the expected range of values observed in historical control data.

- D. Deviations from Standard Procedures - A 9-day expression period was used in experiment #2 because the cells appeared to be growing slowly and a longer expression period is believed to increase the sensitivity of the assay. A 7- to 10-day expression period is considered appropriate for a typical assay (see Ref. #3, Appendix I). Cell cultures were equilibrated with a mixture of 10% CO₂ in air to attempt to alleviate the alkaline effects of the test agent in the medium (buffered with a carbonate-bicarbonate buffer system). A 0.075% solution of trypsin was used to remove the cells from the culture flask rather than 0.05%, as stated in the concurrent SOP, since this results in better cell separation.

E. Conclusions

DETA-Comm. was consistently inactive as a mutagenic agent for CHO cells when tested with and without the incorporation of an S9 metabolic activation system over a 32-fold range of concentrations. No statistically significant increase above the concurrent solvent control was produced at any dose level tested and DETA-Comm. was considered to be non-mutagenic in the CHO test.

SECTION II - SCE TEST - Diethylenetriamine - Commercial Grade (DETA-COMM.)

- A. Test Dates - Initiated: March 5, 1980
Completed: June 16, 1980

B. Selection of Test Concentrations

A maximum concentration of $20 \times 10^{-2}\%$ DETA-Comm. was chosen as the top dose level for testing with and without S9 activation based on cytotoxicity data from the CHO mutation test. Higher concentrations were expected to produce delays in the mitotic cycle and to decrease the number of cells with SCE staining, based on experience in previous studies. A 16-fold range of DETA-Comm. concentrations from $20 \times 10^{-2}\%$ to $1.25 \times 10^{-2}\%$ was examined in SCE experiment #1 without S9 activation. A 32-fold range of concentrations of DETA-Comm. from $20 \times 10^{-2}\%$ to $0.625 \times 10^{-2}\%$ (by volume) was examined in SCE experiment #2 with S9 activation. A wider concentration range of DETA-Comm. was employed in tests without S9, in comparison to tests with S9, because a greater degree of cytotoxicity and delays in cell division were observed at the highest dose level in the tests without a metabolic activation system.

C. Determinations of SCE Induction

1. The data for SCE induction in CHO cells treated with various dose levels of DETA-Comm. or with positive, negative or solvent control agents in tests without S9 metabolic activation are summarized in Table 5. Only a single statistically significant increase in the SCE frequency was produced by the highest dose level of DETA-Comm. tested for direct action in the absence of a metabolic activation system. Because we did not observe any dose-related increase in the number of SCE, this single statistically positive result was not considered to be biologically significant. The test without S9 activation is considered a negative indication for potential direct mutagenic action of DETA-Comm.

The number of SCE produced by the concurrent EMS positive control was highly statistically significant from the concurrent solvent control and these data indicated an appropriate sensitivity of the test system comparable to our historical positive control data. The numbers of SCE obtained with the H₂O solvent and DMSO controls were also in an acceptable range of values included in the variability encountered in our historical control values for this test. However, the numbers of SCE in the H₂O control are higher than the values obtained with the DMSO negative control and over values desirable for maximum sensitivity. Medium purchased from the K. C. Biological Co. has tended to increase our spontaneous mutation values over values obtained in previous studies on other chemicals and using media from other sources. These differences are not considered to be detrimental to the present study.

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2. SCE values obtained following treatments of CHO cells with DETA-Comm. in the presence of an S9 metabolic activation system are presented in Table 6. No statistically significant increase in the SCE values was observed at any of the tested concentrations of DETA-Comm. Also, there was no evidence for dose-response effect of DETA-Comm. treatments. These negative results were consistent with the findings in the test without addition of S9 (Table 5) and DETA-Comm. was considered inactive as a mutagenic agent in the induction of SCE in vitro.

The SCE values for the negative (DMSO) and solvent controls in the test with S9 activation were in an acceptable range of variability as encountered in previous experiments with this test system. Highly statistically significant numbers of SCE were produced by the two concentrations of the DMN positive control tested indicating that the metabolic activation system was suitably active.

D. Deviations from Standard Procedures

Because of the usual cytotoxicity of the liver S9 homogenate, cells treated with the test agent and S9 were incubated for a total of 36 to 40 hours before harvesting cells and preparation of chromosomes for SCE slides. This extended growth period allowed for the cell division delay induced by the liver homogenate. Medium purchased from K. C. Biological was used in this study rather than from Grand Island Biological as stated in the concurrent SOP. Trypsin at 0.075% rather than 0.05% was used to remove cells from culture dishes since this produced better cell suspensions. Culture flasks were equilibrated with 10% CO₂ in air to attempt to counteract the alkalization of the medium by the test agent.

E. Conclusions

DETA-Comm. did not produce a significant increase in the frequency of SCE over the 16- to 32-fold range of concentrations tested with or without addition of an active S9 metabolic activation system. No dose-related effect of DETA-Comm. exposure on the SCE frequency was evident and the test agent was considered to be inactive in the present in vitro assay.

SECTION III - UDS TEST - Diethylenetriamine - Commercial Grade (DETA-COMM.)

- A. Test Dates - Initiated: March 18, 1980
Completed: May 12, 1980

B. Selection of Test Concentrations

Standard procedures were followed and DETA-Comm. was tested over a 3-log range of concentrations from $10 \times 10^{-2}\%$ to $0.01 \times 10^{-2}\%$ by volume. The maximum dose-level was selected with consideration of the cytotoxicity data obtained in the CHO Mutation test which indicated that higher values would result in excessive cell killing. These dose-levels were considered to be appropriate for testing because uptake and incorporation of ^3H -thymidine into hepatocytes are generally even more sensitive to chemical effects than survival measurements with CHO cells (in which cytotoxicity is measured after a 24 hr recovery period following treatment).

C. Determination of UDS Induction

1. Nuclear-Bound Radioactive Label (Data in Table 7)

Induction of "unscheduled" incorporation of radioactive thymidine into nuclei of hepatocytes exposed to DETA-Comm. or to appropriate positive and negative controls is presented in Table 7. In hepatocytes treated with DETA-Comm., no concentration tested for potential activity induced a statistically significant increase in the amounts of ^3H -thymidine incorporation. A gradual decrease in the amounts of radioactive incorporation, corresponding to increased dose levels, particularly at the $10 \times 10^{-2}\%$ dose level, was considered an indication of the cytotoxicity of the test agent a finding consistent with the usually observed higher sensitivity of this assay to cytotoxic effects in comparison to the CHO or SCE tests. The negative results in this test indicated that DETA-Comm. did not produce primary DNA damage in rat hepatocytes. Both of the positive control agents, NQO and DMN, induced numerically elevated and statistically significant increases in UDS over values obtained with the solvent control. With both positive controls the two lowest dose-levels did not induce a statistically significant level of UDS. This finding may suggest that the sensitivity of the test system with measurements using nuclei was lower than observed in our historical data.

2. DNA-Bound Radioactive Label (Data in Table 8)

Analyses of DNA, from aliquots of hepatocyte nuclei used for the UDS studies presented on Table 7, were performed as a second assessment of "unscheduled" incorporation of radioactive thymidine. Values for radioactivity incorporated into the DNA of these hepatocyte nuclei are presented in Table 8.

For hepatocytes treated with DETA-Comm. none of the test concentrations induced levels of UDS which were statistically significant from the solvent control. More critically, there was no dose-response effect upon ^3H -thymidine incorporation following treatments with DETA-Comm. Negative results on ^3H -thymidine incorporation into DNA were consistent with the data obtained in the assessment of nuclei from cells treated with the same range of concentrations. DETA-Comm. was considered to be inactive in the induction of DNA damage discernible by UDS activity in the present study.

The pattern of UDS activity produced by varying concentrations of the positive control agents NQO and DMN were similar and consistent with the data obtained in the assessment of radioactive thymidine incorporated into nuclei presented in Table 7. Again all doses of the positive control agents produced a numerical elevation in the amount of UDS but only the top dose level produced a response which was significantly above the solvent control level. With the DNA method, however, the response at the highest dose level was highly statistically significant for both DMN and NQO which suggests that this second method had greater degree of sensitivity than the method employing nuclei.

D. Deviations from Standard Procedures - None.

E. Conclusion

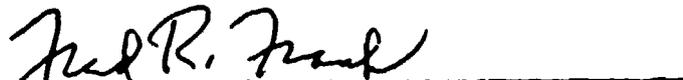
DETA-Comm. failed to stimulate significantly the incorporation of radioactive thymidine in cells treated over a 1000-fold range of test concentrations. DETA-Comm. was considered inactive in the tests with hepatocytes.

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Table 1
 Chinese Hamster Ovary (CHO) Mutation Assay:
 Determination of Toxic Effects of Chemical Treatment During 5 Hr Mutation Induction Period
 Experiment #1

Test Chemicals	Total # Colonies	Total # Cells Plated	% Survival	% of Solvent Control
Without S9 Activation				
[Diethylenetriamine - Commercial] (% v/v)				
40.0 x 10 ⁻²	-	TOXIC -	-	-
20.0 x 10 ⁻²	474	800	59.2	144.5
10.0 x 10 ⁻²	93	400	23.2	56.7
5.0 x 10 ⁻²	173	400	44.5	108.5
2.5 x 10 ⁻²	130	400	32.5	79.3
1.25 x 10 ⁻²	150	400	37.5	91.5
Controls				
H ₂ O - Solvent (20 ul/ml)	164	400	41.0	-
DMSO - (20 ul/ml)	166	400	41.5	101.2
EMS - (200 ug/ml)	134	400	33.5	81.7
With S9 Activation				
[Diethylenetriamine - Commercial] (% v/v)				
40.0 x 10 ⁻²	-	TOXIC -	-	-
20.0 x 10 ⁻²	403	800	50.4	97.3
10.0 x 10 ⁻²	250	400	62.5	120.8
5.0 x 10 ⁻²	162	400	40.5	78.3
2.5 x 10 ⁻²	168	400	42.0	81.2
1.25 x 10 ⁻²	167	400	41.8	80.7
Controls				
H ₂ O - Solvent (20 ul/ml)	207	400	51.8	-
DMSO - (20 ul/ml)	145	400	36.2	70.0
DMN - (3700 ug/ml)	135	400	33.8	65.2

Abbreviations: H₂O - water; S9 - liver homogenate; DMSO - dimethylsulfoxide
 EMS - ethylmethanesulfonate; DMN - dimethylnitrosamine

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Table 2
 Chinese Hamster Ovary (CHO) Mutation Assay:
 Results on Evaluation of Mutant Induction by Diethylenetriamine, Commercial Grade
 Experiment # 1

Test Chemicals	Plating Efficiency			Mutation Induction		
	Total # Colonies	Total # Cells Plated	Viable Fraction	Total # Mutant Colonies	Total # Cells Plated	Mutants ¹ 10 ⁶ Viable Cells
Without S9 Activation						
[Diethylenetriamine - Commercial] (% v/v)						
40.0 x 10 ⁻²	-	-	TOXIC	-	-	-
20.0 x 10 ⁻²	394	400	0.985	8	1 x 10 ⁶	8.1
10.0 x 10 ⁻²	407	400	1.018	0	1 x 10 ⁶	0
5.0 x 10 ⁻²	316	400	0.790	3	1 x 10 ⁶	3.8
2.5 x 10 ⁻²	478	400	1.195	0	1 x 10 ⁶	0
1.25 x 10 ⁻²	576	400	1.440	6	1 x 10 ⁶	4.2
Controls:						
H ₂ O - Solvent (20 ul/ml)	685	400	1.712	0	1 x 10 ⁶	0
DMSO - (20 ul/ml)	435	400	1.088	0	1 x 10 ⁶	0
EMS - (200 ug/ml)	432	400	1.080	119	1 x 10 ⁶	110.2 ^c
With S9 Activation						
[Diethylenetriamine - Commercial] (% v/v)						
40.0 x 10 ⁻²	-	-	TOXIC	-	-	-
20.0 x 10 ⁻²	330	400	0.825	3	1 x 10 ⁶	3.6
10.0 x 10 ⁻²	220	400	0.550	0	1 x 10 ⁶	0
5.0 x 10 ⁻²	391	400	0.978	0	1 x 10 ⁶	0
2.5 x 10 ⁻²	380	400	0.950	0	1 x 10 ⁶	0
1.25 x 10 ⁻²	275	300	0.917	8	1 x 10 ⁶	8.7
Controls:						
H ₂ O - Solvent (20 ul/ml)	393	400	0.982	0	1 x 10 ⁶	0
DMSO - (20 ul/ml)	392	400	0.980	0	1 x 10 ⁶	0
DMN - (3700 ug/ml)	229	400	0.572	31	1 x 10 ⁶	54.1 ^a

¹Total # mutant colonies per 10⁶ cells plated divided by viable fraction.

Statistical significance above solvent control: a: 0.05 > p > 0.01; c: p < 0.001.

No superscript indicates p > 0.05. Data analyzed by Student's t-test.

Abbreviations: H₂O - water; S-9 - liver homogenate; DMSO - dimethylsulfoxide; EMS - ethylmethanesulfonate;
 DMN - dimethylnitrosamine.

Table 3
 Chinese Hamster Ovary (CHO) Mutation Assay:
 Determination of Toxic Effects of Chemical Treatment During 5 Hr Mutation Induction Period
 Experiment #2

Test Chemicals	Total # Colonies	Total # Cells Plated	% Survival	% of Solvent Control
With S9 Activation				
[Diethylenetriamine - Commercial] (% v/v)				
20.0 x 10 ⁻²	177	400	44.2	79.0
10.0 x 10 ⁻²	204	400	51.0	91.1
5.0 x 10 ⁻²	303	400	75.8	135.3
2.5 x 10 ⁻²	243	400	60.8	108.5
1.25 x 10 ⁻²	258	400	64.5	115.2
Controls				
H ₂ O - Solvent (20 ul/ml)	224	400	56.0	-
DMSO - (20 ul/ml)	172	400	43.0	76.8
DMN - (3700 ug/ml)	105	400	26.2	46.9

Abbreviations: H₂O - water; S9 - liver homogenate; DMSO - dimethylsulfoxide
 EMS - ethylmethanesulfonate; DMN - dimethylnitrosamine

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Table 4
 Chinese Hamster Ovary (CHO) Mutation Assay:
 Results on Evaluation of Mutant Induction by Diethylenetriamine, Commercial Grade
 Experiment #2

Test Chemicals	Plating Efficiency			Mutation Induction		
	Total # Colonies	Total # Cells Plated	Viable Fraction	Total # Mutant Colonies	Total # Cells Plated	Mutants ¹ 10 ⁶ Viable Cells
With S9 Activation						
[Diethylenetriamine - Commercial] (% v/v)						
20.0 x 10 ⁻²	307	400	0.768	16	1 x 10 ⁶	20.8
10.0 x 10 ⁻²	368	400	0.920	4	1 x 10 ⁶	4.3
5.0 x 10 ⁻²	348	400	0.870	3	1 x 10 ⁶	3.4
2.5 x 10 ⁻²	251	400	0.628	2	1 x 10 ⁶	3.2
1.25 x 10 ⁻²	382	400	0.955	7	1 x 10 ⁶	7.3
Controls:						
H ₂ O - Solvent (20 ul/ml)	338	400	0.845	2	1 x 10 ⁶	2.4
DMSO - (20 ul/ml)	461	400	1.152	2	1 x 10 ⁶	1.7
DMN - (3700 ug/ml)	279	400	0.698	31	1 x 10 ⁶	44.4 ^b

¹Total # mutant colonies per 10⁶ cells plated divided by viable fraction.
 Statistical significance above solvent control: b: 0.01 > p > 0.001.
 No superscript indicates p > 0.05. Data analyzed by Student's t-test.

Abbreviations: H₂O - water; S-9 - liver homogenate; DMSO - dimethylsulfoxide; EMS - ethylmethanesulfonate;
 DMN - dimethylnitrosamine.

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Table 5
 Sister Chromatid Exchange (SCE) Assay:
 Induction of SCE's by Diethylenetriamine, Commercial Without S9 Metabolic Activation
 5 Hour Treatment

Test Chemicals	Total # of Chromosomes	Total # of SCE	SCE/Cell ¹	Mean Number SCE/Chromo- some ² + S.D.	Significance Above Solvent Control ³
[Diethylenetriamine - Commercial] (% v/v)					
20.0 x 10 ⁻²	297	325	21.67	1.094 + 0.259	b
10.0 x 10 ⁻²	301	254	16.93	0.842 + 0.192	NS
5.0 x 10 ⁻²	297	251	16.73	0.843 + 0.203	NS
2.5 x 10 ⁻²	296	189	12.60	0.642 + 0.298	NS
1.25 x 10 ⁻²	297	231	15.40	0.776 + 0.237	NS
Controls					
H ₂ O (5 ul/ml) - Solvent	294	234	15.60	0.795 + 0.210	-
DMSO (5 ul/ml) -	299	177	11.80	0.590 + 0.225	NS
EMS (100 ug/ml) -	301	375	25.00	1.245 + 0.336	c

¹Fifteen cells examined per dose level.

²Mean value of SCE/chromosome determined from the values of the individual cells examined.

³Statistical significance above solvent control: b: 0.01 > p > 0.001; c: p < 0.001
 NS: p > 0.05. Data analyzed by Duncan's Multiple Range Analysis.

Abbreviations: H₂O - water; S9 - liver homogenate; DMSO - dimethylsulfoxide;
 EMS - ethylmethanesulfonate; S.D. - standard deviation

Table 6
Sister Chromatid Exchange (SCE) Assay:
Induction of SCE's by Diethylenetriamine, Commercial With S9 Metabolic Activation
2 Hour Treatment

Test Chemicals	Total # of Chromosomes	Total # of SCE	SCE/Cell ¹	Mean Number SCE/Chromo- some ² + S.D.	Significance Above Solvent Control ³
[Diethylenetriamine - Commercial] (% v/v)					
20.0 x 10 ⁻²	-	-	TOXIC	-	-
10.0 x 10 ⁻²	298	170	11.33	0.571 + 0.169	NS
5.0 x 10 ⁻²	281	152	10.13	0.549 + 0.207	NS
2.5 x 10 ⁻²	299	149	9.93	0.501 + 0.210	NS
1.25 x 10 ⁻²	295	162	10.80	0.551 + 0.197	NS
0.625 x 10 ⁻²	293	176	11.73	0.600 + 0.156	NS
Controls					
H ₂ O (5 ul/ml) - Solvent	292	178	11.87	0.614 + 0.212	-
DMSO (5 ul/ml) -	293	188	12.53	0.644 + 0.168	NS
DMN (1000 ug/ml) -	296	954	63.60	3.201 + 0.716	c
DMN (800 ug/ml) -	304	883	58.87	2.914 + 0.798	c

¹Fifteen cells examined per dose level.

²Mean value of SCE/chromosome determined from the values of the individual cells examined.

³Statistical significance above solvent control: c: p < 0.001;
NS: p > 0.05. Data analyzed by Student's t-test.

Abbreviations: H₂O - water; S9 - liver homogenate; DMSO - dimethylsulfoxide;
DMN - Dimethylnitrosamine; S.D. - standard deviation

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Table 7
Unscheduled DNA Synthesis in Hepatocytes from Rat Liver

Nuclear-bound label: all DPM values are calculated from nuclei per 10^6 viable hepatocytes. Each average is calculated from duplicate samples, except for DMSO which was done in quadruplicate.

Test Chemical	Concentration	Radioactivity in Nuclei Avg. DPM \pm S.D.	% of Solvent Control \pm S.D.	Significance Above Solvent Control ¹
Solvent - DMSO	2.0%	7808 \pm 198	100.0% \pm 2.5%	-
Positive Controls:				
4 - NQO	3.0 ug/ml	16184 \pm 232	207.3% \pm 3.0%	c
	1.0 ug/ml	8836 \pm 1592	113.2% \pm 20.4%	NS
	0.3 ug/ml	8859 \pm 1111	113.5% \pm 14.2%	NS
DMN	1000 ug/ml	9408 \pm 852	120.5% \pm 10.9%	a
	300 ug/ml	8459 \pm 893	108.3% \pm 11.4%	NS
	100 ug/ml	8380 \pm 804	107.3% \pm 10.3%	NS
<u>Test Chemical:</u> [Diethylenetriamine, Commercial] (X, v,v)	10 x 10 ⁻² %	2780 \pm 311	35.6% \pm 4.0%	NS
	3 x 10 ⁻² %	6840 \pm 1075	87.6% \pm 13.8%	NS
	1 x 10 ⁻² %	7609 \pm 74	97.5% \pm 0.9%	NS
	0.3 x 10 ⁻² %	7819 \pm 65	100.1% \pm 0.8%	NS
	0.1 x 10 ⁻² %	7166 \pm 85	91.8% \pm 1.1%	NS
	0.01 x 10 ⁻² %	6878 \pm 115	88.1% \pm 1.5%	NS

¹Statistical significance above solvent control: a: 0.05 > p > 0.01; c: p < 0.001;
 NS: p > 0.05. Data analyzed by Duncan's Multiple Range Analysis.

Abbreviations: DMSO - dimethylsulfoxide; 4-NQO - 4-nitroquinoline oxide; DMN - dimethylnitrosamine;
 DPM - disintegrations per minute; S.D. - standard deviation

Table 8
Unscheduled DNA Synthesis in Hepatocytes from Rat Liver

DNA-bound label: all DPM values are calculated from DNA precipitated per 10^6 viable hepatocytes. Each average is calculated from duplicate samples, except for DMSO which was done in quadruplicate.

Test Chemical	Concentration	Radioactivity	% of Solvent	Significance Above Solvent Control ¹
		in DNA	Control	
		Avg. DPM \pm S.D.	\pm S.D.	
Solvent - DMSO	2.0%	6345 \pm 442	100.0% \pm 7.0%	-
Positive Controls:				
4 - NQO	3.0 ug/ml	14302 \pm 1260	225.4% \pm 19.9%	c
	1.0 ug/ml	7004 \pm 534	110.4% \pm 8.4%	NS
	0.3 ug/ml	7062 \pm 545	111.3% \pm 8.6%	NS
DMN	1000 ug/ml	8934 \pm 576	140.8% \pm 9.1%	c
	300 ug/ml	7250 \pm 865	114.3% \pm 13.6%	NS
	100 ug/ml	7176 \pm 145	113.1% \pm 2.3%	NS
<u>Test Chemical:</u>				
[Diethylenetriamine, Commercial]	10 x 10 ⁻² %	2373 \pm 381	37.4% \pm 6.0%	NS
(%, v/v)	3 x 10 ⁻² %	5583 \pm 1117	88.0% \pm 17.6%	NS
	1 x 10 ⁻² %	6687 \pm 271	105.4% \pm 4.3%	NS
	0.3 x 10 ⁻² %	6878 \pm 652	108.4% \pm 10.3%	NS
	0.1 x 10 ⁻² %	6261 \pm 343	98.7% \pm 5.4%	NS
	0.01 x 10 ⁻² %	5750 \pm 138	90.6% \pm 2.2%	NS

¹Statistical significance above solvent control: c: p < 0.001;
 NS: p > 0.05. Data analyzed by Duncan's Multiple Range Analysis.

Abbreviations: DMSO - dimethylsulfoxide; 4-NQO - 4-nitroquinoline oxide; DMN - dimethylnitrosamine;
 DPM - disintegrations per minute; S.D. - standard deviation

APPENDIX I

Chinese Hamster Ovary (CHO) Mutation AssayTheoretical Basis

Mutation is a heritable alteration in a cell in which a gene specifying the genetic code for a specific protein is modified in structure and/or function. Mutations, induced by chemical or physical agents, of the HGPRT (hypoxanthine-guanine phosphoribosyltransferase) gene may be detected by the growth of colonies of "autant" cells which are resistant to the purine analogs 6-thioguanine (TG) or 8-azaguanine. Normal cells contain a functional HGPRT enzyme which phosphorylates TG and allows its incorporation into DNA causing the cells to die. Mutant cells with a non-functional HGPRT enzyme are unable to phosphorylate or incorporate TG, thus survive and grow in its presence.

The CHO mutation test is an assay which detects "forward mutations" from TG-sensitivity to TG-resistance caused by a direct loss of the activity of the HGPRT enzyme ($HGPRT^+ \rightarrow HGPRT^-$). An assessment of the ability of several hundred agents to cause gene mutations in vitro indicates that the CHO mutation assay provides a reasonable estimate of the potential genetic activity of the test chemical.

Methods

Cell Culture Procedures: CHO cells used in these studies were obtained from Abraham Hsieh at Oak Ridge National Laboratory with the designation CHO-K1-BH4-D1 (or simply CHO for report purposes). Cells are maintained in active growth by subculturing 2 to 3 times/week in antibiotic-free, Ham's Modified F12 Medium supplemented with 10% (v/v) heat-inactivated, fetal bovine sera (F12-10), and lacking in hypoxanthine. For treatment of cells without metabolic activation, F12 medium with 50 units/ml of penicillin, 50 ug/ml streptomycin and 5% (v/v) of dialyzed bovine serum (F12-D5) is used. For treatments incorporating an S9 metabolic activation system, identical medium, but without serum, is employed. For determination of mutant frequencies, F12-D5 medium containing 2.0 ug/ml TG (6-thioguanine) is used as a "selective medium." Cell numbers are determined routinely with a Coulter Model F electronic cell counter which is standardized periodically with a pre-counted suspension of latex beads. Presence of Mycoplasma cell contaminants is determined by a microscopic fluorescence assay employing Hoechst 33258 dye. All culture procedures and treatments with test chemicals are performed under aseptic conditions in a laminar-flow, biohazard hood.

Positive and Negative Controls: Sterile water or glass-distilled dimethylsulfoxide (DMSO) are the usual solvents for test chemicals and the respective solvent is tested as a control at the maximum concentration used to add the test agent. Dimethylnitrosamine (DMN) or ethylmethanesulfonate (EMS) are used as positive control mutagens for tests with or without an S9 metabolic activation system, respectively. Mutation frequencies obtained with concurrent positive and negative controls are used as the basis for monitoring the sensitivity and stability of the CHO mutation test system. Comparison of concurrent control values with historical controls is used to delineate the range of acceptable variations in the test system.

Metabolic Activation: Rat liver, S9 homogenate prepared from Arochlor-1254 induced, Sprague-Dawley, male rats is purchased from Litton Bionetics, Kensington, MD. Each lot of liver homogenate is prescreened for metabolic capability to activate DMN in our laboratory before use in the testing program. The complete S9 metabolic activation system contains the following: 8 umoles/ml $MgCl_2$, 33 umoles/ml KCl, 5 umoles/ml glucose-6-phosphate, 4 umoles/ml NADP-oxidized (nicotinamide adenine dinucleotide phosphate), 100 umoles/ml Na_2HPO_4 , and between 500 to 4000 ug/ml of S9 protein (depending on metabolic activity); a volume of 1.0 ml of the complete mixture of the above reagents is added to each 4.0 ml of culture medium.

Dose Selection: Toxicity of the test chemical is determined prior to assessment of mutagenic potential to select doses which produce a maximum of 80 to 90% cell killing. Cytotoxicity is determined by either of the following two methods:

- (1) **Clonal assay** - 200 to 400 CHO cells are exposed to a minimum of five dose levels of the test agent at concentrations from 0.1% to $3 \times 10^{-6}\%$ (by weight or volume, as appropriate) with and without the presence of a metabolic activation system. The number of cells which survive the treatment is determined by counting the number of colonies produced after a 7- to 8-day incubation period ($37^\circ C$) in comparison with the colonies formed by cells treated only with appropriate concentrations of solvent (generally 20 ul/ml).
- (2) **Growth Inhibition** - 5×10^5 cells in 25 cm^2 culture flasks are treated for 5 hours with a minimum of five test concentrations both with and without S9 metabolic activation. Following treatment the cells are rinsed, fresh F12-D5 medium is added and the flasks are incubated for an additional 18 to 24 hours. Cytotoxicity is determined by comparing the relative number of cells in control (untreated cells) and in cells treated with various concentrations of the test agent.

If no cytotoxicity is evident at the highest concentrations in the cytotoxicity tests, the test is either repeated at higher concentrations, or mutation testing is performed with a greater number of treatment flasks starting at higher dose levels. If marked toxicity is evident even at the lowest dose, the cytotoxicity test is repeated at a concentration range of 3×10^{-4} to 3×10^{-6} percent by weight or volume, as appropriate.

Dose levels which are moderately toxic but permit survival of at least 10 to 20% of the cells, in comparison to the solvent control, are selected as the maximum dose, and at least four additional one-half dilutions are tested for induction of mutations. If cytotoxicity data are equivocal, a total of 5 to 8 one-half dilutions of the selected, maximum concentration are used to treat cells; but only the highest five concentrations which permit survival of a sufficient number of cells are assessed for mutation induction.

Chemical samples are sterilized by membrane filtration when microbiological tests indicate this is required to assure sterility. Liquid test agents are tested on a percentage by volume basis. Solid chemicals are dissolved in an appropriate solvent by making a 10 to 20% stock solution (by weight) and subsequent dilutions are made from this stock on a volume/volume basis.

Treatment with Test Chemicals: For tests of chemicals which may act directly without incorporation of an S9 metabolic activation system, 5×10^5 cells are inoculated 20 to 24 hours prior to treatment into 25 cm² culture flasks containing F12-D5 medium and incubated at 37°C in a 5 to 6% CO₂ atmosphere. Appropriate concentrations of the test agent or control chemicals are added to the cells and cultures are treated for 5 hr at 37°C. The medium and test agents are removed by suction, cells are rinsed once or twice and fresh F12-D5 medium is added. The cells are allowed a period of 20 to 24 hours of recovery from treatment before survival is determined. Treatment of cells for testing of chemicals which require metabolic activation for mutagenic capacity is performed identically with the procedure above, with the exception that F12 medium without serum and containing 1.0 ml of S9 activation mixture per 4.0 ml of medium is employed.

Determination of Cytotoxicity: The relative survival of treated cells, in comparison to solvent controls, is determined one day after the exposure to the test agents. The level of cytotoxicity is often correlated with the mutation frequencies induced by known chemical mutagens. Thus, excessive cytotoxicity may kill both normal cells and mutants and may depress the actual mutation frequencies; insufficient cytotoxicity may indicate an insufficient concentration of the test agent was employed. The colony-forming potential of 100 to 200 treated cells is used as the measure of treatment-induced cytotoxicity.

Survival values which indicate the cytotoxic effects of the test agents are included in reports in tabular form. Statistical analyses are not performed on these data, since they are only useful to assess whether appropriate doses were employed and are not used to calculate mutation frequencies.

Determination of Mutant Induction: On days 1, 3 and 6 (or alternatively 1, 4 and 6) after treatment with the various test agents, approximately 5×10^5 cells are subcultured in 100 mm tissue culture dishes in F12-D5 medium and incubated at 37°C in a 5 to 6% CO₂ atmosphere. After a total of 7 days to allow "expression" of the mutant phenotype, cells are dissociated with 0.05 to 0.075% trypsin, counted and plated at a concentration of 2.5×10^5 /dish in four culture dishes (1×10^6 total cells) which each contain 5 ml of F12-D5 (TG) selective medium. At this time, cells are diluted and 100 cells/dish are added to four culture plates containing F12-D5 medium (without TG) to assess viability (plating efficiency) of the treated cell population and to determine the surviving fraction. All cultures are then incubated for an additional 6 to 8 days to allow growth of cells; medium is then discarded and colonies are fixed and stained for counting. The number of colonies in selection plates and in the viability test are counted by electronic methods, checked by manual counts and data are recorded both as total mutants, mutants/ 10^6 total cells and mutants/ 10^6 viable cells.

Statistical Analyses: Uniform statistical procedures to evaluate in vitro mutation data have not been developed. The distribution of mutation frequencies from historical controls in at least two laboratories indicates that the frequency distribution and variances encountered do not justify the use of parametric analyses unless data is transformed before application of standard parametric tests. Analysis of mutation frequencies in the CHO test follow the procedure of Irr and Snee (Reference 4) which employs the Box-Cox Transformation (Reference 5) to transform data before parametric analyses. The mutation frequency for each plate is increased by 1.0 (to eliminate zeros) and raised to the 0.15 power. Experience with historical negative control data in our laboratory indicates that a normal probability distribution of the data suitable for parametric analyses is achieved by this transformation. Parametric analysis of mutation data by the Student's t-test is performed with the transformed data. The degree of statistical significance for the mutation values are indicative of a difference from the concurrent solvent control, but these statistical indicators must be viewed conservatively until additional historical control data are available.

Interpretation of Data: The criteria for interpretation of the test results as a positive or negative response depend upon both the level of statistical significance from the concurrent control and the evidence of a dose-response following treatment. When a definite dose-response relationship is not evident but one or more marginally significant values are obtained, a careful examination of the data from the concurrent positive and negative controls and comparisons to historical control data are used to evaluate the possible significance of the responses. Historical control data indicate that a spontaneous mutation frequency in CHO cells of approximately 4 to 5 mutants/ 10^6 viable cells, with a range of 0 to 25 mutants/ 10^6 viable cells, can be obtained in the absence of mutagenic treatment. Statistical comparisons against unusually high or low spontaneous controls are subjectively scrutinized in respect to the above variability.

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APPENDIX II

Determination of Sister Chromatid Exchange (SCE) Frequencies
in Chinese Hamster Ovary (CHO) Cells In VitroTheoretical Basis

Exchanges of genetic material between the individual arms of a chromosome (i.e. sister chromatids) are thought to arise from breakage and physical interchanges in the DNA of a cell during cell division. An increase in the frequency of such interchanges between sister chromatids can be observed in cells treated with physical or chemical mutagenic agents, or in cells exposed to many suspect or proven human carcinogens. Thus, analysis of SCE frequencies in cells treated with a test agent has been suggested as a sensitive screening test for potential mutagenic/carcinogenic chemicals.

The method used in our study to visualize SCE's in CHO cells grown in culture is based on the procedure described by Perry and Wolff (1974). A standard concentration of 3.0 ug/ml of bromodeoxyuridine (BrdU) was used in the growth medium to allow a visualization of SCE's after two cell divisions in the presence of BrdU. Staining of chromosomes with 5.0 ug/ml of 33258-Hoechst fluorescent dye, exposure to light and Giemsa staining was used to differentiate chromatids for SCE analysis.

Methods

Cell Culture Procedures: Chinese hamster ovary (CHO) cells were obtained from Abraham Hsie at Oak Ridge National Laboratory with the designation CHO-K1-BH4-D1 (referred to simply as CHO for report purposes). CHO cells are maintained in active growth by 2 to 3 weekly subcultures into fresh antibiotic-free, Ham's F12 (modified) medium fortified with 10% (v/v) of heat-inactivated fetal bovine serum and lacking hypoxanthine and thymidine. Cell concentrations are determined routinely with a Coulter® Model-F electronic cell counter calibrated with a precounted suspension of latex beads. All cell culture procedures prior to final harvesting of cells for chromosome preparations are performed under aseptic conditions in a laminar flow, biohazard hood. Presence of Mycoplasma cell contaminants is determined using a fluorescent microscopic assay employing Hoechst 33258 dye.

For treatments with test chemicals without S9 metabolic activation, modified F12 medium is used with 50 units/ml of penicillin, 50 ug/ml streptomycin and 5% (v/v) of heat-inactivated, dialyzed fetal bovine serum (F12-D5). Identical medium but without serum is used for treatments incorporating an S9 metabolic activation system.

Positive and Negative Controls: Sterile water or glass-distilled dimethyl sulfoxide (DMSO) are the usual solvents used for test chemicals and the respective solvent is tested as a control at the maximum concentration used to add the test agent. Dimethylnitrosamine (DMN) and ethylmethanesulfonate (EMS) are used as positive control mutagens for tests with or without the addition of an S9 metabolic activation system, respectively. Results from treatments with concurrent control agents are used as a basis of comparison and for demonstrating the sensitivity and stability of the SCE test system. Comparison of concurrent control values with historical controls is used to delineate the range of acceptable variations in the test system. 000028

Metabolic Activation: Rat liver S9 homogenate (prepared from Arochlor 1254 induced, Sprague-Dawley, male rats) is purchased from Litton Bionetics, Kensington, MD. Each lot of liver homogenate is prescreened for activity in our laboratory before use in the testing program. The complete S9 metabolic activation system contains the following: 8 uoles/ml $MgCl_2$, 33 uoles/ml KCl , 5 uoles/ml KCl , 5 uoles/ml glucose-6-phosphate, 4 uoles/ml NADP-oxidized form (nicotinamide adenine dinucleotide phosphate), 100 uoles/ml Na_2HPO_4 and between 500 to 4000 ug/ml of S9 protein (depending on metabolic activity). A volume of 1.0 ml of the complete mixture of the above reagents is added to each 4.0 ml of culture medium.

Dose Selection: Toxicity of the test chemical is determined prior to assessment of mutagenic potential to select doses which produce a maximum of 80 to 90% cell killing. Cytotoxicity is determined by either of the following two methods as part of the CHO mutation testing procedure:

- (1) **Clonal assay** - 200 to 400 CHO cells are exposed to a minimum of five dose levels of the test agent at concentrations from 0.1% to $5 \times 10^{-4}\%$ (by weight or volume, as appropriate) with and without the presence of a metabolic activation system. The number of cells which survive the treatment is determined by counting the number of colonies produced after a 7- to 8-day incubation period ($37^\circ C$) in comparison with the colonies formed by cells treated only with appropriate concentrations of solvent (generally 20 ul/ml).
- (2) **Growth Inhibition** - 5×10^5 cells in 25 cm^2 culture flasks are treated for 5 hours with a minimum of five test concentrations both with and without S9 metabolic activation. Following treatment the cells are rinsed, fresh F12-D5 medium is added and the flasks are incubated for an additional 18 to 24 hours. Cytotoxicity is determined by comparing the relative number of cells in control (untreated cells) and in cells treated with various concentrations of the test agent.

If no cytotoxicity is evident at the highest concentrations in the cytotoxicity tests, the test is either repeated at higher concentrations, or mutation testing is performed with a greater number of treatment flasks starting at higher dose levels. If marked toxicity is evident even at the lowest dose, the cytotoxicity test is repeated at a concentration range of 3×10^{-4} to 3×10^{-6} percent by volume.

Dose levels which are moderately toxic but permit survival of at least 40 to 50% of the cells, in comparison to the solvent control, are selected as the maximum dose, and at least four additional one-half dilutions are tested for induction of mutations. If cytotoxicity data are equivocal, a total of 5 to 8 one-half dilutions of the selected, maximum concentration are used to treat cells; but only the highest five concentrations which permit survival of a sufficient number of mitotic cells with SCE staining, are evaluated for SCE induction.

Chemical samples are sterilized by membrane filtration when microbiological tests indicate this is required to assure sterility. Liquid test agents are tested on a percentage by volume basis. Solid chemicals are dissolved in an appropriate solvent by making a 10 to 20% stock solution (by weight) and subsequent dilutions are made from this stock on a volume/volume basis.

Treatment With Test Chemicals: Testing of chemicals for direct autagenic action (without S9 metabolic activation) is performed first. For chemicals with clearly positive autagenic capabilities by direct action, testing with metabolic activation is generally not performed.

For testing direct acting chemicals for SCE induction, between 1 to 2 x 10⁶ cells are plated into 75 cm² culture flasks in F12-D5 medium at least 20 hrs prior to treatment and incubated at 37°C in a 5 to 6% CO₂ atmosphere. Appropriate concentrations of the test agent or control chemicals are added to the cells and 3 ug/ml BrdU is added to all flasks. Cells are treated with test agents for 5 hrs, media is then removed by suction, cells are rinsed with buffered, physiological salt solution and fresh medium containing 3 ug/ml BrdU is added for at least 24 hrs of additional incubation at 37°C to allow two rounds of cell division. Cells are harvested and chromosomes are prepared for SCE staining.

Treatment of cells for testing of chemicals which require metabolic activation for autagenic effectiveness is performed similarly as for treatments without activation, except for three modifications:

1. Before treatment with the test agents, F12-D5 medium is removed and F12 medium without serum is added.
2. S9 metabolic activation mixture is added to each flask (including solvent and positive controls) before addition of test agents.
3. Cells are treated for a total of 2 hrs (rather than 5 hrs) and then incubated for 38 to 42 additional hours before harvest for chromosome preparation.

Preparation of Chromosomes: Colcemid® (0.1 ug/ml) or Colchicine (0.2 ug/ml) is added to culture flasks 1 to 2 hrs prior to harvesting to arrest cells in mitosis. Cells are then removed from flasks, after a brief incubation with 0.01X DIFCO trypsin, suspended in 0.075M KCl (hypotonic) solution and incubated for 15 to 20 min at 37°C. Cells are centrifuged, fixed with 3 or 4 changes of Carnoy's fixative (3:1 methanol acetic acid) and chromosome spreads are prepared from cells suspended in a small volume of fixative. One slide/dose level is prepared, but fixed cells are saved if needed for preparation of additional slides.

Chromosomes are stained for SCE's by treatment with 5.0 ug/ml of Hoechst 33258 dye for 20 min, rinsed in distilled water, immersed in Sorenson's buffer and exposed to a high intensity sunlamp for 15 to 30 min., as required. Irradiated chromosomes are stained in Gurr's giemsa (diluted 1:25 with water), rinsed in water and dried before application of coverslips.

Examination of SCE's: All slides are coded and read in a blind fashion without indication of the specific treatment or concentration of the test agent. The number of chromosomes and the number of SCE's in a minimum of 15 cells are recorded for each dose level. The mean number of SCE/cell and SCE/chromosome are calculated and recorded. Slides are decoded only after examination of all slides in the experiment has been completed.

Statistical Analyses: Data are analyzed by appropriate parametric statistical procedures which follow BRRC standard operating procedures for analyses of data. Significance values and the statistical test employed are shown for data summarized in tabular form.

Interpretation of Data: The criteria for evaluation of a positive or negative response depend both on the level of statistical significance and subjective analyses of concurrent and historical control data. The key determinant is whether a dose-dependent increase in SCE's is induced by the test agent. When no clear dose-response relationship is evident and when one or more responses of marginal statistical significance are obtained, a careful examination of the data in comparison to the concurrent controls and historical data base is necessary. Testing may be repeated to clarify unusual responses, if data for the concurrent positive or negative controls suggest a defect in the original experiment. Overall assessment will also rely on corroborating data from the other tests in the testing battery. Clearly positive responses will include any of the following: (i) Doubling in the SCE frequency at a minimum of two of the five concentrations tested; (ii) Statistically significant responses of $p < 0.05$ at three concentrations or at 2 concentrations if $p < 0.01$; (iii) Induction of a statistically significant, dose-related increase in the number of SCE.

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APPENDIX III

Unscheduled DNA Synthesis (UDS) in Hepatocytes from Rat LiverTheoretical Basis

Chemicals may interact with both the cellular components and the genetic material of a cell (e.g. DNA and RNA) because of their electrophilic nature or by conversion into reactive electrophiles by the metabolic enzymes of the cell. Damage to the DNA of a cell can result in cell death, mutation or, theoretically, carcinogenic transformation. Studies of agents which are capable of reacting and damaging the cellular DNA have suggested that such methods may be useful as a sensitive screening test for detecting potential mutagenic/carcinogenic chemical properties.

Detection of the relatively small amounts of DNA damage induced by chemical treatment requires a cellular system in which normal, semi-conservative DNA replication, which occurs during cell division, is inhibited. The system employed for the present study uses a suspension culture of primary hepatocyte cells isolated from rat liver according to the general methods of Seglen (1973) and Williams (1976). Hepatocytes do not normally divide in the minimal culture medium employed and stimulation of "unscheduled" incorporation of radioactive DNA precursors can be detected by scintillation spectrometry. The stimulation of incorporation of tritiated thymidine into both purified hepatocyte nuclei and DNA is used as the indicator of chemically induced DNA damage. The amount of unscheduled DNA synthesis (UDS) following treatment is compared with both concurrent positive and negative controls as well as with historical data for similar tests.

Methods

Preparation of Hepatocyte Suspensions: Hilltop-Wistar albino rats are anesthetized with Metafane(R). The abdominal cavity is surgically exposed and 1250 units of heparin is injected intravenously. A catheter is inserted into the portal vein and warm Hanks Balanced Salt Solution (HBSS) is pumped into the vein and through the liver. This first solution contains heparin and EGTA, [ethylene glycol-bis-(beta-aminoethyl-ether)N,N-tetracetic acid], which preferentially chelates calcium; the solution contains no magnesium or calcium. After the liver is blanched, a second solution of HBSS containing 60 units/ml of collagenase is perfused. This solution is pumped through the liver until the liver is digested. The liver is then removed and the cells are freed in cold medium 199 by combing through the lobes with a sterile metal comb. The cell suspension is passed through two nylon meshes to remove cell clumps and the cells are washed once at low centrifugation speed. After resuspension in medium 199, equal volumes of cells and 0.4% trypan blue are mixed together and the cell viability and number of viable cells per ml is determined microscopically.

Preincubation of Hepatocytes: Approximately 2×10^6 viable hepatocytes are added to 5 ml medium 199 containing 10 mM hydroxyurea and 30 mM HEPES (N-2-hydroxyethyl piperazine-N-2 ethane sulfonic acid) buffer. After the cells are dispensed into the tubes, they are placed on a rocker platform and are incubated at 37°C for 1 hour. Although hepatocytes do not normally divide in culture, medium 199 which lacks serum and contains hydroxyurea is used to further block semi-conservative DNA synthesis. Thus, any radioactive thymidine incorporated into the nuclei is expected to result from repair or unscheduled DNA synthesis.

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Selection of Doses of Test Chemical: Initially, the following concentrations $\times 10^{-3}\%$ (by volume) are tested: 100, 30, 10, 3, 1, and 0.1. If these concentrations prove to be cytotoxic, or if additional information is available from other in vitro tests as to the proper dose levels, then an appropriate series of concentrations is used over a 3-log range of concentrations.

Treatment of Hepatocytes: After preincubation, 25 microCuries of tritiated thymidine (20 Curies/millimole) is added to each tube. The test chemical and positive controls are diluted in an appropriate solvent and they are then added to each labeled tube. Generally, at least six concentrations of the test chemical over a 3-log range of concentrations are tested and each concentration is run in duplicate. The tubes are returned to the rocker platform for a 2-hour exposure at 37°C.

Positive and Negative Controls: 4-nitroquinoline oxide (NQO), a direct-acting mutagen, which induces UV-type DNA repair and dimethylnitrosamine (DMN), which requires metabolic activation by microsomal enzymes for activity, are run in duplicate as positive control chemicals. The solvent control is run in quadruplicate and consists of 100 to 150 microliters (concentration specified in individual reports) of the solvent used to dilute the sample. Dimethylsulfoxide (DMSO) or water are the usual solvents for test chemicals.

Harvest: At the end of incubation with the test agent, the cells are centrifuged from the medium at 200 x g at 5°C. The cells are rinsed once in 5 ml of cold medium 199 and are resuspended in 0.25% Triton X-100, 5% citric acid and 3 mM MgCl₂, a lysing solution which liberates the nuclei. The nuclei are rinsed once in this solution and resuspended in 0.25 M sucrose, 2.5% citric acid and 3 mM MgCl₂. The nuclei are then centrifuged at 600 x g for 10 min at 5°C and resuspended in 2 ml of the lysing solution.

Determination of Nuclear-Bound Label: To measure the amount of radioactive thymidine incorporated into the nuclei, 0.25 ml of the nuclear suspension is mixed with 1.0 mg of NCS tissue solubilizer in a scintillation vial. Ten ml of Dimilume® scintillation cocktail is added and the radioactive disintegrations per minute (DPM) are determined by counting twice in a scintillation counter for ten minutes. The measured DPM are then used to calculate the DPM/10⁶ viable hepatocytes presented on tables.

Determination of DNA-Bound Label: The amount of radioactive thymidine incorporated into DNA is quantitated in DNA isolated and precipitated from 1.00 to 1.25 x 10⁵ viable hepatocytes. To 1.25 ml of the nuclear suspension, 2.75 ml of 1% sodium dodecyl sulfate (SDS) and 5 mM Ethylenediaminetetraacetic Acid (EDTA) is added to lyse the nuclei. The DNA is precipitated from this solution with 4 ml of ice-cold 10% trichloroacetic acid (TCA) and the sample tubes are incubated at 0°C for at least 30 minutes. The solution is then poured onto Whatman glass fiber filters under vacuum and the tubes and filters are washed twice with cold 5% TCA. Finally, each filter is rinsed once with methanol, dried and placed in a scintillation vial. The filters are incubated at 50°C for 1 hour with 1 ml of a diluted solution of NCS tissue solubilizer; prepared by adding 1 part solubilizer to 2 parts of Dimilume® cocktail. Dimilume® is then added to each vial and the vials are counted twice in a scintillation counter for ten minutes.

Statistical Analysis: The average DPM is calculated for each dose level and the controls and final results are expressed as DPM/10⁶ viable hepatocytes. Data are also expressed as a percent of the solvent control for purposes of comparison. The original data are statistically analyzed by the appropriate parametric test, following the BRRC standard procedures for statistical analyses and the test(s) employed is indicated on the respective tables. Comparison between the mean for each dose level with the 95% confidence limits of the historical solvent control may also be used in some cases to assess the potential biological significance of the data. Testing may be repeated to clarify unusual responses, if data with the concurrent controls suggest a defect in the original experiment.

Interpretation of Results: The classification of a chemical as a positive, active agent depends upon the production of a statistically significant, dose-related increase in the amount of UDS activity. If a definite dose-response relationship is not evident, or when a few increases with marginal statistical significance are obtained, comparison of the responses to historical control data provides a meaningful assessment of the possibility for random variations which may be statistically significant only in relation to the concurrent control. A key determinant of the reliability of the UDS data is the detection of a similar response with both DNA and isolated nuclei determined at two or three consecutive concentrations.

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Appendix IV

Physical and Chemical Characteristics of Test Material

1. Chemical Name:	Diethylenetriamine
Trade Name and/or Synonyms:	Bis-(2-Aminoethyl)Amine
Molecular Weight:	103.17
Formula:	$H_2NCH_2CH_2NHCH_2CH_2NH_2$
Specific Gravity (@ 20°C):	0.9542
Boiling Point:	206.9°C (760 mm Hg)
Solubility in H ₂ O (% by wt):	Complete at 20°C
Purity	98.5%, minimum (see comments in additional analytical sections)
Vapor Pressure (@ 20°C):	< 1 mm Hg
pH:	Not available
Flash Point:	210°F, tag closed cup
Stability:	Stable
Incompatibility:	Avoid acids
Appearance and Odor:	Water-white liquid; mildly ammoniacal odor
Disposal:	Incineration

2. Protective Measures: Do not get on skin, eyes or clothing. Use rubber gloves, mechanical ventilation or local exhaust when handling. Avoid prolonged breathing of vapor or contact with skin. Safety goggles, organic vapor mask and rubber apron are recommended. Small spills should be flushed with large quantities of water.
3. Health Hazard: Harmful if inhaled; skin and eye contact cause burns. Diethylenetriamine is a sensitizer which may cause skin rash. Prolonged and repeated breathing will be irritating and may cause an asthma-like condition. In case of contact flush with large volumes of water for 15 minutes followed by soap and water. Remove contaminated clothing and wash before wearing again.

ETHYLENEAMINES

PRESHIPMENT ANALYSES OF HIGHER ETHYLENEAMINES
SENT TO MELLON INSTITUTE FOR TOXICOLOGY STUDIES

AUTHORS: P. R. Umberger

DATE: March 14, 1980

PROJECT NO.: 142A40

SUPERVISOR: D. C. Best

FILE NO.: 27479

SUMMARY This memorandum documents the preshipment analyses of the following ethyleneamines recently sent to Mellon Institute for toxicology studies.

- Diethylenetriamine-high purity (DETA-HP)
- Diethylenetriamine-commercial (DETA-Comm) 1 PRU-50
- Diethylenetriamine-(DETA) - Hearts Cut
- Triethylenetetramine (TETA) - Linear
- Tetraethylenepentamine (TEPA) - Hearts Cut

Pertinent
Sections
of Report
On:

The high purity and commercial DETA are Union Carbide commercial products while the other samples were prepared in the laboratory from commercial products.

Over the next two years these materials will be used in either mouse skin painting, pharmacokinetics or mutagenicity tests. Elemental, gas chromatograph and infrared analyses were run on the samples as part of a continuing Union Carbide support program in accordance with the study protocols. The commercial DETA for the skin-painting studies will be returned to the Technical Center after 6, 12 and 24 months to provide storage stability data.

INTRODUCTION UCC initiated a program to determine the toxicological properties of the ethyleneamines. While the studies on ethylenediamine are sponsored by an intra-industry group - UCC, Dow Jefferson and Berol Kemi- the studies on the higher ethyleneamines are funded solely by UCC.

The preshipment analyses of these ethyleneamines previously shipped to Mellon have been reported: (1,2)

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Ethylenediamine (EDA)
Diethylenetriamine-high purity (DETA-HP)
N-Aminoethylethanolamine (AEEA)
N-(2-Aminoethyl) piperazine (AEP)
Triethylenetetramine (TETA)
Tetraethylenepentamine (TEPA)
Polyamine HPA #2
HPA #2 Sludge

Analytical data on the materials shipped to Mellon recently are supplied in this report.

DISCUSSION All of the amines shipped to Mellon were either prepared from materials produced, or produced via the reaction of aqueous ammonia with ethylenedichloride in UCC's commercial facilities - either Taft or Texas City. The DETA-HP for the pharmacokinetics study and the DETA-Comm are commercial products, while the DETA-hearts cut, TETA-linear and TEPA-hearts cut were prepared in the laboratory. (See Appendices A, B and C for preparation procedures.)

The elemental analyses of the samples are given in Table I, together with theoretical C, H, N values for DETA and TETA-linear. Tables II and III show the results of gas chromatographic analyses; the scans are in Figures 1-8. Unfortunately, no single method exists to analyze all the ethyleneamines, so three different methods were used: two to analyze the DETA samples and a third to characterize TETA and TEPA.

The first method used to analyze DETA involves derivatization of the amines with N-methyl-bis (trifluoroacetamide) and is a semi-quantitative method for the major component present. However, the necessary accuracy and reproducibility data required to define the method have not been generated yet. (See Appendix D.) The second method for DETA analysis is a direct method and does not involve derivatization; it utilizes a thermal conductivity detector and area percent data are obtained. (See Appendix E.) If the components of a sample have different response factors, or if the sample contains components which do not elute from the column, the analysis will be in error. Although the two methods give different results, two procedures - with their attendant problems - provide better characterization of the amines.

The gas chromatographic procedure used to characterize TETA and TEPA is also a direct method using a thermal conductivity detector and gives area percent data. The column and instrument parameters are described in Appendix F.

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Figures 9-13 are the infrared scans of the materials sent to Mellon showing the typical bands expected for the ethyleneamines.

FUTURE WORK The DETA-Comm for the skin-painting studies is to be shipped back to the Technical Center from Mellon for re-analysis at the 6, 18 and final (24) month of testing. Additional quantities of the materials are being stored for 36 months at the Technical Center.

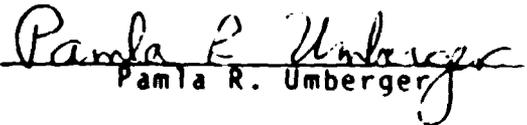
ACKNOWLEDGEMENT C. A. Gibson and A. H. Larch prepared the TETA-linear and TEPA-hearts cut and Jane Montgomery distilled the DETA-hearts cut. Sample analyses were performed by A. H. Larch and W. H. Nelson (G. C.), J. T. Hildebrand (Elemental), L. C. D'Esposito and J. E. Richardson (I.R.)

REFERENCES

1. Umberger, P. R., Memorandum, "Ethyleneamines, Preshipment Analyses of the Ethylenediamine Sent to Mellon Institute for Toxicology Studies", File No. 26942, October 18, 1979.
2. Umberger, P. R., Memorandum, "Ethyleneamines, Analyses of Higher Ethyleneamines Sent to Mellon Institute for Toxicology Studies", File No. 26894, October 8, 1979.

NOTEBOOK REFERENCE

99CAG and 103CAG (Charles A. Gibson)
1PRU and 5PRU (Pamla R. Umberger)
UCC Technical Center
South Charleston, WV


Pamla R. Umberger

Manuscript Date: February 5, 1980

Date Typed: February 15, 1980

PRU:s11

Attachments: 3 Tables
13 Figures
Appendices A, B, C, D, E and F

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TABLE I

ELEMENTAL ANALYSES*

<u>Sample</u>	<u>Reference</u>	<u>Analysis (Wt. %)</u>		
		<u>N</u>	<u>C</u>	<u>H</u>
DETA-HP	1 PRU-72	40.80	46.68	13.25
DETA-Comm	1 PRU-50	39.84	47.13	13.14
DETA-Hearts cut	1 PRU-47	40.05	46.12	13.13
TETA-Linear	99CAG-84-28	37.97	49.72	12.51
TEPA-Hearts cut	103CAG-25-5	35.14	53.16	12.20

	<u>Theoretical Wt. %</u>		
	<u>N</u>	<u>C</u>	<u>H</u>
DETA	40.71	46.53	12.76
TETA-Linear	38.32	49.28	12.40

TEPA is a complex mixture so theoretical C, H, N analyses cannot be calculated.

*Performed by J. T. Hildebrand

NOTEBOOK REFERENCE: 1 PRU and
5 PRU

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TABLE II
GAS CHROMATOGRAPH ANALYSES OF DETA SAMPLES (AREA %)

Method	DETA-HP 1 PRU-72		DETA-Comm 1 PRU-50		DETA-Hearts Cut 1 PRU-47	
	<u>1</u>	<u>2</u>	<u>1</u>	<u>2</u>	<u>1</u>	<u>2</u>
Component (%)						
EDA	0.21	-	0.34	-	Trace	
DETA	98.79	99.18	90.75	92.74	100	99.97
AEP	1.00	0.82	8.91	7.26	Trace	0.03

1 = Derivative Method

See Appendix D.

2 = 30M Column

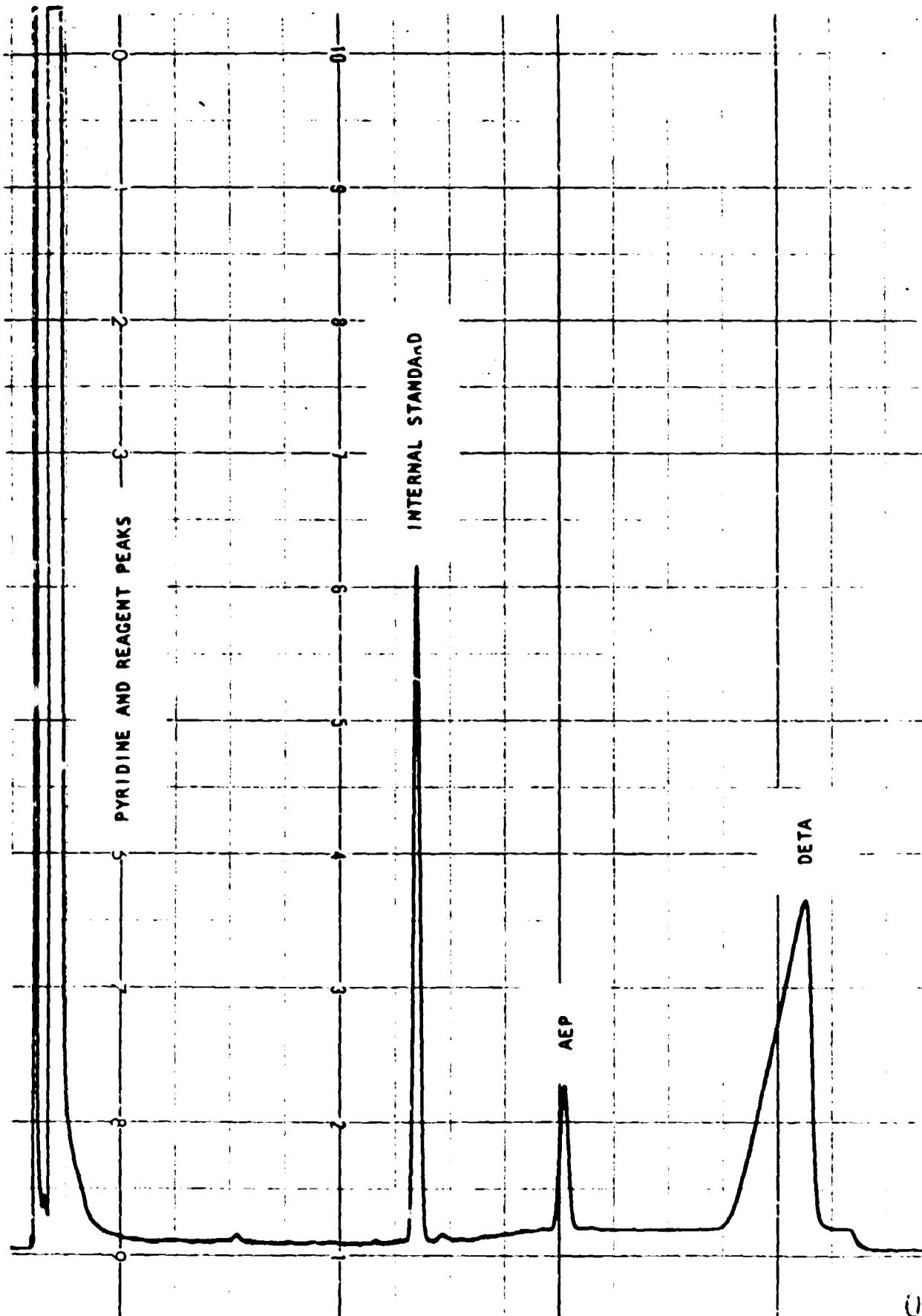
See Appendix E.

NOTEBOOK REFERENCE: 5 PRU

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FIGURE 3

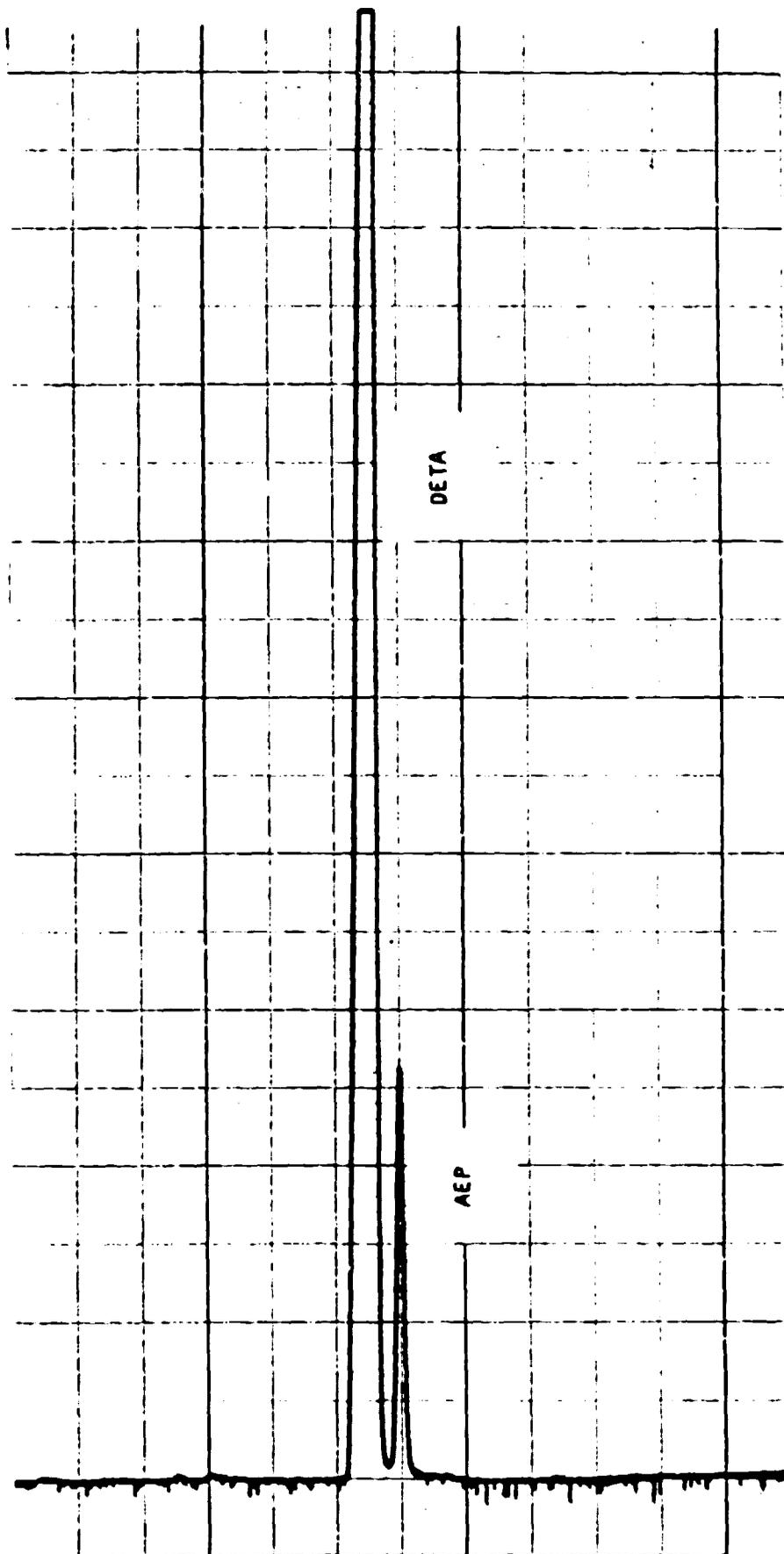
CHROMATOGRAM OF MBTFA DERIVATIVE OF DETA-COMM (17RU-50)



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FIGURE 4

DETA - COMM (IPRU-50) CHROMATOGRAM (30M-COLUMN)



Quality Assurance Unit Study Inspection Summary

Test Substance: Diethylenetriamine -- Commercial Grade

Study: In Vitro Mutagenesis Studies: 3-Test Battery

Study Director: R. S. Slesinski

The Quality Assurance Unit conducted the following inspections and reported the results to the Study Director and to Management on the dates indicated.

<u>Date</u>	<u>Inspection</u> <u>Type</u>	<u>Date QAU Report Issued</u>	
		<u>To Study Director</u>	<u>To Management</u>
10/31 to 11/13/80	Final Ongoing and Final Report	11/6/80	11/13/80


Quality Assurance Office

11/13/80
Date

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	IN VITRO MUTAGENESIS STUDIES: 3-TEST BATTERY			
Chemical Name (300 per name)	DIETHYLENETRIAMINE		CAS No. (10)	111-4φ-φ

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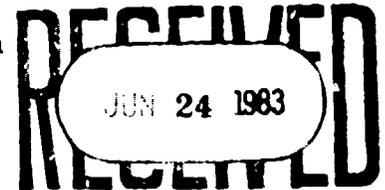
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Project Report 43-120
21 Pages
Tel: (412) 327-1020
December 12, 1980

Diethylenetriamine-Hearts Cut (DETA-HC)In Vitro Mutagenesis Studies: 3-Test Battery

Authors: R. S. Siesinski, M. W. Gaunt, P. J. Guzzie, W. C. Hengler

Sponsor: Union Carbide Corporation

SUMMARY

Diethylenetriamine-Hearts Cut (DETA-HC) was evaluated for potential mutagenic activity with a battery of three in vitro tests, which were: the Chinese Hamster Ovary (CHO) Mutation test, the Sister Chromatid Exchange (SCE) test and an assay for induction of Unscheduled DNA Synthesis (UDS) in rat liver cells. The data indicated that DETA-HC did not produce a mutagenic effect typical of known chemical mutagens and it lacked significant mutagenic potential in the three in vitro tests performed.

RESULTS AND INTERPRETATION

Selection of Test Concentrations - Previous tests on other DETA samples indicated that a maximum concentration of $40 \times 10^{-2}\%$ (by volume) was an appropriate dose level which would allow survival of approximately 10% of the cells. In previous tests, higher concentrations of DETA killed > 99% of the treated cells.

CHO Mutation Test - DETA-HC did not produce either statistically significant or dose-related increases in the mutation frequency of CHO cells at any concentration between $40 \times 10^{-2}\%$ to $1.25 \times 10^{-2}\%$ (by volume). The test in the presence of an S9 activation system was repeated to assure the reproducibility of the negative results in the first test and those data were also consistently negative. DETA-HC was considered non-mutagenic to CHO cells in the present in vitro test.

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SCE Test - DETA-HC did not produce dose-related increases in the frequency of SCE in tests both with and without the incorporation of an S9 metabolic activation system. Single, statistically significant increases in the SCE frequency were produced in both tests with and without S9 activation but the lack of a dose-related effect suggested that these results were not biologically significant. DETA-HC was considered inactive in the present SCE in vitro assay.

UDS Test - DETA-HC did not produce dose-related increases in the amount of ^3H -thymidine incorporation in tests over a range of concentrations from $10 \times 10^{-2}\%$ to $0.01 \times 10^{-2}\%$ (by volume). Production of numerically elevated levels of UDS in comparison to the concurrent solvent control were only marginally statistically significant and the data did not show a relationship to the treatment dose. DETA-HC was considered inactive in the present test with the hepatocyte test system.

Comparative Mutagenicity - The pattern of negative results produced by DETA-HC in the 3-test battery of mutagenicity assays indicated that DETA-HC was non-mutagenic in the in vitro tests performed. The data from each of the three cell culture tests were consistent in demonstrating a lack of mutagenic activity for the DETA-HC test sample in tests both with and without a metabolic activation system. The high degree of alkalinity of this chemical limited the range of doses which could be employed, but the test concentrations for these studies were considered appropriate because only a 2-fold increase generally produced excessive cytotoxicity observed as cell death or lack of SCE staining.

One literature reference reported that DETA was positive for inducing mutations in the Salmonella typhimurium (Ames) test (Hedenstedt, 1978). However, an active alkylating contaminant was found in the DETA sample used in that test and no conclusion on the potential activity of pure DETA was possible from that study.

SAMPLE

Quantity: 4 oz

CHF Sample No.: 42-449

Submitted by: D. C. Best

Date Received: November 12, 1979

Division: Specialty Chemicals
and Plastics
South Charleston, WV

Identification: Diethylenetriamine
Hearts Cut; liquid

Ref.: 1PRU-47

CAS#: 111-40-0

000003

Diethylenetriamine-Hearts Cut (DETA-HC)

In Vitro Mutagenesis Studies: 3-Test Battery

Sponsor: Union Carbide Corporation

* * * * *

OBJECTIVE

The purpose of this study was to evaluate the potential of DETA-HC to induce genetic damage in mammalian cells at the gene, chromosome and/or DNA (deoxyribonucleic acid) level of molecular organization. A battery of three in vitro, short-term tests which detect each of these genetic endpoints was employed to evaluate DETA-HC for potential mutagenic activity. A general description of the theoretical basis for each of these tests is presented in Appendices I, II and III attached to this report.

SAMPLE CHARACTERISTICS

A typical, commercial sample of DETA-HC was received for testing on November 12, 1979. The physical and chemical characteristics of this chemical available from the Toxicology Data Bank or Material Safety Data Sheet, are attached to this report in Appendix IV.

Additional analytical data on the sample received for testing was obtained from P. R. Umberger, UCC, S. Charleston, WV (Appendix V - pertinent data attached to the complete report). Two different analytical methods were used: with a derivative method, the percentage by weight of DETA was essentially 100%, with traces of ethylenediamine (EDA) and N-(2-aminoethyl)piperazine (AEP); with a second method using a column of 10% CARBOWAX 30 M on 750 Chromosorb 60/80 mesh, the area % of DETA was 99.97% with 0.03% AEP (Data in Table II, Appendix IV).

METHODS

A description of the technical procedures used in the CHO test, the SCE test and the UDS assay are presented in greater detail in Appendices I, II and III, respectively (attached to the complete report). Testing was performed in compliance with Standard Operating Procedures used for these tests at the Bushy Run Research Center and deviations from procedures are noted in the individual test results. Diethylenetriamine-Hearts Cut is abbreviated DETA-HC in this report.

1. CHO Test (Detailed procedures in Appendix I): SOP #7.2.4A; 7.2.5; 7.2.11
- A. Dose Selection - Data obtained from testing DETA-High Purity for cytotoxicity in an earlier test (BRRC Report #43-90) were used to select the doses of DETA-HC for testing. The earlier study on DETA-High Purity evaluated cytotoxicity at six concentrations from 0.1% to $0.3 \times 10^{-2}\%$ (by volume), both in the presence and absence of a liver S9 metabolic activation system.

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Selection of a maximum concentration for testing depended upon an estimate of dose levels which would permit survival of approximately 10% of the treated cells. Sterile water was used as the solvent and solvent control; glass-distilled dimethylsulfoxide (DMSO) was used as the negative control.

To simplify tables and to allow comparisons between different tests, concentrations of DETA-HC in the following sections of the report are given in terms of volume percentages $\times 10^{-2}$ to eliminate zeros in the lower concentration values (eg., 0.0001% = $0.01 \times 10^{-2}\%$).

- B. Mutation - CHO cells were exposed for 5 hours to seven concentrations of DETA-HC both with and without the addition of an S9 metabolic activation system. Only the top five concentrations which allowed sufficient cell survival are typically assessed for survival and induction of mutants. Dilutions of DETA-HC for testing were prepared by either direct addition of various aliquots of the test agent into the cell culture media or by making sequential one-half dilutions in sterile H₂O from the stock solution for the highest dose level. The surviving fraction was determined at 20 to 24 hours after treatment and the mutant fraction was determined after a 7- to 9-day period to allow "expression" of the mutant phenotype. The percentage of cells surviving the treatment, the frequencies of mutant colonies and the number of mutants/ 10^6 viable cells are presented in tabular form. The test with an S9 metabolic activation system was repeated with five concentrations of DETA-HC to assure the reproducibility of the DETA-HC data in consideration of the relatively low values obtained with the DMN positive control.
2. SCE Test (Detailed procedures in Appendix II): SOP #7.2.12A

Production of SCE's following exposure to various concentrations of DETA-HC was studied in CHO cells both with and without the incorporation of an S9 metabolic activation system. Selection of dose levels which would permit survival of approximately 50% of the treated cells was based on the prescreening test for cytotoxicity performed as part of the CHO Mutation test. Dilutions of DETA-HC for testing, were prepared either by direct addition of various aliquots into the culture medium or by making sequential one-half dilutions in sterile H₂O from the stock solution for the highest dose level. For determination of direct mutagenic action, CHO cells were exposed to DETA-HC and appropriate controls for 5 hours without S9 activation. Indirect mutagenic action, requiring metabolic activation by liver S9 homogenate, was studied with a 2-hour exposure period. Bromodeoxyuridine (BrdU) required to differentiate between the individual "sister" chromatids by SCE staining, was present at a concentration of 3 $\mu\text{g}/\text{ml}$ in the growth medium during treatment and during the culture period following exposure. A total of 20 cells/dose level and 5 dose levels, with or without metabolic activation, were studied. The number of SCE/cell, mean number of SCE/chromosome and the level of statistical significance of the increases above concurrent solvent control values are presented in tabular form.

3. UDS Test (Detailed procedures in Appendix III): SOP #7.2.6; 7.2.7; 7.2.8A; 7.2.9A

Induction of primary DNA damage in rat liver cells (hepatocytes), was studied at a minimum of six dose levels which spanned a 1000-fold range of concentrations. Cells were treated with DETA-HC for 2 hours in culture medium containing ^3H -thymidine, hydroxyurea and appropriate dilutions of DETA-HC prepared in DMSO. Determination of UDS activity was performed by analyses of incorporation of ^3H -thymidine into isolated hepatocyte nuclei or in DNA (precipitated from aliquots of the isolated nuclei) using a Searle Analytic Model 81 or Packard Model 2650 scintillation spectrometer. Data are presented in tabular form with an indication of the level of statistical significance above the concurrent solvent control values.

4. Controls - Positive, negative and solvent controls were tested concurrently with the test chemical to assure the sensitivity of the test system and concurrence of the results to previous test performance. In the CHO and SCE assays, dimethylnitrosamine (DMN) and ethylmethanesulfonate (EMS) were used as positive control agents to assure the sensitivity of the test system for detecting indirect- and direct-acting chemicals, respectively. Deionized water, sterilized by membrane filtration and glass-distilled dimethylsulfide (DMSO) were used as the solvent and negative controls, respectively.

In the UDS assay, DMN and 4-nitroquinoline oxide (4-NQO) were used as positive controls for indirect- or direct-acting mutagens, respectively. DMSO was used as the solvent and the solvent control.

5. Metabolic Activation - S9 liver homogenate, prepared from Arochlor 1254-induced, Sprague-Dawley male rats, was purchased from Litton Bionetics. The S9 preparation used for the CHO test in experiment 1 contained 27.0 mg/ml protein and had a benzo(a)pyrene hydroxylase activity of 9.97 nmol hydroxybenzpyrene/20 min/mg protein, (assayed by Litton). A concentration of 1200 ug of S9 protein was added to 5 ml of culture media. In the repeat test (experiment #2), the S9 contained 40 mg/ml protein and had a benzo(a)pyrene hydroxylase activity of 13.93 nmol hydroxybenzpyrene 20 min/mg protein. A concentration of 3600 ug of S9 protein was added to 5 ml of culture media.

For the SCE test, a third lot of S9 homogenate, containing 40 mg/ml protein and a benzo(a)pyrene hydroxylase activity of 15.0 nmol hydroxybenzpyrene/20 min/mg protein (assayed by Litton), was used at a concentration of 600 ug of S9 protein/5 ml culture media.

6. Statistical Analyses - Data from the SCE and UDS tests were analyzed by appropriate parametric tests following Standard Operating Procedures for statistical analyses at the Bushy Run Research Center. Data from the CHO test do not follow a normal distribution according to experience with historical controls. Thus, the Student's t-test was employed after transformation of the mutation frequencies (MF) following the procedure of Irr and Snee: $(MF + 1)^{0.15}$ (Irr, J. D. and R. Snee, Proceedings of the Cold Spring Harbor-Banbury Conference, II (1979), 263-274).

Rounding of data to either two decimal places or to the appropriate number of significant figures was performed for presentation on tables. Although statistically significant decreases in mutation indices can occur because of cytotoxic responses, only statistically significant increases in responses above control values are indicated on tables for simplicity. The degree of statistical significance is denoted by: a: $0.05 > p > 0.01$, b: $0.01 > p > 0.001$, or c: $p < 0.001$. No superscript (or NS) indicates $p > 0.05$.

7. Raw Data Storage - Copies of the final report, statistical analyses, analytical data and data used to prepare the final report are stored in the BRRC Archives. Slides are stored in the Genetic Toxicology slide storage area.

RESULTS

SECTION I - CHO MUTATION TEST - Diethylenetriamine - Hearts Cut (DETA-HC)

A. Test Dates - Initiated: March 11, 1980
Completed: August 11, 1980

B. Selection of Test Concentration (Data not shown in tables)

Data from an earlier test of DETA-High Purity (BRRC Report #43-90) were used to select concentrations for testing of DETA-HC. Based upon the survival and cytotoxicity data of this earlier study, a concentration of $40 \times 10^{-2}\%$ was selected as the maximum concentration for testing with and without S9 activation.

C. Determination of Mutation Induction

1. Survival (Cytotoxicity)

Table 1 presents the cytotoxicity data for CHO cells treated with DETA-HC in the presence and absence of a liver S9 metabolic activation system. A steep dose-response effect with the test agent was suggested from the high degree of cytotoxicity observed for the top concentration ($40 \times 10^{-2}\%$) tested with or without S9 activation, in comparison to the markedly lower cytotoxicity obtained at only one-half the top dose-level.

2. Mutation

Table 2 presents the data for induction of mutants by DETA-HC and control agents. DETA-HC did not produce a dose-related increase in the frequency of mutants/ 10^6 viable cells over the range of concentrations tested for potential mutagenic action either with or without the presence of an S9 metabolic activation system.

Only the lowest concentration tested with S9 activation system in experiment 1 produced any mutant colonies and this result was not statistically significant. The experiment with S9 was repeated to assure the negative response to DETA-HC exposure and because of the low value for the DMN positive control. The mutation data from experiment #2, shown in Table 4, again showed no significant response to DETA-HC or any indication of a dose-related increase in the frequency of mutants. The test agent was considered to be non-mutagenic to CHO cells in the present tests.

Mutation frequencies for the solvent controls for tests both with and without S9 activation, in experiments 1 and 2, were in an acceptable and low range based upon experience with historical control values. Mutation frequencies obtained for the DMN positive control for both experiments were highly statistically significant in comparison to the concurrent Solvent Control but these values were outside the expected range of variability observed in historical control data.

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D. Deviations from Standard Procedures - Due to the alkalinity of the test chemical, the cell culture flasks were equilibrated with 10% CO₂ and sealed to help neutralize the alkalization of the media by the test chemical.

E. Conclusions

DETA-HC was consistently inactive as a mutagenic agent for CHO cells when evaluated for mutagenicity with or without an S9 metabolic activation system over a 16-fold range of concentrations. The production of mutants by DETA-HC was not statistically significant from the concurrent solvent control and all values were within the expected variation in mutant frequencies observed in historical control data.

SECTION II - SCE TEST - Diethylenetriamine - Hearts Cut (DETA-HC)

A. Test Dates - Initiated: March 31, 1980
Completed: June 16, 1980

B. Selection of Test Concentrations

A maximum concentration of $20 \times 10^{-2}\%$ of DETA-HC was chosen as the top dose level for testing with or without S9 activation based on cytotoxicity data from the CHO mutation test. Higher concentrations were expected to produce delays in the mitotic cycle and to decrease the number of cells with SCE staining. A 16-fold range of concentrations from $20 \times 10^{-2}\%$ to $1.25 \times 10^{-2}\%$ was tested in SCE experiment #1 without S9 activation. An identical range of concentrations of DETA-HC was tested in SCE experiment #2 with S9 activation.

C. Determinations of SCE Induction

1. The data for SCE induction in CHO cells treated without metabolic activation using various dose levels of DETA-HC and with positive, negative or solvent control agents are summarized in Table 5. DETA-HC did not produce a dose-related effect on the SCE frequency in this test. Only one concentration produced a statistically significant increase in the SCE frequency ($10 \times 10^{-2}\%$). Because both the highest dose-level ($20 \times 10^{-2}\%$) and all lower concentrations did not produce significant responses, this single statistically significant value was considered to be a random event, without probable biological significance. The test without S9 activation was considered a negative indication of potential direct mutagenic action of DETA-HC.

Induction of SCE by the concurrent EMS positive control was highly statistically significant from the concurrent solvent control and these data indicated an appropriate sensitivity of the test system comparable to our historical positive control data. The numbers of SCE obtained with the H₂O solvent and DMSO controls were also in an acceptable range of values included in the variability encountered in our historical control values for this test.

2. SCE values obtained from treatments of CHO cells with DETA-HC in the presence of an S9 metabolic activation system are presented in Table 6. DETA-HC did not produce a dose-related effect on the SCE frequency in this test. Only one statistically significant increase in the SCE values was produced by the $1.25 \times 10^{-2}\%$ concentration, the lowest concentration tested. Because no other concentration produced a significant response the test results were considered to be negative and consistent with the findings in the test without addition of S9 (Table 5). DETA-HC was considered inactive as a mutagenic agent in the induction of SCE in vitro.

Although the negative control, DMSO, was statistically different from the concurrent control (Table 6), it was not outside the 95% confidence interval of the historical control data. The indication of a statistical difference of the DMSO data in comparison to the concurrent solvent control was considered a chance event without biological significance. A highly statistically significant increase in the numbers of SCE was produced by the two concentrations of the DMN positive control. This result indicated that the metabolic activation system was functioning in an appropriate manner.

D. Deviations from Standard Procedures

Because of the cytotoxicity of the liver S9 homogenate, cells treated with the test agent and S9 were incubated for a total of 38 to 42 hours before harvesting cells and preparation of chromosomes for SCE slides. This extended growth period circumvented the cell division delay induced by the liver homogenate. Two concentrations of DMN were tested to assure the activity of the metabolic activation system.

Due to the alkalinity of the test chemical, the flasks were equilibrated with 10% CO₂ and sealed to help neutralize the pH of the media. Colcemid® at a concentration of 0.1 µg/ml was used rather than colchicine to increase the number of cells in mitosis. Media purchased from K. C. Biological Co. rather than from Grand Island Biological Co. was used. A concentration of 0.075% trypsin, rather than 0.05% as stated in the current SOP, was used to improve cell dissociation.

E. Conclusions

DETA-HC did not produce either statistically significant or dose-related increases in the mutation of CHO cells in tests over a 16-fold range of concentrations both with and without addition of an active S9, metabolic activation system. The test agent was considered to be inactive in the present in vitro assay.

SECTION III - UDS TEST - Diethylenetriamine - Hearts Cut (DETA-HC)

A. Test Dates - Initiated: April 2, 1980
Completed: May 12, 1980

B. Selection of Test Concentrations

Standard procedures were followed and DETA-HC was tested over a 3-log range of concentrations from $10 \times 10^{-2}\%$ to $0.01 \times 10^{-2}\%$ by volume. The maximum dose-level was selected in consideration of the cytotoxicity data obtained in the CHO Mutation test, which indicated that higher values would result in excessive cell killing. These dose-levels were considered to be appropriate for testing because uptake and incorporation of ^3H -thymidine into hepatocytes are generally even more sensitive to chemical effects than survival measurements with CHO cells (in which cytotoxicity is measured after a 24-hr recovery period following treatment).

C. Determination of UDS Induction

1. Nuclear-Bound Radioactive Label (Data in Table 7)

Data for "unscheduled" incorporation of ^3H -thymidine into nuclei of hepatocytes exposed to DETA-HC or to appropriate positive and negative controls are presented in Table 7.

In hepatocytes treated with DETA-HC, only one concentration ($0.3 \times 10^{-2}\%$) produced a statistically significant increase in the amounts of ^3H -thymidine incorporation. The single statistically significant value at $0.3 \times 10^{-2}\%$ DETA-HC was considered to have limited biological significance because there was no indication of a dose-related effect of DETA-HC treatments. DETA-HC was considered to be non-mutagenic in the present test.

Both of the positive control agents, NQO and DMN, induced numerically elevated increases in UDS over values obtained with the solvent control. With both positive controls a moderate increase in the amount of UDS in relation to the concentration indicated the responsiveness of the test system with measurements using nuclei. Although only the highest doses of DMN or NQO produced statistically significant increases above the concurrent solvent control, all concentrations produced numerical increases above the solvent control indicative of a positive response.

2. DNA-Bound Radioactive Label (Data in Table 8)

Analyses of DNA, from aliquots of hepatocyte nuclei used for the UDS studies presented on Table 7, were performed as a second assessment of "unscheduled" incorporation of radioactive thymidine. Values for radioactivity incorporated into the DNA of these hepatocyte nuclei are presented in Table 8.

Two concentrations of DETA-HC, $0.3 \times 10^{-2}\%$ and $0.01 \times 10^{-2}\%$ produced statistically significant increases in UDS above the solvent control. These same two concentrations also produced similar quantitative increases in UDS in measurements of ^3H -thymidine incorporated into nuclei (Table 7). However, neither method of measurement of UDS indicated that DETA-HC produced either highly statistically significant or dose-related increases in UDS. Thus, the responses were considered to be without biological significance and DETA-HC was considered to be inactive in the hepatocyte test system.

The positive control agents NQO and DMN both induced numerically elevated levels of UDS at all dose-levels and highly statistically significant levels of UDS at the top concentrations tested. The lack of a statistically significant response at the two lower dose-levels may suggest that the present test was either insufficiently responsive to detect low levels of activity or that an insufficient dose of each positive control was employed. The test was considered acceptable based on the highly significant responses produced by the top dose-levels of each positive control agent.

D. Deviations from Standard Procedures - None

E. Conclusion

DETA-HC did not stimulate significantly the incorporation of ^3H -thymidine in cells treated over a 1000-fold range of test concentrations. DETA-HC was considered inactive in the present tests with hepatocytes.

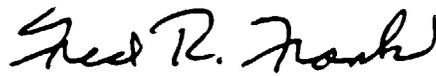
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Table 1
 Chinese Hamster Ovary (CHO) Mutation Assay:
 Determination of Toxic Effects of Chemical Treatment During 5 Hr Mutation Induction Period
 Experiment #1

Test Chemicals	Total # Colonies	Total # Cells Plated	% Survival	% of Solvent Control
Without S9 Activation				
[Diethylenetriamine-Hearts Cut] (% v/v)				
40.0 x 10 ⁻²	-	TOXIC	-	-
20.0 x 10 ⁻²	393	400	98.2	98.0
10.0 x 10 ⁻²	410	400	102.5	102.2
5.0 x 10 ⁻²	427	400	106.8	106.5
2.5 x 10 ⁻²	398	400	99.5	99.3
1.25 x 10 ⁻²	421	400	105.2	105.0
<u>Controls</u>				
H ₂ O Solvent (20 ul/ml)	401	400	100.2	-
DMSO (20 ul/ml)	403	400	100.8	100.5
EMS (200 ug/ml)	333	400	83.2	83.0
With S9 Activation				
[Diethylenetriamine-Hearts Cut] (% v/v)				
40.0 x 10 ⁻²	102	400	25.5	29.0
20.0 x 10 ⁻²	391	400	97.8	111.1
10.0 x 10 ⁻²	417	400	104.2	118.5
5.0 x 10 ⁻²	422	400	105.5	119.9
2.5 x 10 ⁻²	443	400	110.8	125.9
<u>Controls</u>				
H ₂ O Solvent (20 ul/ml)	352	400	88.0	-
DMSO (20 ul/ml)	484	400	121.0	137.5
DMN (3700 ug/ml)	259	400	64.8	73.6

Abbreviations: H₂O - water; S9 - liver homogenate; DMSO - dimethylsulfoxide
 EMS - ethylmethanesulfonate; DMN - dimethylnitrosamine

Table 2
 Chinese Hamster Ovary (CHO) Mutation Assay:
 Results on Evaluation of Mutant Induction by Diethylenetriamine - Hearts Cut
 Experiment #1

Test Chemicals	Plating Efficiency			Mutation Induction		
	Total # Colonies	Total # Cells Plated	Viable Fraction	Total # Mutant Colonies	Total # Cells Plated	Mutants ¹ 10 ⁶ Viable Cells
Without S9 Activation						
[Diethylenetriamine-Hearts Cut] (% v/v)						
40.0 x 10 ⁻²	-	-	TOXIC	-	-	-
20.0 x 10 ⁻²	361	400	0.902	0	1 x 10 ⁶	0
10.0 x 10 ⁻²	378	400	0.945	0	1 x 10 ⁶	0
5.0 x 10 ⁻²	604	400	1.510	0	1 x 10 ⁶	0
2.5 x 10 ⁻²	390	400	0.975	0	1 x 10 ⁶	0
1.25 x 10 ⁻²	479	400	1.198	0	1 x 10 ⁶	0
Controls:						
H ₂ O Solvent (20 ul/ml)	447	400	1.118	0	1 x 10 ⁶	0
DMSO (20 ul/ml)	504	400	1.260	0	1 x 10 ⁶	0
EMS (200 ug/ml)	398	400	0.995	62	1 x 10 ⁶	62.3 ^b
With S9 Activation						
[Diethylenetriamine-Hearts Cut] (% v/v)						
40.0 x 10 ⁻²	376	400	0.940	0	1 x 10 ⁶	0
20.0 x 10 ⁻²	445	400	1.112	0	1 x 10 ⁶	0
10.0 x 10 ⁻²	465	400	1.162	0	1 x 10 ⁶	0
5.0 x 10 ⁻²	543	400	1.358	0	1 x 10 ⁶	0
2.5 x 10 ⁻²	376	400	0.940	1	1 x 10 ⁶	1.1
Controls:						
H ₂ O Solvent (20 ul/ml)	508	400	1.270	0	1 x 10 ⁶	0
DMSO (20 ul/ml)	633	400	1.582	1	1 x 10 ⁶	0.6
DMN (3700 ug/ml)	294	400	0.735	33	1 x 10 ⁶	44.9 ^b

¹Total # mutant colonies per 10⁶ cells plated divided by viable fraction.
 Statistical significance above solvent control: b: 0.01 > p > 0.001.
 No superscript indicates p > 0.05. Data analyzed by Student's t-test.

Abbreviations: H₂O - water; S-9 - liver homogenate; DMSO - dimethylsulfoxide; EMS - ethylmethanesulfonate;
 DMN - dimethylnitrosamine.

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Table 3
 Chinese Hamster Ovary (CHO) Mutation Assay:
 Determination of Toxic Effects of Chemical Treatment During 5 Hr Mutation Induction Period
 Experiment #2

Test Chemicals	Total # Colonies	Total # Cells Plated	% Survival	% of Solvent Control
With S9 Activation				
(Diethylenetriamine-Hearts Cut) (% v/v)				
40.0 x 10 ⁻²	-	TOXIC	-	-
20.0 x 10 ⁻²	247	400	61.8	110.3
10.0 x 10 ⁻²	219	400	54.8	97.8
5.0 x 10 ⁻²	220	400	55.0	98.2
2.5 x 10 ⁻²	258	400	64.5	115.2
1.25 x 10 ⁻²	228	400	57.0	101.8
<u>Controls</u>				
H ₂ O - Solvent (20 ul/ml)	224	400	56.0	-
DMSO - (20 ul/ml)	172	400	43.0	76.8
DMN - (3700 ug/ml)	105	400	26.2	46.9

Abbreviations: H₂O - water; S9 - liver homogenate; DMSO - dimethylsulfoxide
 EMS - ethylmethanesulfonate; DMN - dimethylnitrosamine

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Table 4
 Chinese Hamster Ovary (CHO) Mutation Assay:
 Results on Evaluation of Mutant Induction by Diethylenetriamine - Hearts Cut
 Experiment #2

Test Chemicals	Plating Efficiency		Mutation Induction			
	Total # Colonies	Total # Cells Plated	Viable Fraction	Total # Mutant Colonies	Total # Cells Plated	Mutants ¹ 10 ⁶ Viable Cells
With S9 Activation						
[Diethylenetriamine-Hearts Cut] (% v/v)						
40.0 x 10 ⁻²	-	-	TOXIC	-	-	-
20.0 x 10 ⁻²	308	400	0.770	2	1 x 10 ⁶	2.6
10.0 x 10 ⁻²	488	400	1.220	4	1 x 10 ⁶	3.3
5.0 x 10 ⁻²	322	400	0.805	1	1 x 10 ⁶	1.2
2.5 x 10 ⁻²	458	400	1.145	4	1 x 10 ⁶	3.5
1.25 x 10 ⁻²	333	400	0.832	2	1 x 10 ⁶	2.4
Controls:						
H ₂ O Solvent (20 ul/ml)	338	400	0.845	2	1 x 10 ⁶	2.4
DMSO (20 ul/ml)	461	400	1.152	2	1 x 10 ⁶	1.7
DMN (3700 ug/ml)	279	400	0.698	31	1 x 10 ⁶	44.4 ^b

¹Total # mutant colonies per 10⁶ cells plated divided by viable fraction.
 Statistical significance from solvent control: b: 0.01 > p > 0.001.
 No superscript indicates p > 0.05. Data analyzed by Students t-test.

Abbreviations: H₂O - water; S-9 - liver homogenate; DMSO - dimethylsulfoxide; EMS - ethylmethanesulfonate;
 DMN - dimethylnitrosamine.

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Table 5
Sister Chromatid Exchange (SCE) Assay:
Induction of SCE's by Diethylenetriamine-Hearts Cut Without S9 Metabolic Activation
5 Hour Treatment

Test Chemicals	Total # of Chromosomes	Total # of SCE	SCE/Cell ¹	Mean Number SCE/Chromo- some ² + S.D.	Significance Above Solvent Control ³
[Diethylenetriamine-Hearts Cut] (% v/v)					
20.0 x 10 ⁻²	390	304	15.20	0.779 + 0.261	NS
10.0 x 10 ⁻²	400	340	17.00	0.851 + 0.235	b
5.0 x 10 ⁻²	392	270	13.50	0.693 + 0.203	NS
2.5 x 10 ⁻²	391	254	12.70	0.648 + 0.184	NS
1.25 x 10 ⁻²	397	257	12.85	0.640 + 0.277	NS
Controls					
H ₂ O (5 ul/ml) - Solvent	394	249	12.45	0.633 + 0.203	-
DMSO (5 ul/ml)	405	187	9.35	0.462 + 0.160	NS
EMS (100 ug/ml)	390	574	28.70	1.472 + 0.487	c

¹Twenty cells examined per dose level.

²Mean value of SCE/chromosome determined from the values of the individual cells examined.

³Statistical significance above solvent control: b: 0.01 > p > 0.001; c: p < 0.001
NS: p > 0.05.

Abbreviations: H₂O - water; S9 - liver homogenate; DMSO - dimethylsulfoxide;
EMS - ethylmethanesulfonate; S.D. - standard deviation

Table 6
Sister Chromatid Exchange (SCE) Assay:
Induction of SCE's by Diethylenetriamine - Hearts Cut With S9 Metabolic Activation
2 Hour Treatment

Test Chemicals	Total # of Chromosomes	Total # of SCE	SCE/Cell ¹	Mean Number SCE/Chromo- some ² + S.D.	Significance Above Solvent Control ³
[Diethylenetriamine-Hearts Cut] (% v/v)					
20.0 x 10 ⁻²	388	179	8.95	0.462 + 0.139	NS
10.0 x 10 ⁻²	394	183	9.15	0.467 + 0.120	NS
5.0 x 10 ⁻²	394	170	8.50	0.433 + 0.139	NS
2.5 x 10 ⁻²	364	156	7.80	0.427 + 0.160	NS
1.25 x 10 ⁻²	384	192	9.60	0.504 + 0.147	a
Controls					
H ₂ O (5 ul/ml) - Solvent	383	147	7.35	0.384 + 0.166	-
DMSO (5 ul/ml)	378	191	9.55	0.510 + 0.179	a
DMN (600 ug/ml)	390	830	41.50	2.110 + 0.692	c
DMN (800 ug/ml)	391	1043	52.15	2.661 + 0.725	c

¹Twenty cells examined per dose level.

²Mean value of SCE/chromosome determined from the values of the individual cells examined.

³Statistical significance above solvent control: a: 0.05 > p > 0.01; c: p < 0.001;
NS: p > 0.05.

Abbreviations: H₂O - water; S9 - liver homogenate; DMSO - dimethylsulfoxide;
DMN - Dimethylnitrosamine; S.D. - standard deviation

Table 7
 Unscheduled DNA Synthesis in Hepatocytes from Rat Liver

Nuclear-bound label: all DPM values are calculated from nuclei per 10^6 viable hepatocytes. Each average is calculated from duplicate samples, except for DMSO which was done in quadruplicate.

Test Chemical	Concentration	Radioactivity in Nuclei Avg. DPM \pm S.D.	% of Solvent Control \pm S.D.	Significance Above Solvent Control ¹
Solvent - DMSO	2.0%	3195 \pm 679	100.0% \pm 21.2%	-
Positive Controls:				
4 - NQO	3.0 ug/ml	7950 \pm 1662	248.8% \pm 52.0%	c
	1.0 ug/ml	3925 \pm 1291	122.8% \pm 40.4%	NS
	0.3 ug/ml	4369 \pm 1321	136.7% \pm 41.4%	NS
DMN	1000 ug/ml	5341 \pm 59	167.2% \pm 1.9%	a
	300 ug/ml	4066 \pm 317	127.3% \pm 9.9%	NS
	100 ug/ml	3850 \pm 617	120.5% \pm 19.3%	NS
Test Chemical:				
[Diethylenetriamine-Hearts Cut]	10 x 10 ⁻² %	2855 \pm 1195	89.4% \pm 37.4%	NS
(%, v/v)	3 x 10 ⁻² %	3787 \pm 584	118.5% \pm 18.3%	NS
	1 x 10 ⁻² %	2877 \pm 116	90.1% \pm 3.6%	NS
	0.3 x 10 ⁻² %	4987 \pm 69	156.1% \pm 2.2%	a
	0.1 x 10 ⁻² %	3087 \pm 411	96.6% \pm 12.9%	NS
	0.01 x 10 ⁻² %	4608 \pm 130	144.2% \pm 4.1%	NS

¹Statistical significance above solvent control: a: 0.05 > p > 0.01; c: p < 0.001; NS: p > 0.05.
 Data analyzed by Duncan's Multiple Range Analysis.

Abbreviations: DMSO - dimethylsulfoxide; 4-NQO - 4-nitroquinoline oxide; DMN - dimethylnitrosamine;
 DPM - disintegrations per minute; S.D. - standard deviation

Table 8
Unscheduled DNA Synthesis in Hepatocytes from Rat Liver

DNA-bound label: all DPM values are calculated from DNA precipitated per 10^6 viable hepatocytes. Each average is calculated from duplicate samples, except for DMSO which was done in quadruplicate.

Test Chemical	Concentration	Radioactivity in DNA Avg. DPM \pm S.D.	% of Solvent Control \pm S.D.	Significance Above Solvent Control ¹
Solvent - DMSO	2.0%	2907 \pm 524	100.0% \pm 18.0%	-
Positive Controls:				
4 - NQO	3.0 ug/ml	9035 \pm 1461	310.8% \pm 50.2%	c
	1.0 ug/ml	4270 \pm 1440	146.9% \pm 49.5%	NS
	0.3 ug/ml	4424 \pm 930	152.2% \pm 32.0%	NS
DMN	1000 ug/ml	5225 \pm 389	179.7% \pm 13.4%	b
	300 ug/ml	4356 \pm 610	149.8 \pm 21.0	NS
	100 ug/ml	3769 \pm 1047	129.6% \pm 36.0%	NS
Test Chemical: [Diethylenetriamine-Hearts Cut] (%, v/v)	10 x 10 ⁻² %	2433 \pm 840	83.7% \pm 28.9%	NS
	3 x 10 ⁻² %	3688 \pm 769	126.8% \pm 26.5%	NS
	1 x 10 ⁻² %	2487 \pm 206	85.5% \pm 7.1%	NS
	0.3 x 10 ⁻² %	4777 \pm 307	164.3% \pm 10.5%	a
	0.1 x 10 ⁻² %	3032 \pm 239	104.3% \pm 8.2%	NS
	0.01 x 10 ⁻² %	4554 \pm 206	156.6% \pm 7.1%	a

¹Statistical significance above solvent control: a: 0.05 > p > 0.01; b: 0.01 > p > 0.001; c: p < 0.001; NS: p > 0.05.

Data analyzed by Duncan's Multiple Range analysis.

Abbreviations: DMSO - dimethylsulfoxide; 4-NQO - 4-nitroquinoline oxide; DMN - dimethylnitrosamine; DPM - disintegrations per minute; S.D. - standard deviation

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APPENDIX I

Chinese Hamster Ovary (CHO) Mutation AssayTheoretical Basis

Mutation is a heritable alteration in a cell in which a gene specifying the genetic code for a specific protein is modified in structure and/or function. Mutations, induced by chemical or physical agents, of the HGPRT (hypoxanthine-guanine phosphoribosyltransferase) gene may be detected by the growth of colonies of "mutant" cells which are resistant to the purine analogs 6-thioguanine (TG) or 8-azaguanine. Normal cells contain a functional HGPRT enzyme which phosphorylates TG and allows its incorporation into DNA causing the cells to die. Mutant cells with a non-functional HGPRT enzyme are unable to phosphorylate or incorporate TG, thus survive and grow in its presence.

The CHO mutation test is an assay which detects "forward mutations" from TG-sensitivity to TG-resistance caused by a direct loss of the activity of the HGPRT enzyme (HGPRT⁺ → HGPRT⁻). An assessment of the ability of several hundred agents to cause gene mutations in vitro indicates that the CHO mutation assay provides a reasonable estimate of the potential genetic activity of the test chemical.

Methods

Cell Culture Procedures: CHO cells used in these studies were obtained from Abraham Hsie at Oak Ridge National Laboratory with the designation CHO-K1-BH4-D1 (or simply CHO for report purposes). Cells are maintained in active growth by subculturing 2 to 3 times/week in antibiotic-free, Ham's Modified F12 Medium supplemented with 10% (v/v) heat-inactivated, fetal bovine sera (F12-10), and lacking in hypoxanthine. For treatment of cells without metabolic activation, F12 medium with 50 units/ml of penicillin, 50 ug/ml streptomycin and 5% (v/v) of dialyzed bovine serum (F12-D5) is used. For treatments incorporating an S9 metabolic activation system, identical medium, but without serum, is employed. For determination of mutant frequencies, F12-D5 medium containing 2.0 ug/ml TG (6-thioguanine) is used as a "selective medium." Cell numbers are determined routinely with a Coulter Model F electronic cell counter which is standardized periodically with a pre-counted suspension of latex beads. Presence of Mycoplasma cell contaminants is determined by a microscopic fluorescence assay employing Hoechst 33258 dye. All culture procedures and treatments with test chemicals are performed under aseptic conditions in a laminar-flow, biohazard hood.

Positive and Negative Controls: Sterile water or glass-distilled dimethylsulfoxide (DMSO) are the usual solvents for test chemicals and the respective solvent is tested as a control at the maximum concentration used to add the test agent. Dimethylnitrosamine (DMN) or ethylmethanesulfonate (EMS) are used as positive control mutagens for tests with or without an S9 metabolic activation system, respectively. Mutation frequencies obtained with concurrent positive and negative controls are used as the basis for monitoring the sensitivity and stability of the CHO mutation test system. Comparison of concurrent control values with historical controls is used to delineate the range of acceptable variations in the test system.

Metabolic Activation: Rat liver, S9 homogenate prepared from Arochlor-1254 induced Sprague-Dawley, male rats is purchased from Litton Bionetics, Kensington, MD. Each lot of liver homogenate is prescreened for metabolic capability to activate DMN in our laboratory before use in the testing program. The complete S9 metabolic activation system contains the following: 8 umoles/ml $MgCl_2$, 33 umoles/ml KCl, 5 umoles/ml glucose-6-phosphate, 4 umoles/ml NADP-oxidized (nicotinamide adenine dinucleotide phosphate), 100 umoles/ml Na_2HPO_4 , and between 500 to 4000 ug/ml of S9 protein (depending on metabolic activity); a volume of 1.0 ml of the complete mixture of the above reagents is added to each 4.0 ml of culture medium.

Dose Selection: Toxicity of the test chemical is determined prior to assessment of mutagenic potential to select doses which produce a maximum of 80 to 90% cell killing. Cytotoxicity is determined by either of the following two methods:

- (1) **Clonal assay** - 200 to 400 CHO cells are exposed to a minimum of five dose levels of the test agent at concentrations from 0.1% to $3 \times 10^{-4}\%$ (by weight or volume, as appropriate) with and without the presence of a metabolic activation system. The number of cells which survive the treatment is determined by counting the number of colonies produced after a 7- to 8-day incubation period ($37^\circ C$) in comparison with the colonies formed by cells treated only with appropriate concentrations of solvent (generally 20 ul/ml).
- (2) **Growth Inhibition** - 5×10^5 cells in 25 cm^2 culture flasks are treated for 5 hours with a minimum of five test concentrations both with and without S9 metabolic activation. Following treatment the cells are rinsed, fresh F12-D5 medium is added and the flasks are incubated for an additional 18 to 24 hours. Cytotoxicity is determined by comparing the relative number of cells in control (untreated cells) and in cells treated with various concentrations of the test agent.

If no cytotoxicity is evident at the highest concentrations in the cytotoxicity tests, the test is either repeated at higher concentrations, or mutation testing is performed with a greater number of treatment flasks starting at higher dose levels. If marked toxicity is evident even at the lowest dose, the cytotoxicity test is repeated at a concentration range of 3×10^{-4} to 3×10^{-6} percent by weight or volume, as appropriate.

Dose levels which are moderately toxic but permit survival of at least 10 to 20% of the cells, in comparison to the solvent control, are selected as the maximum dose, and at least four additional one-half dilutions are tested for induction of mutations. If cytotoxicity data are equivocal, a total of 5 to 8 one-half dilutions of the selected, maximum concentration are used to treat cells; but only the highest five concentrations which permit survival of a sufficient number of cells are assessed for mutation induction.

Chemical samples are sterilized by membrane filtration when microbiological tests indicate this is required to assure sterility. Liquid test agents are tested on a percentage by volume basis. Solid chemicals are dissolved in an appropriate solvent by making a 10 to 20% stock solution (by weight) and subsequent dilutions are made from this stock on a volume/volume basis.

Treatment with Test Chemicals: For tests of chemicals which may act directly without incorporation of an S9 metabolic activation system, 5×10^5 cells are inoculated 20 to 24 hours prior to treatment into 25 cm² culture flasks containing F12-D5 medium and incubated at 37°C in a 5 to 6% CO₂ atmosphere. Appropriate concentrations of the test agent or control chemicals are added to the cells and cultures are treated for 5 hr at 37°C. The medium and test agents are removed by suction, cells are rinsed once or twice and fresh F12-D5 medium is added. The cells are allowed a period of 20 to 24 hours of recovery from treatment before survival is determined. Treatment of cells for testing of chemicals which require metabolic activation for mutagenic capacity is performed identically with the procedure above, with the exception that F12 medium without serum and containing 1.0 ml of S9 activation mixture per 4.0 ml of medium is employed.

Determination of Cytotoxicity: The relative survival of treated cells, in comparison to solvent controls, is determined one day after the exposure to the test agents. The level of cytotoxicity is often correlated with the mutation frequencies induced by known chemical mutagens. Thus, excessive cytotoxicity may kill both normal cells and mutants and may depress the actual mutation frequencies; insufficient cytotoxicity may indicate an insufficient concentration of the test agent was employed. The colony-forming potential of 100 to 200 treated cells is used as the measure of treatment-induced cytotoxicity.

Survival values which indicate the cytotoxic effects of the test agents are included in reports in tabular form. Statistical analyses are not performed on these data, since they are only useful to assess whether appropriate doses were employed and are not used to calculate mutation frequencies.

Determination of Mutant Induction: On days 1, 3 and 6 (or alternatively 1, 4 and 6) after treatment with the various test agents, approximately 5×10^5 cells are subcultured in 100 mm tissue culture dishes in F12-D5 medium and incubated at 37°C in a 5 to 6% CO₂ atmosphere. After a total of 7 days to allow "expression" of the mutant phenotype, cells are dissociated with 0.05 to 0.075% trypsin, counted and plated at a concentration of 2.5×10^5 /dish in four culture dishes (1×10^6 total cells) which each contain 5 ml of F12-D5 (TG) selective medium. At this time, cells are diluted and 100 cells/dish are added to four culture plates containing F12-D5 medium (without TG) to assess viability (plating efficiency) of the treated cell population and to determine the surviving fraction. All cultures are then incubated for an additional 6 to 8 days to allow growth of cells; medium is then discarded and colonies are fixed and stained for counting. The number of colonies in selection plates and in the viability test are counted by electronic methods, checked by manual counts and data are recorded both as total mutants, mutants/ 10^6 total cells and mutants/ 10^6 viable cells.

Statistical Analyses: Uniform statistical procedures to evaluate in vitro mutation data have not been developed. The distribution of mutation frequencies from historical controls in at least two laboratories indicates that the frequency distribution and variances encountered do not justify the use of parametric analyses unless data is transformed before application of standard parametric tests. Analysis of mutation frequencies in the CHO test follow the procedure of Irr and Snee (Reference 4) which employs the Box-Cox Transformation (Reference 5) to transform data before parametric analyses. The mutation frequency for each plate is increased by 1.0 (to eliminate zeros) and raised to the 0.15 power. Experience with historical negative control data in our laboratory indicates that a normal probability distribution of the data suitable for parametric analyses is achieved by this transformation. Parametric analysis of mutation data by the Student's t-test is performed with the transformed data. The degree of statistical significance for the mutation values are indicative of a difference from the concurrent solvent control, but these statistical indicators must be viewed conservatively until additional historical control data are available.

Interpretation of Data: The criteria for interpretation of the test results as a positive or negative response depend upon both the level of statistical significance from the concurrent control and the evidence of a dose-response following treatment. When a definite dose-response relationship is not evident but one or more marginally significant values are obtained, a careful examination of the data from the concurrent positive and negative controls and comparisons to historical control data are used to evaluate the possible significance of the responses. Historical control data indicate that a spontaneous mutation frequency in CHO cells of approximately 4 to 5 mutants/ 10^6 viable cells, with a range of 0 to 25 mutants/ 10^6 viable cells, can be obtained in the absence of mutagenic treatment. Statistical comparisons against unusually high or low spontaneous controls are subjectively scrutinized in respect to the above variability.

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APPENDIX II

Determination of Sister Chromatid Exchange (SCE) Frequencies
in Chinese Hamster Ovary (CHO) Cells In VitroTheoretical Basis

Exchanges of genetic material between the individual arms of a chromosome (i.e. sister chromatids) are thought to arise from breakage and physical interchanges in the DNA of a cell during cell division. An increase in the frequency of such interchanges between sister chromatids can be observed in cells treated with physical or chemical mutagenic agents, or in cells exposed to many suspect or proven human carcinogens. Thus, analysis of SCE frequencies in cells treated with a test agent has been suggested as a sensitive screening test for potential mutagenic/carcinogenic chemicals.

The method used in our study to visualize SCE's in CHO cells grown in culture is based on the procedure described by Perry and Wolff (1974). A standard concentration of 3.0 ug/ml of bromodeoxyuridine (BrdU) was used in the growth medium to allow a visualization of SCE's after two cell divisions in the presence of BrdU. Staining of chromosomes with 5.0 ug/ml of 33258-Hoechst fluorescent dye, exposure to light and Giemsa staining was used to differentiate chromatids for SCE analysis.

Methods

Cell Culture Procedures: Chinese hamster ovary (CHO) cells were obtained from Abraham Hsie at Oak Ridge National Laboratory with the designation CHO-K1-BH4-D1 (referred to simply as CHO for report purposes). CHO cells are maintained in active growth by 2 to 3 weekly subcultures into fresh antibiotic-free, Ham's F12 (modified) medium fortified with 10% (v/v) of heat-inactivated fetal bovine serum and lacking hypoxanthine and thymidine. Cell concentrations are determined routinely with a Coulter® Model-F electronic cell counter calibrated with a precounted suspension of latex beads. All cell culture procedures prior to final harvesting of cells for chromosome preparations are performed under aseptic conditions in a laminar flow, biohazard hood. Presence of Mycoplasma cell contaminants is determined using a fluorescent microscopic assay employing Hoechst 33258 dye.

For treatments with test chemicals without S9 metabolic activation, modified F12 medium is used with 50 units/ml of penicillin, 50 ug/ml streptomycin and 5% (v/v) of heat-inactivated, dialyzed fetal bovine serum (F12-D5). Identical medium but without serum is used for treatments incorporating an S9 metabolic activation system.

Positive and Negative Controls: Sterile water or glass-distilled dimethyl sulfoxide (DMSO) are the usual solvents used for test chemicals and the respective solvent is tested as a control at the maximum concentration used to add the test agent. Dimethylnitrosamine (DMN) and ethylmethanesulfonate (EMS) are used as positive control mutagens for tests with or without the addition of an S9 metabolic activation system, respectively. Results from treatments with concurrent control agents are used as a basis of comparison and for demonstrating the sensitivity and stability of the SCE test system. Comparison of concurrent control values with historical controls is used to delineate the range of acceptable variations in the test system.

Metabolic Activation: Rat liver S9 homogenate (prepared from Arochlor 1254 induced, Sprague-Dawley, male rats) is purchased from Litton Bionetics, Kensington, MD. Each lot of liver homogenate is prescreened for activity in our laboratory before use in the testing program. The complete S9 metabolic activation system contains the following: 8 μ moles/ml $MgCl_2$, 33 μ moles/ml KCl, 5 μ moles/ml KCl, 5 μ moles/ml glucose-6-phosphate, 4 μ moles/ml NADP-oxidized form (nicotinamide adenine dinucleotide phosphate), 100 μ moles/ml Na_2HPO_4 and between 500 to 4000 μ g/ml of S9 protein (depending on metabolic activity). A volume of 1.0 ml of the complete mixture of the above reagents is added to each 4.0 ml of culture medium.

Dose Selection: Toxicity of the test chemical is determined prior to assessment of mutagenic potential to select doses which produce a maximum of 80 to 90% cell killing. Cytotoxicity is determined by either of the following two methods as part of the CHO mutation testing procedure:

- (1) Clonal assay - 200 to 400 CHO cells are exposed to a minimum of five dose levels of the test agent at concentrations from 0.1% to 3×10^{-4} % (by weight or volume, as appropriate) with and without the presence of a metabolic activation system. The number of cells which survive the treatment is determined by counting the number of colonies produced after a 7- to 8-day incubation period ($37^\circ C$) in comparison with the colonies formed by cells treated only with appropriate concentrations of solvent (generally 20 μ l/ml).
- (2) Growth Inhibition - 5×10^5 cells in 25 cm^2 culture flasks are treated for 5 hours with a minimum of five test concentrations both with and without S9 metabolic activation. Following treatment the cells are rinsed, fresh F12-D5 medium is added and the flasks are incubated for an additional 18 to 24 hours. Cytotoxicity is determined by comparing the relative number of cells in control (untreated cells) and in cells treated with various concentrations of the test agent.

If no cytotoxicity is evident at the highest concentrations in the cytotoxicity tests, the test is either repeated at higher concentrations, or mutation testing is performed with a greater number of treatment flasks starting at higher dose levels. If marked toxicity is evident even at the lowest dose, the cytotoxicity test is repeated at a concentration range of 3×10^{-4} to 3×10^{-6} percent by volume.

Dose levels which are moderately toxic but permit survival of at least 40 to 50% of the cells, in comparison to the solvent control, are selected as the maximum dose, and at least four additional one-half dilutions are tested for induction of mutations. If cytotoxicity data are equivocal, a total of 5 to 8 one-half dilutions of the selected, maximum concentration are used to treat cells; but only the highest five concentrations which permit survival of a sufficient number of mitotic cells with SCE staining, are evaluated for SCE induction.

Chemical samples are sterilized by membrane filtration when microbiological tests indicate this is required to assure sterility. Liquid test agents are tested on a percentage by volume basis. Solid chemicals are dissolved in an appropriate solvent by making a 10 to 20% stock solution (by weight) and subsequent dilutions are made from this stock on a volume/volume basis. 001029

Treatment With Test Chemicals: Testing of chemicals for direct mutagenic action (without S9 metabolic activation) is performed first. For chemicals with clearly positive mutagenic capabilities by direct action, testing with metabolic activation is generally not performed.

For testing direct acting chemicals for SCE induction, between 1 to 2 x 10⁶ cells are plated into 75 cm² culture flasks in F12-D5 medium at least 20 hrs prior to treatment and incubated at 37°C in a 5 to 6% CO₂ atmosphere. Appropriate concentrations of the test agent or control chemicals are added to the cells and 3 ug/ml BrdU is added to all flasks. Cells are treated with test agents for 5 hrs, media is then removed by suction, cells are rinsed with buffered, physiological salt solution and fresh medium containing 3 ug/ml BrdU is added for at least 24 hrs of additional incubation at 37°C to allow two rounds of cell division. Cells are harvested and chromosomes are prepared for SCE staining.

Treatment of cells for testing of chemicals which require metabolic activation for mutagenic effectiveness is performed similarly as for treatments without activation, except for three modifications:

1. Before treatment with the test agents, F12-D5 medium is removed and F12 medium without serum is added.
2. S9 metabolic activation mixture is added to each flask (including solvent and positive controls) before addition of test agents.
3. Cells are treated for a total of 2 hrs (rather than 5 hrs) and then incubated for 38 to 42 additional hours before harvest for chromosome preparation.

Preparation of Chromosomes: Colceid® (0.1 ug/ml) or Colchicine (0.2 ug/ml) is added to culture flasks 1 to 2 hrs prior to harvesting to arrest cells in mitosis. Cells are then removed from flasks, after a brief incubation with 0.01% DIFCO trypsin, suspended in 0.075M KCl (hypotonic) solution and incubated for 15 to 20 min at 37°C. Cells are centrifuged, fixed with 3 or 4 changes of Carnoy's fixative (3:1 methanol acetic acid) and chromosome spreads are prepared from cells suspended in a small volume of fixative. One slide/dose level is prepared, but fixed cells are saved if needed for preparation of additional slides.

Chromosomes are stained for SCE's by treatment with 5.0 ug/ml of Hoechst 33258 dye for 20 min, rinsed in distilled water, immersed in Sorenson's buffer and exposed to a high intensity sunlamp for 15 to 30 min., as required. Irradiated chromosomes are stained in Gurr's Giemsa (diluted 1:25 with water), rinsed in water and dried before application of coverslips.

Examination of SCE's: All slides are coded and read in a blind fashion without indication of the specific treatment or concentration of the test agent. The number of chromosomes and the number of SCE's in a minimum of 15 cells are recorded for each dose level. The mean number of SCE/cell and SCE/chromosome are calculated and recorded. Slides are decoded only after examination of all slides in the experiment has been completed.

Statistical Analyses: Data are analyzed by appropriate parametric statistical procedures which follow BRRC standard operating procedures for analyses of data. Significance values and the statistical test employed are shown for data summarized in tabular form.

Interpretation of Data: The criteria for evaluation of a positive or negative response depend both on the level of statistical significance and subjective analyses of concurrent and historical control data. The key determinant is whether a dose-dependent increase in SCE's is induced by the test agent. When no clear dose-response relationship is evident and when one or more responses of marginal statistical significance are obtained, a careful examination of the data in comparison to the concurrent controls and historical data base is necessary. Testing may be repeated to clarify unusual responses, if data for the concurrent positive or negative controls suggest a defect in the original experiment. Overall assessment will also rely on corroborating data from the other tests in the testing battery. Clearly positive responses will include any of the following: (i) Doubling in the SCE frequency at a minimum of two of the five concentrations tested; (ii) Statistically significant responses of $p < 0.05$ at three concentrations or at 2 concentrations if $p < 0.01$; (iii) Induction of a statistically significant, dose-related increase in the number of SCE.

General References

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APPENDIX III

Unscheduled DNA Synthesis (UDS) in Hepatocytes from Rat LiverTheoretical Basis

Chemicals may interact with both the cellular components and the genetic material of a cell (e.g. DNA and RNA) because of their electrophilic nature or by conversion into reactive electrophiles by the metabolic enzymes of the cell. Damage to the DNA of a cell can result in cell death, mutation or, theoretically, carcinogenic transformation. Studies of agents which are capable of reacting and damaging the cellular DNA have suggested that such methods may be useful as a sensitive screening test for detecting potential mutagenic/carcinogenic chemical properties.

Detection of the relatively small amounts of DNA damage induced by chemical treatment requires a cellular system in which normal, semi-conservative DNA replication, which occurs during cell division, is inhibited. The system employed for the present study uses a suspension culture of primary hepatocyte cells isolated from rat liver according to the general methods of Seglen (1973) and Williams (1976). Hepatocytes do not normally divide in the minimal culture medium employed and stimulation of "unscheduled" incorporation of radioactive DNA precursors can be detected by scintillation spectrometry. The stimulation of incorporation of tritiated thymidine into both purified hepatocyte nuclei and DNA is used as the indicator of chemically induced DNA damage. The amount of unscheduled DNA synthesis (UDS) following treatment is compared with both concurrent positive and negative controls as well as with historical data for similar tests.

Methods

Preparation of Hepatocyte Suspensions: Hilltop-Wistar albino rats are anesthetized with Metafane(R). The abdominal cavity is surgically exposed and 1250 units of heparin is injected intravenously. A catheter is inserted into the portal vein and warm Hanks Balanced Salt Solution (HBSS) is pumped into the vein and through the liver. This first solution contains heparin and EGTA, [ethylene glycol-bis-(beta-aminoethyl-ether)N,N-tetracetic acid], which preferentially chelates calcium; the solution contains no magnesium or calcium. After the liver is blanched, a second solution of HBSS containing 60 units/ml of collagenase is perfused. This solution is pumped through the liver until the liver is digested. The liver is then removed and the cells are freed in cold medium 199 by combing through the lobes with a sterile metal comb. The cell suspension is passed through two nylon meshes to remove cell clumps and the cells are washed once at low centrifugation speed. After resuspension in medium 199, equal volumes of cells and 0.4% trypan blue are mixed together and the cell viability and number of viable cells per ml is determined microscopically.

Preincubation of Hepatocytes: Approximately 2×10^6 viable hepatocytes are added to 5 ml medium 199 containing 10 mM hydroxyurea and 30 mM Hepes (N-2-hydroxyethyl piperazine-N-2 ethane sulfonic acid) buffer. After the cells are dispensed into the tubes, they are placed on a rocker platform and are incubated at 37°C for 1 hour. Although hepatocytes do not normally divide in culture, medium 199 which lacks serum and contains hydroxyurea is used to further block semi-conservative DNA synthesis. Thus, any radioactive thymidine incorporated into the nuclei is expected to result from repair or unscheduled DNA synthesis.

Selection of Doses of Test Chemical: Initially, the following concentrations $\times 10^{-3}\%$ (by volume) are tested: 100, 30, 10, 3, 1, and 0.1. If these concentrations prove to be cytotoxic, or if additional information is available from other in vitro tests as to the proper dose levels, then an appropriate series of concentrations is used over a 3-log range of concentrations.

Treatment of Hepatocytes: After preincubation, 25 microCuries of tritiated thymidine (20 Curies/millimole) is added to each tube. The test chemical and positive controls are diluted in an appropriate solvent and they are then added to each labeled tube. Generally, at least six concentrations of the test chemical over a 3-log range of concentrations are tested and each concentration is run in duplicate. The tubes are returned to the rocker platform for a 2-hour exposure at 37°C.

Positive and Negative Controls: 4-nitroquinoline oxide (NQO), a direct-acting mutagen, which induces UV-type DNA repair and dimethylnitrosamine (DMN), which requires metabolic activation by microsomal enzymes for activity, are run in duplicate as positive control chemicals. The solvent control is run in quadruplicate and consists of 100 to 150 microliters (concentration specified in individual reports) of the solvent used to dilute the sample. Dimethylsulfoxide (DMSO) or water are the usual solvents for test chemicals.

Harvest: At the end of incubation with the test agent, the cells are centrifuged from the medium at 200 x g at 5°C. The cells are rinsed once in 5 ml of cold medium 199 and are resuspended in 0.25% Triton X-100, 5% citric acid and 3 mM MgCl₂, a lysing solution which liberates the nuclei. The nuclei are rinsed once in this solution and resuspended in 0.25 M sucrose, 2.5% citric acid and 3 mM MgCl₂. The nuclei are then centrifuged at 600 x g for 10 min at 5°C and resuspended in 2 ml of the lysing solution.

Determination of Nuclear-Bound Label: To measure the amount of radioactive thymidine incorporated into the nuclei, 0.25 ml of the nuclear suspension is mixed with 1.0 mg of NCS tissue solubilizer in a scintillation vial. Ten ml of Dimilume® scintillation cocktail is added and the radioactive disintegrations per minute (DPM) are determined by counting twice in a scintillation counter for ten minutes. The measured DPM are then used to calculate the DPM/10⁶ viable hepatocytes presented on tables.

Determination of DNA-Bound Label: The amount of radioactive thymidine incorporated into DNA is quantitated in DNA isolated and precipitated from 1.00 to 1.25 x 10⁵ viable hepatocytes. To 1.25 ml of the nuclear suspension, 2.75 ml of 1% sodium dodecyl sulfate (SDS) and 5 mM Ethylenediaminetetraacetic Acid (EDTA) is added to lyse the nuclei. The DNA is precipitated from this solution with 4 ml of ice-cold 10% trichloroacetic acid (TCA) and the sample tubes are incubated at 0°C for at least 30 minutes. The solution is then poured onto Whatman glass fiber filters under vacuum and the tubes and filters are washed twice with cold 5% TCA. Finally, each filter is rinsed once with methanol, dried and placed in a scintillation vial. The filters are incubated at 50°C for 1 hour with 1 ml of a diluted solution of NCS tissue solubilizer; prepared by adding 1 part solubilizer to 2 parts of Dimilume® cocktail. Dimilume® is then added to each vial and the vials are counted twice in a scintillation counter for ten minutes.

Statistical Analysis: The average DPM is calculated for each dose level and the controls and final results are expressed as DPM/10⁶ viable hepatocytes. Data are also expressed as a percent of the solvent control for purposes of comparison. The original data are statistically analyzed by the appropriate parametric test, following the BRRC standard procedures for statistical analyses and the test(s) employed is indicated on the respective tables. Comparison between the mean for each dose level with the 95% confidence limits of the historical solvent control may also be used in some cases to assess the potential biological significance of the data. Testing may be repeated to clarify unusual responses, if data with the concurrent controls suggest a defect in the original experiment.

Interpretation of Results: The classification of a chemical as a positive, active agent depends upon the production of a statistically significant, dose-related increase in the amount of UDS activity. If a definite dose-response relationship is not evident, or when a few increases with marginal statistical significance are obtained, comparison of the responses to historical control data provides a meaningful assessment of the possibility for random variations which may be statistically significant only in relation to the concurrent control. A key determinant of the reliability of the UDS data is the detection of a similar response with both DNA and isolated nuclei determined at two or three consecutive concentrations.

General References

1. Lampidis, T. J. and J. B. Little. The Enhancement of UV-Induced Unscheduled DNA Synthesis by Hydroxyurea. Experimental Cell Research, 110, (1977), 41-46.
2. Muramatsu, M. Isolation of Nuclei and Nucleoli. In: Methods in Cell Physiology, Vol. IV. 1970. Editor: D. M. Prescott. Academic Press, New York.
3. Seglen, P. O. Preparation of Rat Liver Cells. III. Enzymatic Requirements for Tissue Dispersion. Experimental Cell Research, 82, (1973), 391-398.
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APPENDIX IV

The available information from the Toxicology Data Bank or from "Material Safety Data Sheets" for this product specify the following chemical and physical characteristics:

Chemical Name:	Diethylenetriamine
Trade Name and/or Synonyms:	Bis-(2-aminoethyl)amine
Molecular Weight:	103.17
Formula:	$H_2NCH_2CH_2NHCH_2CH_2NH_2$
Specific Gravity (@ 20°C):	0.9542
Boiling Point:	206.9°C (760 mm Hg)
Solubility in H ₂ O (% by wt):	Complete at 20°C
Purity:	99.97%, minimum (see comments in additional analytical section)
Vapor Pressure (@ 20°C):	< 1 mm Hg
pH:	Not available
Flash Point:	210°F, tag closed cup
Stability:	Stable
Incompatibility:	Avoid acids
Appearances and Odor:	Water-white liquid; mild ammoniacal odor
Disposal:	Incineration

Protective Measures: Do not get on skin, eyes or clothing. Use rubber gloves, mechanical ventilation or local exhaust when handling. Avoid prolonged breathing of vapor or contact with skin. Safety goggles, organic vapor mask and rubber apron are recommended. Small spills should be flushed with large quantities of water.

Health Hazard: Harmful if inhaled; skin and eye contact causes burns. DETA-HC is a sensitizer which may cause skin rash. Prolonged and repeated breathing of DETA-HC will be irritating and may cause an asthma-like condition. In case of contact, flush with large volumes of water for 15 minutes followed by soap and water. Remove contaminated clothing and wash before wearing again.

ETHYLENEAMINES

PRESHIPMENT ANALYSES OF HIGHER ETHYLENEAMINES
SENT TO MELLON INSTITUTE FOR TOXICOLOGY STUDIES

AUTHOR: P. R. Umberger

DATE: March 14, 1980

PROJECT NO.: 142A40

SUPERVISOR: D. C. Best

FILE NO.: 27479

SUMMARY This memorandum documents the preshipment analyses of the following ethyleneamines recently sent to Mellon Institute for toxicology studies.

- Diethylenetriamine-high purity (DETA-HP)
- Diethylenetriamine-commercial (DETA-Comm)
- Diethylenetriamine-(DETA) - Hearts Cut
- Triethylenetetramine (TETA) - Linear
- Tetraethylenepentamine (TEPA) - Hearts Cut

Pertinent
Sections from
Report for Analysis
of:



← HIRU-47

The high purity and commercial DETA are Union Carbide commercial products while the other samples were prepared in the laboratory from commercial products.

Over the next two years these materials will be used in either mouse skin painting, pharmacokinetics or mutagenicity tests. Elemental, gas chromatograph and infrared analyses were run on the samples as part of a continuing Union Carbide support program in accordance with the study protocols. The commercial DETA for the skin-painting studies will be returned to the Technical Center after 6, 12 and 24 months to provide storage stability data.

INTRODUCTION UCC initiated a program to determine the toxicological properties of the ethyleneamines. While the studies on ethylenediamine are sponsored by an intra-industry group - UCC, Dow Jefferson and Beroi Kemi - the studies on the higher ethyleneamines are funded solely by UCC.

The preshipment analyses of these ethyleneamines previously shipped to Mellon have been reported: (1,2)

Ethylenediamine (EDA)
Diethylenetriamine-high purity (DETA-HP)
N-Aminoethylethanolamine (AEEA)
N-(2-Aminoethyl) piperazine (AEP)
Triethylenetetramine (TETA)
Tetraethylenepentamine (TEPA)
Polyamine HPA #2
HPA #2 Sludge

Analytical data on the materials shipped to Mellon recently are supplied in this report.

DISCUSSION All of the amines shipped to Mellon were either prepared from materials produced, or produced via the reaction of aqueous ammonia with ethylenedichloride in UCC's commercial facilities - either Taft or Texas City. The DETA-HP for the pharmacokinetics study and the DETA-Comm are commercial products, while the DETA-hearts cut, TETA-linear and TEPA-hearts cut were prepared in the laboratory. (See Appendices A, B and C for preparation procedures.)

The elemental analyses of the samples are given in Table I, together with theoretical C, H, N values for DETA and TETA-linear. Tables II and III show the results of gas chromatographic analyses; the scans are in Figures 1-8. Unfortunately, no single method exists to analyze all the ethyleneamines, so three different methods were used: two to analyze the DETA samples and a third to characterize TETA and TEPA.

The first method used to analyze DETA involves derivatization of the amines with N-methyl-bis (trifluoroacetamide) and is a semi-quantitative method for the major component present. However, the necessary accuracy and reproducibility data required to define the method have not been generated yet. (See Appendix D.) The second method for DETA analysis is a direct method and does not involve derivatization; it utilizes a thermal conductivity detector and area percent data are obtained. (See Appendix E.) If the components of a sample have different response factors, or if the sample contains components which do not elute from the column, the analysis will be in error. Although the two methods give different results, two procedures - with their attendant problems - provide better characterization of the amines.

The gas chromatographic procedure used to characterize TETA and TEPA is also a direct method using a thermal conductivity detector and gives area percent data. The column and instrument parameters are described in Appendix F.

Figures 9-13 are the infrared scans of the materials sent to Mellon showing the typical bands expected for the ethyleneamines.

FUTURE WORK The DETA-Comm for the skin-painting studies is to be shipped back to the Technical Center from Mellon for re-analysis at the 6, 18 and final (24) month of testing. Additional quantities of the materials are being stored for 36 months at the Technical Center.

ACKNOWLEDGEMENT C. A. Gibson and A. H. Larch prepared the TETA-linear and TEPA-hearts cut and Jane Montgomery distilled the DETA-hearts cut. Sample analyses were performed by A. H. Larch and W. H. Nelson (G. C.), J. T. Hildebrand (Elemental), L. C. D'Esposito and J. E. Richardson (I.R.)

REFERENCES

1. Umberger, P. R., Memorandum, "Ethyleneamines, Preshipment Analyses of the Ethylenediamine Sent to Mellon Institute for Toxicology Studies", File No. 26942, October 18, 1979.
2. Umberger, P. R., Memorandum, "Ethyleneamines, Analyses of Higher Ethyleneamines Sent to Mellon Institute for Toxicology Studies", File No. 26894, October 8, 1979.

NOTEBOOK REFERENCE

99CAG and 103CAG (Charles A. Gibson)
1PRU and 5PRU (Pamla R. Umberger)
UCC Technical Center
South Charleston, WV


Pamla R. Umberger

Manuscript Date: February 5, 1980

Date Typed: February 15, 1980

PRU:s11

Attachments: 3 Tables
13 Figures
Appendices A, B, C, D, E and F

TABLE I

ELEMENTAL ANALYSES*

<u>Sample</u>	<u>Reference</u>	<u>Analysis (Wt. %)</u>		
		<u>N</u>	<u>C</u>	<u>H</u>
DETA-HP	1 PRU-72	40.87	46.68	13.25
DETA-Comm	1 PRU-50	39.84	47.13	13.14
DETA-Hearts cut	1 PRU-47	40.05	46.12	13.13
TETA-Linear	99CAG-84-2B	37.97	49.72	12.51
TEPA-Hearts cut	103CAG-25-5	35.14	53.16	12.20

	<u>Theoretical Wt. %</u>		
	<u>N</u>	<u>C</u>	<u>H</u>
DETA	40.71	46.53	12.76
TETA-Linear	38.32	49.28	12.40

TEPA is a complex mixture so theoretical C, H, N analyses cannot be calculated.

*Performed by J. T. Hildebrand

NOTEBOOK REFERENCE: 1 PRU and 5 PRU

TABLE II
GAS CHROMATOGRAPH ANALYSES OF DETA SAMPLES (AREA %)

Method Component (%)	DETA-HP 1 PRU-72		DETA-Comm 1 PRU-50		DETA-Hearts Cut 1 PRU-47	
	<u>1</u>	<u>2</u>	<u>1</u>	<u>2</u>	<u>1</u>	<u>2</u>
EDA	0.21	-	0.34	-	Trace	
DETA	98.79	99.18	90.75	92.74	100	99.97
AEP	1.00	0.82	8.91	7.26	Trace	0.03

1 = Derivative Method

See Appendix D.

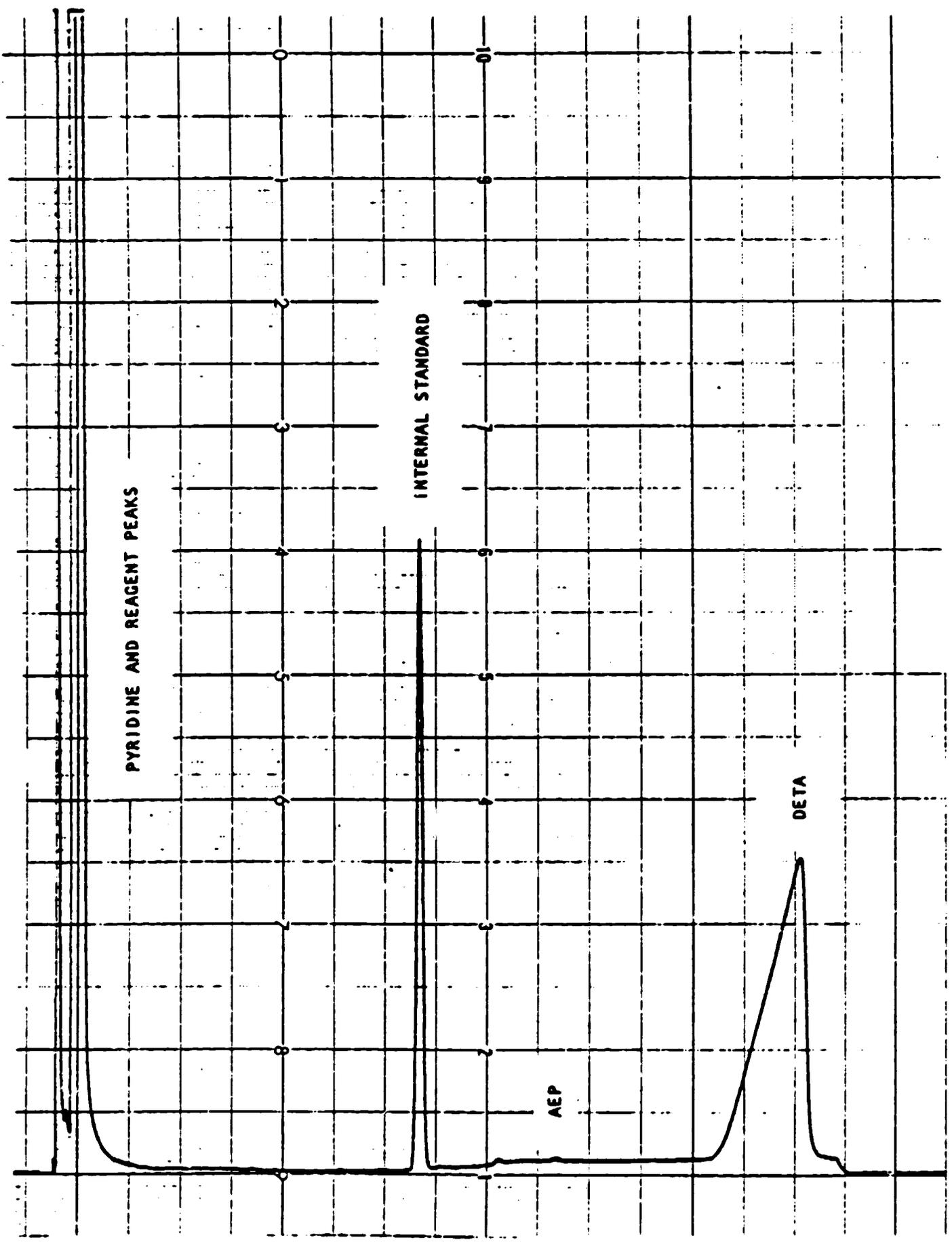
2 = 30M Column

See Appendix E.

NOTEBOOK REFERENCE: 5 PRU

FIGURE 5

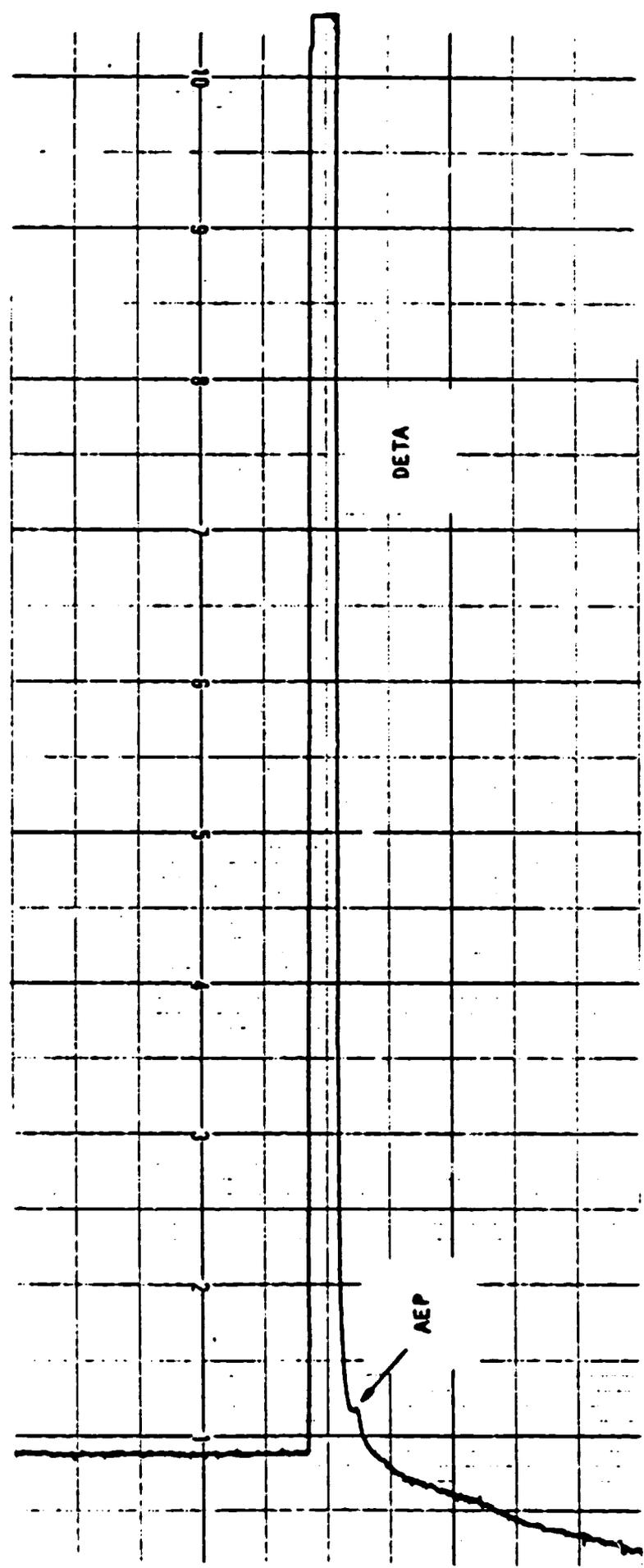
CHROMATOGRAM OF MBTFA DERIVATIVE OF DETA - HEARTS CUT (IPRU-47)



001041

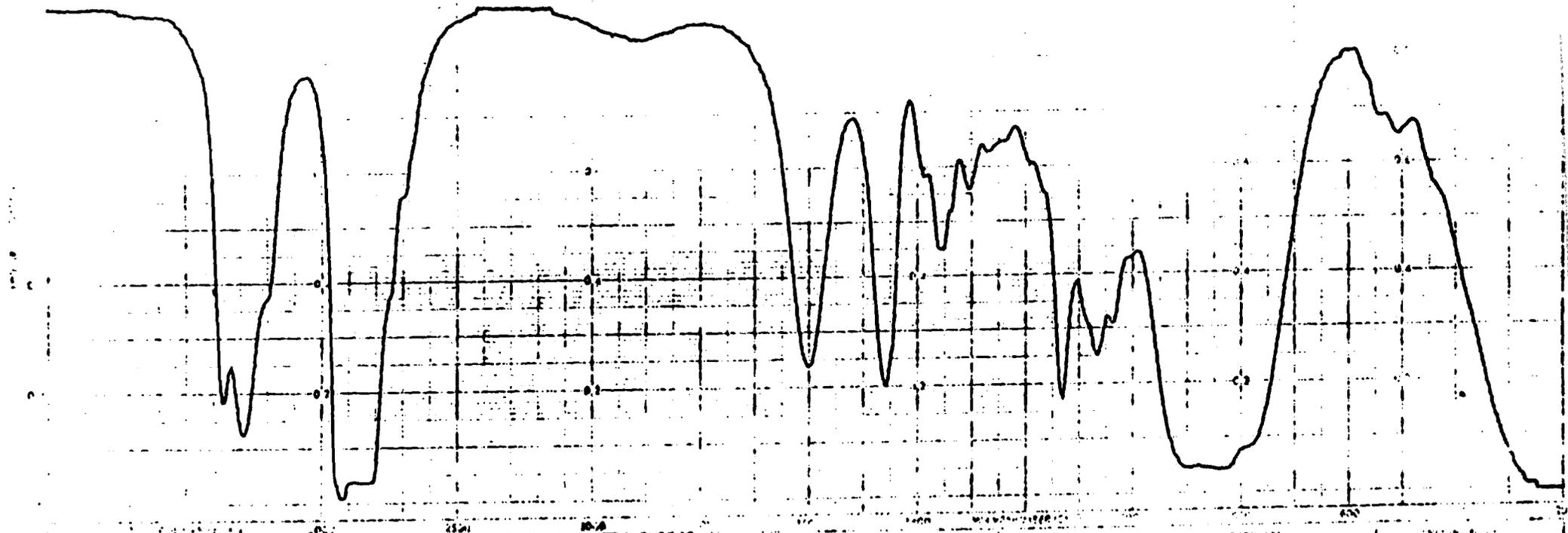
FIGURE 6

DETA-HEARTS CUT (IPRU-47) CHROMATOGRAM (30M COLUMN)



X-1600

FIGURE 11
INFRARED SPECTRA OF BESA-HEARTS CUT (1PRU-47)



Eulynha Gemma X-1600

Umlerger 1PRU-47

APERTURE	1	SCANNING	12	RESOLUTION	1	NO. OF	1
CONCENTRATION	0.0156	WAVENUMBER	1000	WAVENUMBER	1000	DATE	1-10-47
CELL PATH	0.0156	WAVENUMBER	1000	WAVENUMBER	1000	TIME	1-10-47
WAVENUMBER		WAVENUMBER		WAVENUMBER			

001043

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APPENDIX A
ETHYLENEAMINES
PREPARATION OF DETA-HEARTS CUT (CONT)

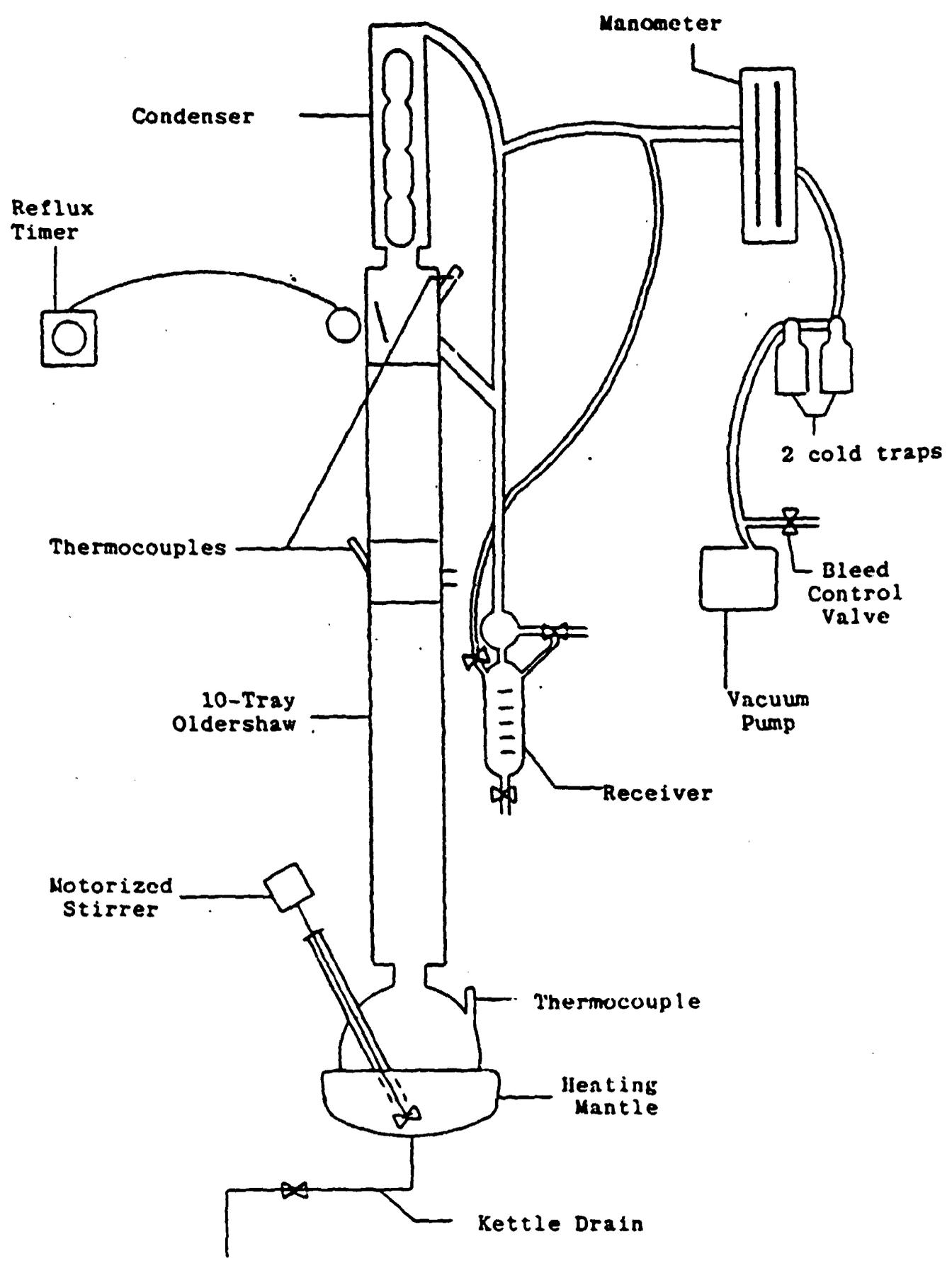
The analyses of the starting material, head and hearts cut and the kettle residues are given below:

<u>Component</u>	<u>Starting Material</u>	<u>Head Cut</u>	<u>Hearts Cut</u>	<u>Kettle Residue</u>
Unknown (1)		0.203	-	0.112
MEA	0.849	1.357	0.393	2.100
Unknown (2)		0.117	-	0.210
Unknown (3)	0.150	0.178	-	0.264
AEP	1.033	2.762	0.167	2.801
DETA	97.468	95.382	99.441	94.514
% of charge		23.53	40.33	33.80

One hundred gram of the hearts cut was shipped to Dr. R. S. H. Yang, Carnegie Mellon Institute.

NOTEBOOK REFERENCE: 1 PRU-47

APPENDIX A
FIGURE 1



Quality Assurance Unit Study Inspection Summary

Test Substance: Diethylenetriamine - Hearts Cut

Study: In Vitro Mutagenesis Studies. 3-Test Battery

Study Director: R. S. Slesinski

The Quality Assurance Unit conducted the following inspections and reported the results to the Study Director and to Management on the dates indicated.

<u>Date</u>	<u>Inspection</u> <u>Type</u>	<u>Date QAU Report Issued</u>	
		<u>To Study Director</u>	<u>To Management</u>
12/1 to 12/4/80	Final Ongoing and Final Report	12/4/80	12/10/80

Daniel L. Geary 12/11/80
Quality Assurance Officer Date

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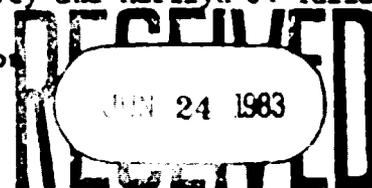
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Pharmacokinetics and Metabolism of Diethylenetriamine in the Rat

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Sponsor: Union Carbide Corporation

Summary

The metabolism and disposition of diethylenetriamine (DETA) in rats was studied with regard to the route of administration and the dosage level. The pharmacokinetics of the compound was studied only with respect to the route of administration. Finally, a comparison of the pharmacokinetics and metabolism was made between DETA and ethylenediamine (EDA).

The route of administration, oral or endotracheal, had little effect on the distribution within the body or the elimination of radioactivity from the rat. Feces and urine were the primary routes of excretion with less than 2% of the administered dose being expired as $^{14}\text{CO}_2$. More than 96% of the recovered dose was eliminated within 48 hours after dosing. In comparing results from animals receiving the compound at 500 mg per kg with those receiving it at 50 mg per kg there was a significant increase in the percentage of radioactivity excreted in the urine and a significant decrease in that eliminated as $^{14}\text{CO}_2$ at the higher dosage level. The route of dose administration did not affect the following pharmacokinetic parameters at the 50 mg/kg level: bioavailability, total clearance or terminal half-life.

A number of major differences were elucidated in a comparison of the metabolism and pharmacokinetics of DETA and EDA. DETA was excreted through the feces to a greater extent than EDA and degraded to $^{14}\text{CO}_2$ to a lesser extent. Tissues contained one-fifth to one-twentieth the concentration of radioactivity from DETA than from EDA when administered at comparable levels. These factors may have important implications in the comparative toxicities of the two chemicals.

Introduction

Ethylenediamine (EDA) is currently being investigated at the Bushy Run Research Center in a comprehensive toxicity testing program. This program consists of chronic and specialized tests along with extensive metabolism and pharmacokinetic studies. Diethylenetriamine (DETA) is structurally related to EDA and is used in similar commercial applications. This compound is also likely to be subjected to a comprehensive toxicological testing program. Metabolism and pharmacokinetic studies have been initiated with the DETA to assist in the design of these tests, particularly with respect to dosages to be used and routes of administration. Information obtained in these metabolism and pharmacokinetic studies will also be useful in the interpretation of results from both the EDA and the proposed DETA toxicology programs.

An attempt was made to conduct the DETA metabolism and pharmacokinetic studies in a manner consistent with that of the EDA studies (Yang, 1978; Yang, et al., 1981). This allowed for a more valid comparison of data obtained from the two chemicals.

Methods

Experimental Schedule: This study was initiated on June 17, 1980 and experimental work was completed by July 17, 1980.

Animals: Male Fischer 344 rats obtained from the Charles River Breeding Laboratories, Portage, Michigan facility were used exclusively in these studies. The animals were 36 days of age upon receipt. They were held in wire-bottom and -front stainless steel cages, three animals per cage, for 9 to 14 days prior to the start of each study. During this time period, the animals were provided free access to ground feed (NIH-07 diet, Zeigler Brothers, Gardner, PA) and tap water supplied by the Municipal Authority of Westmoreland County. Each animal was identified using a toe-clip procedure. A randomization procedure was used to select animals from the shipment groups prior to the initiation of each study, i.e., material balance and pharmacokinetic studies. Some of the rats not selected by this procedure were later used as replacements for animals which died or were not used because of technical difficulties. The remaining culls were sacrificed at the termination of the studies.

Test substance: Diethylenetriamine·3HCl [1,2-¹⁴C] (DETA-¹⁴C) was prepared by the Midwest Research Institute, Kansas City, MO. The material was prepared in a seven step sequence of synthetic reactions ending with the catalytic decomposition of bis(2-phthalimidoethyl)amine to the final product. Details of the synthesis can be found in the MRI final report included in Appendix I. The overall radioactive yield from the starting material, Ba¹⁴CO₃, to DETA-¹⁴C was a disappointingly low 0.73% (2.2 millicuries of final yield); however, this was sufficient to complete the disposition and pharmacokinetic studies. MRI judged the purity of the final product to be greater than or equal to 98% using a thin layer chromatography procedure. Cation exchange chromatography of this material gave a minimum purity of 92%. The radiochemical was assigned BRRC sample number 43-121.

High purity DETA was obtained from P. R. Umberger, Union Carbide Corporation under reference IPRU-72 and assigned a BRRC sample number of 42-186. This chemical has been assigned CAS number 111-40-0.

Dose Preparation: DETA-¹⁴C was mixed with unlabeled DETA in physiological saline solution at concentrations sufficient to dose animals with the appropriate quantity of chemical and amount of radioactivity. The dosing solutions were adjusted to pH 7.4 using hydrochloric acid or ammonium hydroxide, as required. For the material balance study, two dosing solutions were prepared, one for dosing animals at the 50 mg per kg level and the other for the 500 mg per kg group. For the pharmacokinetic study, two solutions were also prepared, one for dosing rats by the oral and endotracheal routes and the second for dosing them intravenously (iv). All solutions were formulated so that each animal would receive approximately 0.25 ml total volume. Standardization was accomplished by weighing samples of the solutions, approximately 10 ul in volume, into 5 ml volumetric flasks, bringing to volume with methanol and counting aliquots of these dilutions for radioactivity.

Dose Administration: All rats were dosed using the weighed syringe procedure. A one ml tuberculin syringe was filled to the 0.25 ml mark with the appropriate dosing solution. The syringe and contents were weighed, the animal dosed and the syringe reweighed. The administered dose was calculated from the weight difference. Animals were orally dosed using a stainless steel animal feeding needle. Endotracheal instillation was accomplished after lightly anesthetizing the rats with Metofane®. The dose was injected into the trachea through a small polyethylene tube. Intravenous administration was carried out via tail vein injection.

Study Design: The material balance study was conducted with 16 rats: four per dosage level and each dosage level administered by two routes of administration. Immediately after dosing, the animals were placed in all-glass Roth metabolism cages. Air was drawn into the cages through Drierite® (W. A. Hammond Drierite Co., Xenia, OH) and Ascarite® (Arthur H. Thomas Co., Philadelphia, PA) traps to remove moisture and CO₂. Air was drawn from the cages through two consecutive traps, each trap containing 2-methoxyethanol and ethanolamine (7:3 v/v) to remove expired CO₂. The flow rate through the system was approximately 250 ml/min. The CO₂ trapping agent was collected and sampled for radioactivity determination at 6, 24 and 48 hours after dose administration.

Urine and feces were obtained separately, the urine chilled with dry ice upon collection. Both were collected at 24 and 48 hours after dose administration with samples of urine taken immediately for radiometric analyses. The feces were frozen until analyses could be conducted. Also, at these same time intervals, 24 and 48 hours postdose, the cages were washed with a solution of 50% acetone in water and samples of the wash solution were also taken for radiometric analyses.

Forty-eight hours after dose administration the rats were anesthetized with Metofane® and blood samples were obtained from the abdominal aorta. After exsanguination, selected tissues (Table 3) were removed, sampled and analyzed for radioactivity. The remaining carcasses were solubilized in 10N NaOH and samples of this solution were also analyzed for radioactivity.

For the pharmacokinetic study, at least six animals per dosage route were surgically implanted with venous cannulae in the right external jugular vein one day prior to dose administration. The surgery was carried out under Metofane® anesthesia. Following an overnight resting period, four rats were selected on the basis of the condition of the cannula and administered DETA. The rats were placed in stainless steel wire metabolism cages and blood samples (approximately 0.2 ml) were taken via the cannulae at 5, 15, 30 and 45 minutes and at 1, 2, 3, 4, 5, 6, 8, 10, 12, 14, 16 and 24 hours. For each blood sample collected, the following operations were conducted: The plasma was separated from the blood cells in microhematocrit capillary tubes (usually two or more capillary tubes/sample) by centrifugation. Part of the plasma was transferred to a tared liquid scintillation vial. The weight of the plasma was obtained and 10 ml of Aquasol II (New England Nuclear) were added to each vial for determination of radioactivity by standard liquid scintillation counting technique.

Analytical Methods: All radioactivity measurements were conducted using a Searle Analytic Mark III Model 6820 liquid scintillation counter. All aqueous samples were counted in liquid scintillation vials containing 10 ml of Aquasol® (New England Nuclear Corp., Boston, MA). One ml samples of cage washing solutions, column eluates and a 0.1 ml sample of urine were all counted in this counting solution. One ml samples of the CO₂ trapping agent and a ten-fold dilution of the solubilized carcass solution were counted in a solution consisting of 2-methoxyethanol, dioxane and xylene (1:1:1, v/v/v) which contained 187 g of naphthalene, 140 g of PPO (2,5-diphenyloxazole) and 1.17 g of POPOP (2,2-p-Phenylenebis-(5-phenyloxazole)].

Samples of feces and tissues were analyzed in a manner similar to that described by Yang et al., (1978). This procedure consisted of solubilizing approximately 50 mg of material with one ml of Soluene 350® (Packard Instrument Co., Downers Grove, IL) in a liquid scintillation counting vial. After standing overnight at 60°C, 0.2 ml of 30% H₂O₂ was added to each vial and they were again left to stand overnight. The following morning 10 ml of Dimilume 30® (Packard Instrument Co., Downers Grove, IL) were added to each vial prior to counting.

Chromatographic Methods: A paper chromatography procedure (modification of Markiw, 1975) was employed for the separation of DETA from other radiolabeled compounds in the plasma. The procedure consisted of banding 10 ul samples of plasma on Whatman #4 paper strips (2 or 4 cm in width by 20 cm in length) and developing them ascendingly in a solvent system of n-butanol, acetic acid and water (4:1:1, v/v) for two hours. The developed strips were cut into 1 cm segments starting 0.5 cm below the origin and extending to the solvent front. Generally 15 sections were obtained from each strip. The radioactivity in each section was determined by liquid scintillation counting. In this system, DETA migrated up the strip with an R_f value of approximately 0.8. EDA has an R_f value in the same system of approximately 0.2. The percentage of total radioactivity present as DETA was lower in plasma obtained at the later times after dosing. This was expected as metabolite concentrations would presumably be higher in these samples. It was also noted that radioactivity in these samples was spread over a wider area of the paper strip. In fact, in some strips no DETA could be detected. These factors led to a greater error in estimating DETA concentrations in plasma at these later time intervals.

Separation of radiolabeled compounds excreted in urine was carried out using cation exchange chromatography. AG-50-X8 (400 mesh or less) cation exchange resin (BioRad Laboratories, Richmond, CA) in the ammonium form was used to achieve these separations. Ammonium chloride solutions were used as the eluting agents. Concentration gradients of these solutions were produced in an apparatus identical to that described by Knask et al., (1965). This apparatus consisted of two graduated cylinders connected at their bases with glass tubing. A stopcock placed in the tubing served to control flow of fluid between the two cylinders. A magnetic stirring bar was used to mix the resulting gradient solution of ammonium chloride formed by allowing the high concentration solution to flow from the second cylinder into the first (which contained ammonium chloride at the lower concentration). This gradient mixture was pumped onto the resin

column. The gradients consisted of 300 ml of each solution in the following pairs: 0.01N to 0.10N and 0.10N to 1.0N ammonium chloride adjusted to pH 9.0 with either NH_4OH or HCl as required. The column was cleared with 500 ml of 2N ammonium chloride solution to complete the chromatograms. Following the 2N elution, at least 500 ml of deionized water and 200 ml of 0.01N ammonium chloride were run through the column prior to the next separation. Ten ml fractions of the column eluent were collected and one ml samples of the first 10 fractions and every other fraction thereafter were analyzed for radioactivity. Results of the radioactivity determinations were plotted as percentages of applied radioactivity against fraction numbers. The column eluent from fractions under the radioactivity peaks in the chromatogram was collected and samples from the resulting pools were analyzed for radioactivity. The percentage of radioactivity in the different chromatogram peaks was determined in this manner.

Anion exchange chromatography was carried out in a manner analogous to that described above with the exception that AG-1-X4 resin in the acetate form was used to achieve separations. Also, in this case, the elution gradients consisted of 0.01N to 0.10N and 0.1N to 1.0N ammonium acetate adjusted to pH 7.0. Only 300 ml of the final 2N ammonium acetate solution was used to clear the columns.

Calculations: Most calculations used in converting original data into meaningful information were conducted using Minitab programs (Ryan *et al.*, 1976) implemented on Carnegie-Mellon University's DEC 20 Computer. Subtraction of background radioactivity from sample radioactivity and conversion of counts per minute (cpm) to disintegrations per minute (dpm) is an integral part of the Searle Mark III liquid scintillation counter's operation. Other values were determined by the following equations:

1. Total dpm (T).
$$T = \text{net dpm per unit weight (or volume)} \times \text{total weight (or volume) of material analyzed.}$$
2. Percentage of dosed radioactivity (PDR).
$$\text{PDR} = (\text{total dpm in fraction analyzed} + \text{dpm dosed}) \times 100.$$
3. Radioactivity concentration in dosing solutions (dpm/mg dosing solution).
$$\text{dpm/mg dosing solution} = (\text{dpm in aliquot dosing solution std.} \times \text{dilution factor}) + \text{mg dosing solution in std.}$$
4. Specific activity DETA (dpm/ug DETA).
$$\text{dpm/ug DETA} = \text{dpm/mg dosing solution} + \text{ug DETA/mg dosing solution.}$$
5. Radioactivity equivalent to ug's of DETA per g (or ml) sample (ug/g).
$$\text{ug/g} = \text{net dpm per unit weight of sample} + \text{specific activity of DETA.}$$

Curve fitting of the pharmacokinetic data was carried out both by hand feathering and by use of the ESTIP curve fitting computer program (Brown and Manno, 1978). Only data from the ESTRIP analysis were used for final calculations. Area under the curve (AUC) was obtained from the program output

and calculated by the trapezoidal rule. This value was corrected to take into account that area from the final sample point to infinity by adding the following correction factor (CF):

$$6. \text{ CF} = \text{DETA plasma concentration of final point} \div \text{terminal rate constant}$$

to the AUC value given by the program. Bioavailability (BA) was calculated from the following equation:

$$7. \text{ BA} = \frac{\int_0^{\infty} \text{AUC}(\text{endotracheal or oral})}{\int_0^{\infty} \text{AUC}(\text{iv})}$$

Mean AUC values for dosage route groups were used in this calculation. Total clearance (TC) was calculated for individual animals using the following equation:

$$8. \text{ TC} = (\text{fraction absorbed} \times \text{total dose in ug}) \div \int_0^{\infty} \text{AUC}$$

where fraction absorbed is equivalent to BA.

Finally, the terminal half-life was calculated by equation 9.

$$9. \text{ Terminal } 1/2 \text{ life} = 0.693 \div \text{terminal rate constant.}$$

Statistical Procedures: Where possible a two way analysis of variance was used to analyze data using dosage route and dosage level as the two main effects. In the case of pharmacokinetics, a one-way analysis of variance (dosage routes being the main effect) was used to analyze the data. The Minitab program was utilized for the two way analyses while the Statistical Package for Social Sciences (SPSS) was used for the one-way analyses (Nie et al., 1975). Bartlett's test for homogeneity of variance was used to determine the validity of these approaches. If the variances were heterogeneous (using the fiducial limit of 0.01) Cochran's t-test was employed to test differences between treatment (dosage route, dosage level) means. Multiple regression analysis (Minitab) was used to analyze radioactivity concentrations over all tissue types. Dosage route, dosage level and tissue type were used as factors in this analysis.

Storage of Data: All raw data, specimens and final report are stored in the BRRC archives.

Results

Material Balance Study: Table 1 contains a summary of rat weights and mean dosages of DETA and radioactivity administered to these animals. Details for individual rats are found in Appendix II. The animal weights ranged from 161 g to 220 g. The dosage level goals for this study were 50 and 500 mg per kg body weight and it can be seen from Table 1 that dosage levels attained were in close agreement with these targets. DETA administered at these levels by either the oral or endotracheal routes did not produce noticeable signs of toxicity. One animal, rat number 313, did succumb to suffocation as a result of a block in the air supply to the Roth metabolism cage. No data for this animal is included in the report. This animal was replaced with rat number 308 which came from the same shipment as the rest of the animals in the study and was administered DETA from the same dosing solution and in an identical manner as the other animals in this treatment group.

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A summary of the radioactivity balance is found in Table 2. Appendix III contains the radioactivity balance for individual rats. Fecal excretion was the major route of excretion followed closely by elimination through the urine. Only a small percentage of the administered radioactivity was recovered as $^{14}\text{CO}_2$ or was retained in the tissues and carcass 48 hours post-dose. No significant differences in the percentages of radioactivity excreted in the urine, feces or CO_2 were observed between animals dosed by the oral route and those dosed endotracheally. There was significantly more radioactivity retained in the carcass of the endotracheally dosed animals. The magnitude of this difference was small, 0.6%, and probably has little biological significance. There was a significantly greater recovery of administered radioactivity in the animals receiving the 500 mg per kg dosage level. This higher recovery is more likely the result of error in the standardization of the dosing solution than it is of a dosage level related effect. The percent recovery ranged from 79% to 104% for individual animals. The animals receiving the 500 mg per kg dosage level excreted a significantly higher percentage of dosed radioactivity in the urine than did the groups receiving the lower dosage level, 41% and 32%, respectively. In contrast, a significantly greater percentage of the dose was eliminated as $^{14}\text{CO}_2$ in the group receiving 50 mg per kg, 1.2% as compared to 0.5%, and retained in the tissues and carcass, 2.5% as compared to 2.0%.

The mean concentrations of radioactivity found in various tissues 48 hours post-dose are listed by dosage level and route of administration in Table 3. The values in this table are expressed as radioactivity equivalent to micrograms of DETA per gram of wet tissue. This method of expression is not meant to imply that the values given are actual concentrations of DETA in these tissues. In actuality, the radioactivity could be present as metabolites; moieties bound to proteins, nucleic acids or other macromolecules or even natural body constituents arising from the fixation of $^{14}\text{CO}_2$. These values do, however, give a relative meaning to the radioactivity concentrations in that they represent the actual mass of DETA from which this radioactivity originated.

Analysis of all tissue data by multiple regression techniques indicated that concentrations of radioactivity in tissues of animals receiving the chemical at 500 mg per kg were significantly higher than in animals receiving it at the lower level. The mean concentration for all tissues was 7.5 times higher in the animals receiving DETA at the higher dosage level. This is slightly lower than the 10-fold difference in dosage level administered. Multiple regression analysis gave no indication that route of administration had any effect on tissue concentrations of radioactivity. Further examination of radioactivity concentrations in lung and trachea tissues gave no indication of an increase in those animals receiving the dose endotracheally. Similarly, examination of esophagus and stomach tissue gave no indication of increased radioactivity concentrations in orally dosed animals. This suggests that there is no direct tissue fixation reaction with this triamine.

The percentage of urinary radioactivity recovered in fractions obtained by cation exchange chromatography are found in Table 4. The data are arranged by route of administration and dosage level. Similar data for individual

animals are recorded in Appendix IV. The chromatograms of radioactivity from the urine of individual animals are found in Appendix V. The radioactivity in the urine was eluted from the columns in three major fractions. These fractions have been designated CV₁, CV₂₂ and CV₃₆; the C representing cation exchange, the V representing the volume of eluting solution given in column volume (subscripted number) representing the number of column volumes of eluting solution in which the fraction appeared. The percentage of radioactivity recovered in a minor fraction, CV₈, which was observed in most urine samples is also included in Table 4. Another fraction designated CV₃₃ was observed in a number of urine samples but had chromatographic characteristics similar to a minor impurity in the labeled DETA preparation. Data for this fraction does not appear in Table 4 nor are data presented for a number of other minor fractions which did not demonstrate consistent patterns among the urine samples. The cation exchange column used in these separations was able to resolve DETA from EDA, the former eluting with 36 and the latter with 29 column volumes of eluting solution. There was no evidence that EDA was present in the urines of the DETA treated animals.

The percentage of radioactivity recovered in fractions CV₁ and CV₂₂ was significantly higher in the 50 mg per kg group while, conversely, the percentage in fraction CV₃₆ was significantly higher in the 500 mg per kg group. The percentage of radioactivity recovered in fraction CV₈ was too small to be meaningful with regard to group differences in a study of this size. A comparison between the rats receiving DETA by the oral route and those receiving it by the endotracheal route revealed no significant differences in the percentage of radioactivity recovered in the major fractions.

A 24-hour fecal homogenate from one rat in each dosage group was also subjected to cation exchange chromatography. These chromatograms are found in Appendix VI. In the animals receiving DETA at 50 mg per kg the majority of radioactivity was recovered in the neutral fraction, CV₁. The remainder of the chromatogram, which was somewhat nondescript in appearance, did not possess the characteristic pattern seen in urine. The chromatograms of fecal homogenate from animals receiving 500 mg per kg, on the other hand, were similar in appearance to the urinary chromatograms. An attempt was also made to obtain the chromatogram of radioactivity in rat plasma (Appendix VII). This was not successful, possibly because of protein interference in the separation process. Characteristics of the radioactivity in the neutral fraction from a urinary chromatogram were investigated using anion exchange chromatography (Appendix VIII). This radioactivity was not retained on the resin indicating that these metabolites do not possess acidic properties.

Pharmacokinetic Study: A summary of data pertinent to weights and dosages received by animals in this study is contained in Table 5. Data for individual animals are found in Appendix IX. The animals used in this study ranged in weight from 160 to 196 g and received a mean dosage level of 49 mg per kg (the dosage level goal was 50 mg per kg). Each animal received approximately 50 microcuries of radioactivity. Three of the rats died after dosage administration, one after receiving an oral dose and the other two after receiving DETA by the intravenous route. The three deaths did not appear to be related to DETA administration, two probably resulted from surgical complications and blood loss, the third from a broken cannula with resulting blood loss which occurred after taking the 6th-hour blood sample.

Mean values for pharmacokinetic parameters are listed in Table 6 by dosage route groups. Values for individual animals are given in Appendix X. The pharmacokinetic parameters were obtained from the analyses of the log linear plots of plasma concentration vs. time after dose administration. These plots, for individual animals, are found in Appendix XI. Using the ESTRIP program, it was found that the data gave results which best fit an equation containing three exponential terms. The solutions to these equations for each set of data points is represented by the solid line in each figure. The choice of the three pharmacokinetic parameters listed in Table 6 is based upon the rationale of Yang et al. (1981). These parameters are independent of a specific pharmacokinetic model and are therefore independent of the assumptions inherent in the mathematical analysis of such models. Because of limitations in the analytical methodology these data did not lend themselves to more sophisticated pharmacokinetic analysis.

The bioavailability parameter is the fraction of perorally or endotracheally administered dose which enters the animals' general circulation. This is determined from the calculated AUC and the assumption that the entire iv dose enters the general circulation. In this study, mean AUC's for each dosage group were used to determine apparent bioavailability since paired observations were not possible. DETA is mostly absorbed into the general circulation when administered either orally or endotracheally, 95% and 90% of the dose, respectively.

Total clearance is an estimate of the efficiency with which an animal can remove a substance from its central compartment. In the present study, this parameter was not affected by route of administration. The terminal half-life is an index of the overall rate of removal of the chemical from the animal. Again, this parameter was not significantly affected by route of administration.

The urine from one of the pharmacokinetic study rats dosed by the iv route was subjected to cation exchange chromatography to determine if this route of DETA administration greatly altered the chromatogram of radioactivity. It can be seen from the chromatogram (Appendix XII) that the pattern is very similar to that seen in the urine of rats dosed by other routes.

Discussion

The results from this study lend themselves to a comparison between the metabolism and pharmacokinetics of DETA in rats and that of its close congener EDA. In comparing the two chemicals, with respect to elimination pathways, a greater percentage of DETA was eliminated through feces than was EDA. At the 50 mg per kg level 40 to 46 percent of radioactivity from DETA was eliminated by this route whereas only 12 to 24 percent of EDA radioactivity was eliminated through feces under similar conditions (Yang, 1978). A similar trend was also observed at the 500 mg per kg level. In the case of urine, the percentage of radioactivity eliminated was somewhat less than that for EDA. Urine still remained a primary pathway of excretion, however. Although radioactivity eliminated as $^{14}\text{CO}_2$ represents a minor pathway (less than 10% of the administered dose), there was a difference between the two chemicals with respect to this route:

A much smaller percentage was eliminated in the case of DETA, 1% vs. 7% to 8% for EDA. The radioactivity remaining in the tissues and carcasses was also much lower in the case of DETA; 2% to 3% of the administered dose as compared to 10% to 20% for EDA. This latter observation is further emphasized in an examination of tissue concentrations of the chemicals. In general, DETA tissue concentrations were one-fifth to one-twentieth those of EDA when administered at similar dosage levels. Furthermore, the target tissues (tissues with the highest concentration of radioactivity) were not the same for the two amines. The major target tissues for EDA, in order, were thyroid, liver, bone marrow, adrenal and kidney, whereas the kidney, liver, bladder, trachea and large intestine contained the highest concentrations of radioactivity from DETA. The route of administration did not appear to affect the ordering of tissues with respect to radioactivity concentration for either compound.

There is some evidence from the results of the disposition study which suggest that saturation levels of DETA were reached at the 500 mg per kg dosage level. This saturation phenomena was suggested by the shift in percentages of radioactivity in the urinary chromatogram fractions. At the 500 mg per kg level, a significantly higher percentage of radioactivity was recovered in the CV₃₆ fraction with correspondingly lower percentages in the CV₁ and CV₂₂ fractions. The CV₃₆ fraction appears chromatographically identical to unchanged DETA. Thus, this pattern of excretion may represent an overloading of DETA metabolizing enzymes with a resulting increase in the excretion of unchanged chemical. A similar dose-related trend was observed in the case of EDA.

The pharmacokinetic investigation of DETA in rats indicated that the compound was absorbed into the general circulatory system when administered by either the oral or endotracheal routes. This has also been shown to be the case with EDA at the 50 and 500 mg per kg dosage level (Yang et al., 1981). The efficiency with which rats could remove EDA from their central compartment, as indicated by the total clearance, was higher than that with which they could remove DETA. This suggests that, in the rat, EDA is more rapidly metabolized than DETA. The radioactive balance studies conclusively demonstrate that 48 hours after dosing less radioactivity from DETA remains in the animal than radioactivity from EDA. Coupled with results from the pharmacokinetic studies, this suggests that the parent amines are more readily eliminated from rats than are metabolites of these amines. These observations may have relevance to the chronic toxicity of these two compounds; particularly since they suggest that metabolism may be responsible for the persistent radioactivity which accumulates in animal tissue. Mechanistic studies could provide further information regarding this point.

Conclusions

When DETA was administered to rats either orally or endotracheally, it was readily absorbed into the general circulatory system. The primary routes of elimination of radioactivity from the compound were feces and urine with smaller amounts leaving by way of ¹⁴CO₂. Forty-eight hours after dosing more than 96% of the recovered radioactivity had been eliminated from the animals.

The concentration of radioactivity in tissues of rats receiving DETA at the 500 mg per kg level were 4 to 10 times higher than in rats receiving the compound at 50 mg per kg. There were no significant differences in the concentrations in tissues of rats receiving the compound by different routes of administration. The higher level of dose administration did produce a shift in the pattern of radioactivity in urinary chromatograms with a greater percentage associated with a fraction having characteristics similar to those of the unchanged amine.

There were no apparent biologically important differences in material balance parameters (excretion pattern, tissue distribution patterns or urinary chromatographic profiles) or in the pharmacokinetic parameters among animals receiving DETA by different routes of administration. These observations would give metabolic support for predicting risks from an inhalation exposure based upon data from a chronic feeding toxicity study.

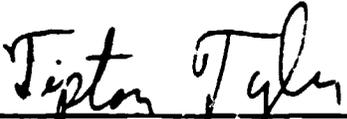
Although DETA and EDA both appear to be readily absorbed, the former is excreted more readily in the feces, retained to a lesser degree in the carcass and not as readily degraded as the latter. At similar dosage levels, tissues had one-fifth to one-twentieth the concentration of DETA than that of EDA. These facts may have important ramifications in the toxicities of the two chemicals.

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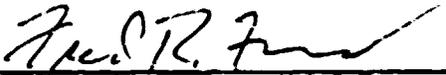
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7. Yang, R. S. H. (1978). "Pharmacokinetics and Metabolism of Ethylenediamine - Interim Report No. 1: Material Balance Studies of Ethylenediamine in the Rat Following Single Oral, Endotracheal and Intravenous Dosing." BRRC Report #41-144.
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Table 1
Experimental Parameters for DETA Material Balance Studies

Parameter	Oral		Endotracheal	
	50 mg/kg	500 mg/kg	50 mg/kg	500 mg/kg
Mean Rat Weight (g)	169	202	171	202
Weight Range (g)	161-175	191-220	163-177	197-206
Mean Quantity DETA Received (mg)	8.16	103	8.46	97.2
Mean Quantity Radioactivity Received (uCi)	7.5	10.2	7.8	9.6
Mean Dosage Level Adminis- trated (mg/kg body weight)	48.2	511	49.5	481

WPC/1138-2

Table 2
Radioactivity Balance in Animals Dosed with ¹⁴C Labeled DETA

	Oral		Endotracheal	
	50 mg/kg	500 mg/kg	50 mg/kg	500 mg/kg
<u>Percentage of Dosed Radioactivity</u>				
Urine	31	43	32	40
Feces	46	44	40	45
CO ₂	1.1	0.5	1.3	0.6
Cage Washings	5.4	10	8.0	14
Carcass	2.0	1.8	3.1	2.1
Total	85	100	84	102

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Table 3
Concentrations of Radioactivity From ¹⁴C Labeled DETA in Rat Tissues

Tissue	Oral		Endotracheal	
	50 ug/kg	500 ug/kg	50 ug/kg	500 ug/kg
	<u>Radioactivity Equivalent to ug DETA per g Tissue</u>			
Adrenal	1.2	9.0	1.6	8.9
Bladder	1.9	13.6	2.0	14.3
Blood	0.9	5.3	0.9	5.9
Blood Cells	1.1	8.1	1.1	8.4
Bone Marrow	1.0	8.6	1.7	8.7
Brain	0.2	1.1	0.2	1.1
Cecum	1.0	11.7	1.5	11.1
Esophagus	1.5	12.1	1.8	9.6
Fat	0.3	1.3	0.2	1.3
G. I. Contents	0.4	7.0	1.1	27.4
Heart	0.9	5.4	1.1	5.4
Kidney	2.3	25.2	3.0	26.1
Large Intestine	1.2	12.7	2.1	15.7
Liver	2.6	13.6	3.0	17.4
Lung	1.1	10.5	1.4	8.5
Lymph Nodes	1.3	10.9	1.2	9.8
Muscle	0.6	4.8	0.7	4.6
Pancreas	0.6	3.4	0.7	5.2
Plasma	0.4	2.4	0.5	2.6
Salivary Glands	0.7	5.9	1.0	5.7
Skin	0.7	10.0	1.4	9.1
Spleen	1.1	10.8	1.8	11.0
Small Intestine	2.1	5.3	0.8	5.9
Stomach	0.8	9.2	1.3	6.8
Testes	0.4	3.7	0.5	3.5
Thymus	1.2	11.8	1.8	11.1
Thyroid	1.1	6.6	1.4	7.3
Trachea	1.4	13.0	2.6	8.5

WPC/1138-2

000017

Table 4
Percentage of Recovered Radioactivity Under Peaks of Urinary Chromatograms

Peak Designation	Oral		Endotracheal	
	50 mg/kg	500 mg/kg	50 mg/kg	500 mg/kg
CV ₁	15	3.8	18	4.8
CV ₈	1.8	1.0	2.0	0.8
CV ₂₂	20	13	17	14
CV ₃₆	48	74	44	71

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Table 5
Experimental Parameters for DETA Pharmacokinetic Study

<u>Parameter</u>	<u>Oral</u>	<u>Endotracheal</u>	<u>Intravenous</u>
Mean Rat Weight (g)	178	183	185
Weight Range (g)	160-196	181-186	178-189
Mean Quantity DETA Received (mg)	8.33	8.41	10.3
Mean Quantity Radioactivity Received (uCi)	30.3	30.6	48.7
Mean Dosage Level Administered (mg/kg body weight)	46.8	45.8	55.6

WPC/1138-1

Table 6
DETA Pharmacokinetic Parameters in Rats

<u>Parameter</u>	<u>Oral</u>	<u>Endotracheal</u>	<u>Intravenous</u>
Bioavailability	0.95	0.90	1
Total Clearance (ml hr ⁻¹)	134	138	174
Terminal Half-Life (hr)	13.9	7.1	5.7

WPC/1138-2

APPENDIX I

Final Midwest Research Institute Report on Synthesis of ^{14}C Labeled DETA

(9 Pages)

000021

June 3, 1980

Carnegie-Mellon Institute of Research
 Bushy Run Laboratories
 4400 Fifth Avenue
 Pittsburgh, Pennsylvania 15213

Attn: Dr. Tipton R. Tyler

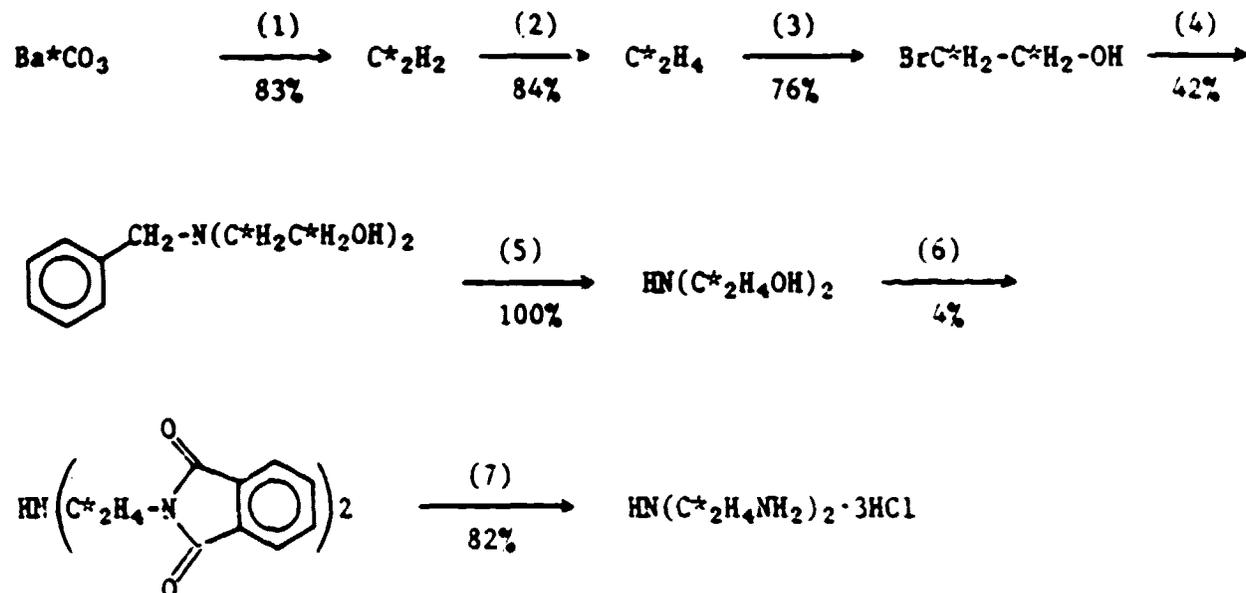
Subject: Final Report, MRI Project No. 4874-C, "Synthesis of ^{14}C -Labeled
 Diethylenetriamine (DETA- ^{14}C)."

Gentlemen:

This letter report briefly describes the results of the subject
 synthesis effort, performed for Carnegie-Mellon under Purchase Order No.
 PR-90318, dated January 25, 1980. As a result of this effort MRI shipped
 to Carnegie-Mellon 2.2 mCi of DETA- ^{14}C on April 7, 1980.

Experimental

The overall reaction sequence used in this synthesis is shown
 below. The yields given reflect actual results obtained.



Step (1): Ba/fusion; (2): CrCl_2/HCl ; (3): NBA/ H_2SO_4 ; (4): N-Benzylethanol-
 amine; (5): $\text{H}_2/10\% \text{Pd/C}$; (6): 1) SOCl_2 ; 2) potassium phthalimide/DMF;
 (7): $\text{HCl}/\text{H}_2\text{O}$.

Dr. Tipton R. Tyler
Carnegie-Mellon Institute of Research 2

June 3, 1980

Step 1

A total of 5.55 mM (300 mCi) of $\text{BaCO}_3\text{-}^{14}\text{C}$ was fused with Ba metal filings in argon. To the fusion product (Ba^{14}C_2) water was added and the acetylene gas was collected in a spiral trap on a vacuum manifold. Drying over P_2O_5 yielded 2.34 mM (250 mCi) of acetylene-(1,2- ^{14}C).

Reference: MRI-79-244-25

Step 2

A solution of 32 g of chromous chloride in 122 ml of 6N hydrochloric acid was added slowly to 30 g of mossy zinc under an argon gas atmosphere. After all of the zinc was dissolved (2 hr), acetylene (10 mM, 250 mCi) was introduced by vacuum transfer. The reaction mixture was stirred for 42 hr at ambient temperature. The gas was dried (Ascarite), affording 8.4 mM (210 mCi, 84%) of pure ethylene-1,2- ^{14}C (see Figure 1).

Reference: MRI-79-244-26

Step 3

A solution of 3.8 g of N-bromoacetamide in 80 ml of 0.0038 M sulfuric acid was degassed (in vacuo). The ethylene (8.5 mM, 210 mCi) was transferred under static vacuum to the reaction vessel containing the N-bromoacetamide solution, and the reaction mixture was stirred for 66 hr at ambient temperature. The ethylene bromohydrin was extracted into chloroform and dried (MgSO_4). Removal of the solvent at 760 mm afforded 159 mCi (76%) of product.

Reference: MRI-79-244-27

Step 4

In a typical run 5 mM (62 mCi) of bromoethanol-1,2- ^{14}C and 10 mM of N-benzylethanolamine were heated for 9 hr at 80-90°C. The resulting reaction mixture was diluted with 3 ml of acetone, and enough ether was added to obtain an oily precipitate. The cloudy solution was pipetted from the oil, which was subsequently dissolved in acetone; the product was then reprecipitated with ether. Purification of the combined product was done by TLC using eight Whatman PLKF 1,000 μ plates (hexanes:acetone, 12:5; developed three times), affording 26 mCi of 98% radiochemically and chemically pure product (see Figure 2). This reaction was repeated several times.

Reference: MRI-79-244-7

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June 3, 1980

Step 5

A 10 mM (67 mCi) portion of N-benzyl diethanolamine-[1,2-¹⁴C] was dissolved in 30 ml of ethanol and reduced in a Parr shaker using 200 mg of 10% palladium on charcoal catalyst and 45 lb of hydrogen gas pressure. After 2 hr the reduction was complete; the catalyst was filtered and the solvent was removed (in vacuo), affording 67 mCi of $\geq 96\%$ pure diethanolamine-1,2-¹⁴C (see Figure 3).

Reference: MRI-79-244-29

Steps 6 and 7

A 10 mM (67 mCi) portion of N-diethanolamine-1,2-¹⁴C was dissolved in 3 ml of chloroform and cooled in an ice bath. A solution of 8 ml of freshly distilled thionyl chloride in 8 ml of chloroform was added slowly at first, then later on much faster (~ 5 min). The reaction mixture was refluxed for 8 min to dissolve all solids and then cooled in an ice bath. The solvent was removed in vacuo, ice chips were added to the oily residue, and the mixture was made basic by addition of 20% KOH solution. The bis(2-chloroethyl)amine-[1,2-¹⁴C₂] was extracted into ether and dried (K₂CO₃/MgSO₄). To the ether solution were added 10 ml of DMF and 12 mmoles of potassium phthalimide. After the ether was removed (in vacuo), the reaction mixture was heated for 5 hr at 150-155°C. The reaction mixture was then diluted with 80 ml of water and stored in the refrigerator for 60 hr. The resulting crystals were filtered, washed with water, then ether, then dried (in vacuo), affording 260 mg of product. The crude product was recrystallized from ethanol to furnish 18 mg (1.8 mCi, SA: 8.45 mCi/mM) of white crystals, m.p. 177-179°C (lit. 178-180°C). The mother liquor was purified by preparative TLC (tetrahydrofuran:hexanes:ether, 3:1:1), affording an additional 38 mg (0.9 mCi) of pure product. The total yield was 116 mg (2.7 mCi) of $\geq 97\%$ radiochemically pure product as judged by TLC (see Figure 4).

Reference: MRI-79-244-14

Step 8

A mixture of the bis(2-phthalimidoethyl)amine (116 mg, 2.7 mCi) and dilute hydrochloric acid (7.5 cc, 1:1 by volume) was heated under reflux for 6 hr. The product was chilled and the phthalic acid separated by filtration, yielding 2.2 mCi of product solution. The material was diluted with a cold standard to a specific activity of 6 mCi/mole. The solution was evaporated (in vacuo), and the residue was dissolved in water and filtered through a Millipore filter, affording 2.2 mCi of product judged to be $\geq 98\%$ pure by TLC (silica gel, methanol:conc. NH₄OH, 1:1). Other TLC systems resulted in considerable trailing; i.e., no really suitable solvent system was identified for the hydrochloride. However, the TLC of the diphtalimidoethylamine-(1,2-¹⁴C) is considered to be quite representative of the purity of the final product.

Dr. Tipton R. Tyler
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Reference: MRI-79-244-23

If you have any questions and/or comments regarding this report,
please do not hesitate to call.

Sincerely,

MIDWEST RESEARCH INSTITUTE



J. Richard Heys, Ph.D.
Senior Radiochemist

Approved:



William P. Duncan, Ph.D.
Head, Organic and Radiochemical
Synthesis Section

Brockman

INFRARED SPECTROPHOTOMETER

SPECTRUM NO 2
PATH 100 mm
ANALYST Ed Clark

DATE 1-23 80

SAMPLE Ethylene (1,2-¹⁴C)

SOURCE MK1-79-244-26

SOLVENT

CONCENTRATION 60 mm tube PHASE gas

WAVELENGTH IN MICRONS

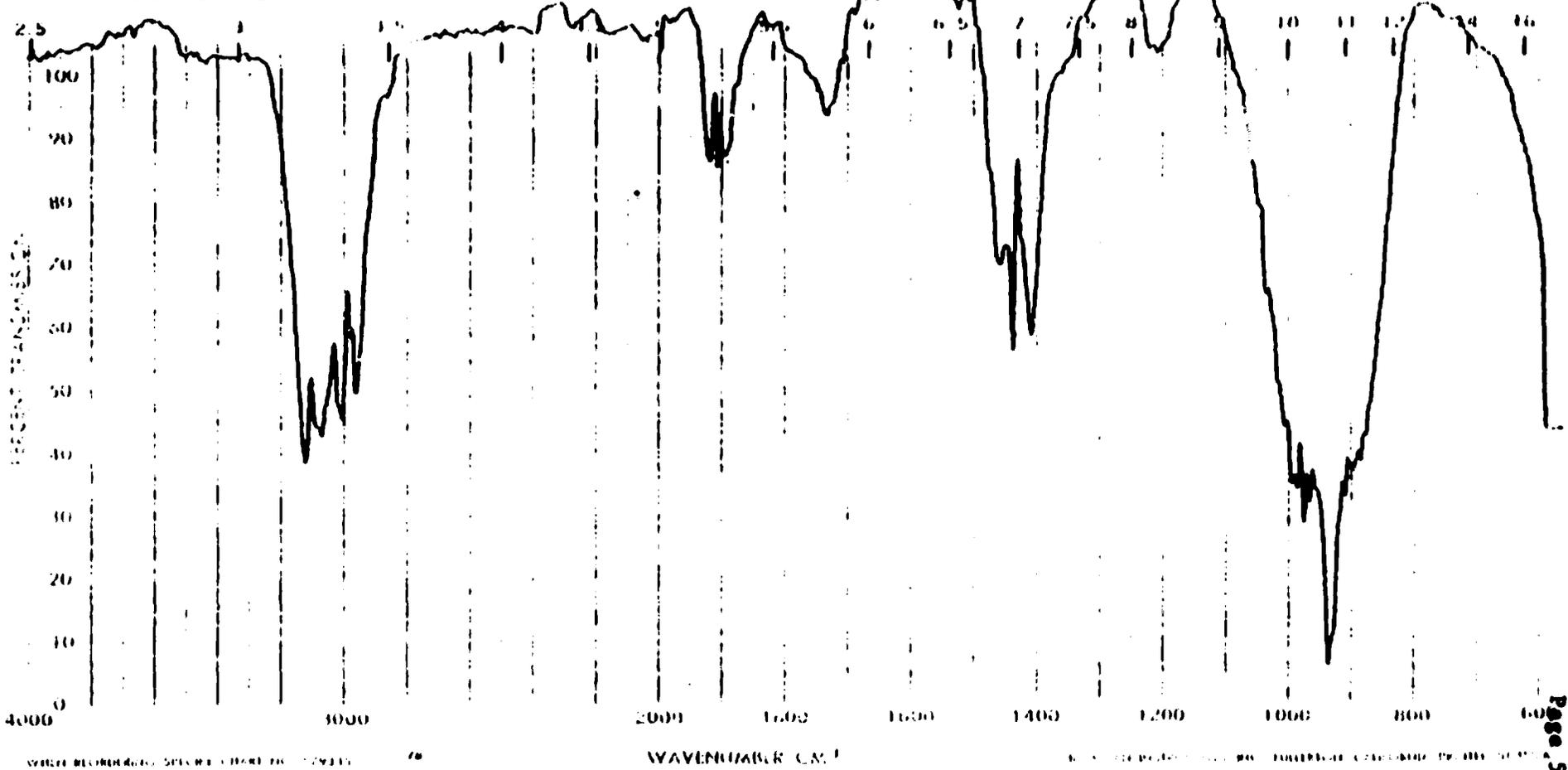


Figure 1 - IR Spectrum of Ethylene-(1,2-¹⁴C)

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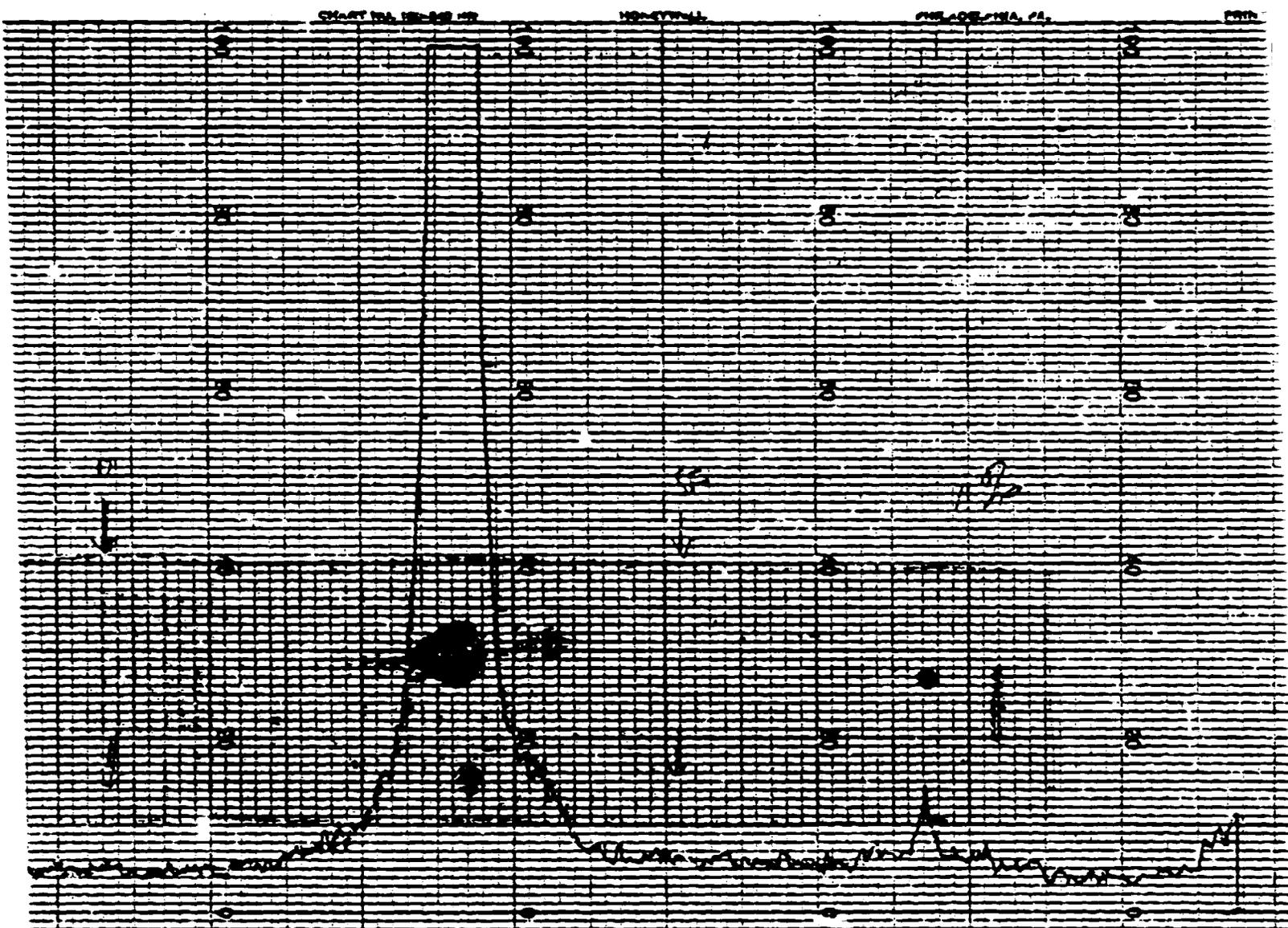


Figure 2 - Radiochromatogram Scan/Autoradiography Film of
N-Benzyl-diethanolamine-(1,2-¹⁴C);
Silica Gel/Chloroform:Acetone:conc. Ammonium Hydroxide (1:8:1)

000627

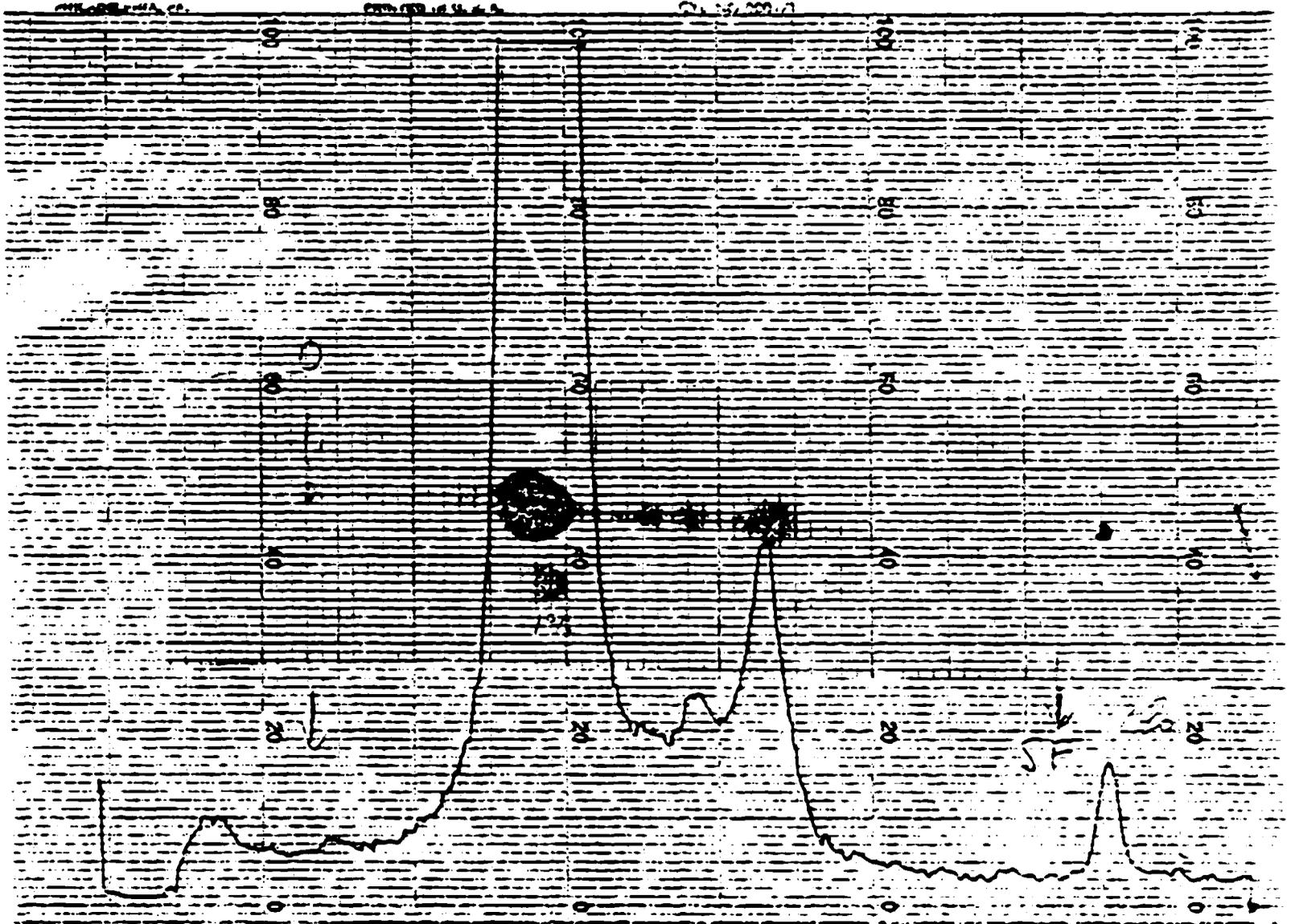


Figure 3 - Radiochromatogram Scan/Autoradiography Film of
Diethanolamine-(1,2-¹⁴C); Silica Gel/
Isopropyl Alcohol:conc. Ammonium Hydroxide:Chloroform (3:1:1)

700128

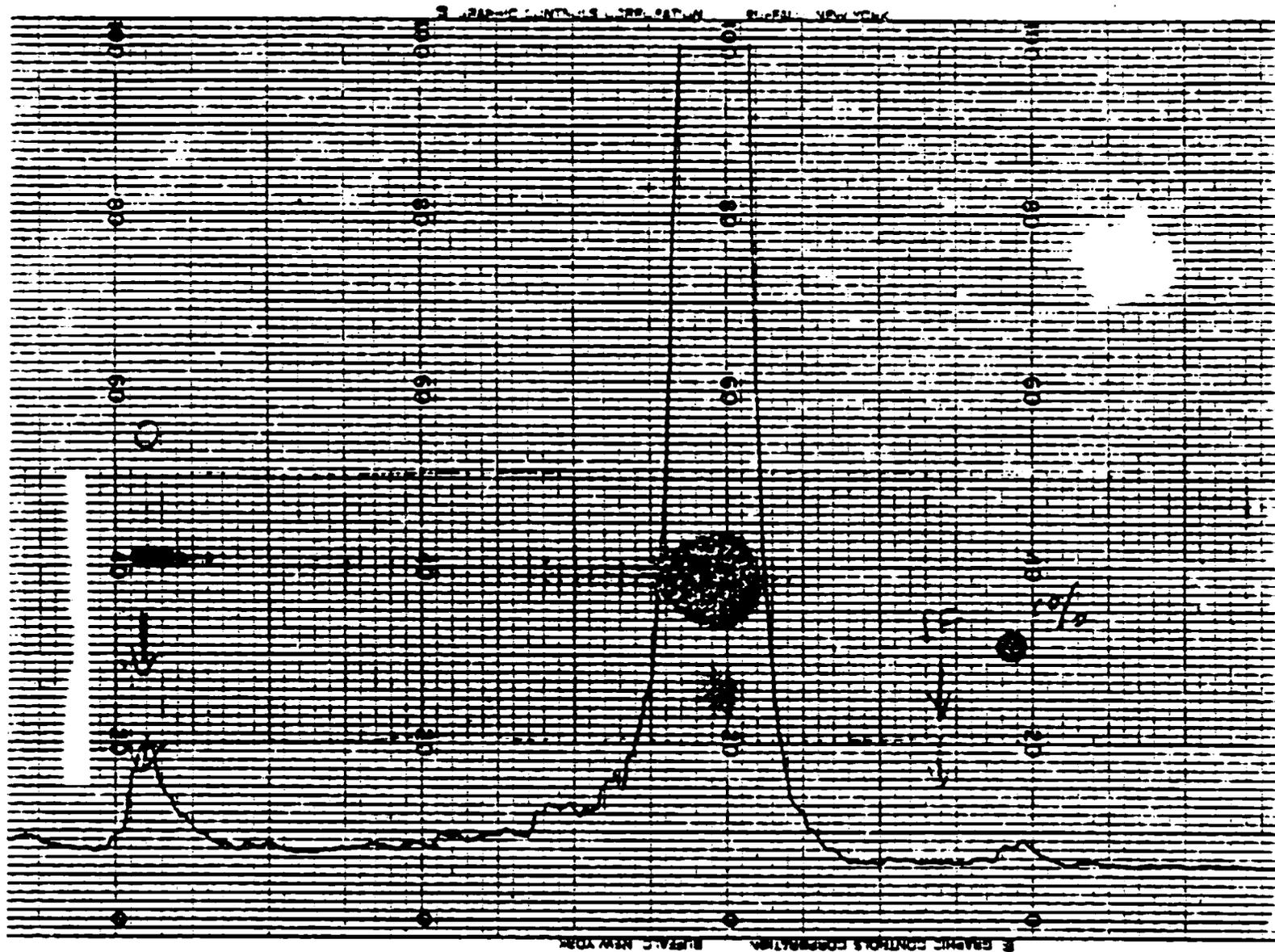


Figure 4 - Radiochromatogram Scan/Autoradiography Film of
3,3'-Diphthalimidodiethylamine-(1,2-¹⁴C);
Silica Gel/Tetrahydrofuran:Ether:Hexanes (4:1:1)

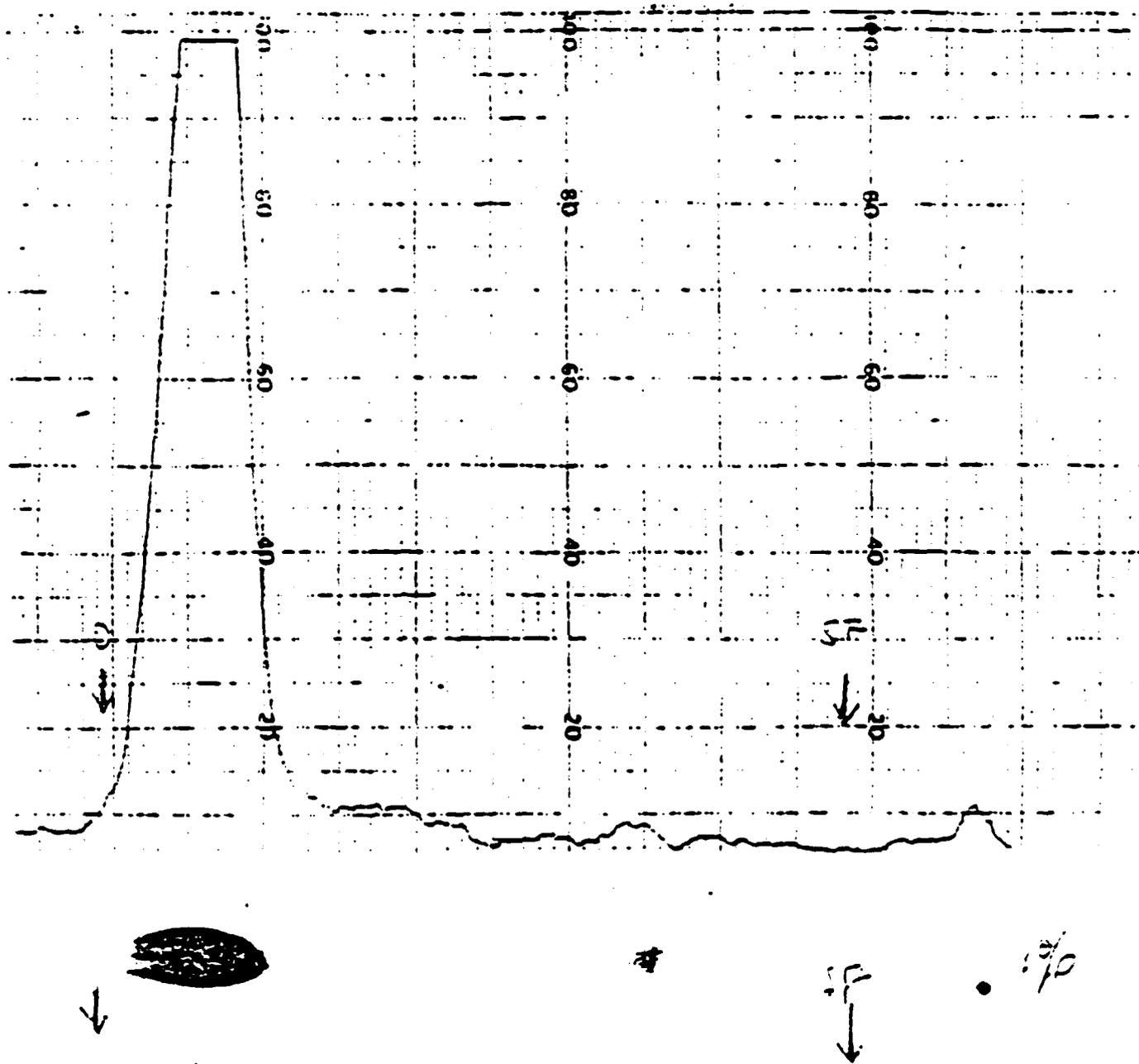


Figure 5 - Radiochromatogram Scan and Autoradiography Film
of Diethylenetriamine·3 HCl [1,2-¹⁴C]
TLC: Silica Gel/MeOH:Conc. NH₄OH (1:1)

APPENDIX II

Experimental Parameters for Individual Animals
used in DETA Material Balance Study

(1 Page)

000031

Experimental Parameters for Individual Animals
used in DETA Material Balance Study

<u>Animal Number</u>	<u>Dosage Route</u>	<u>Dosage Level (mg/kg)</u>	<u>Animal Weight (g)</u>	<u>Dose DETA Received (ug)</u>	<u>Radioactivity Received (uCi)</u>
291	Oral	45.0	171	7.70	7.1
299	Oral	50.7	161	8.17	7.5
305	Oral	46.9	175	8.21	7.5
311	Oral	50.2	170	8.54	7.9
Mean \pm SD ^a		48.2 \pm 2.72	169 \pm 5.91	8.16 \pm 0.34	7.5 \pm 0.33
292	Endotracheal	47.2	176	8.31	7.6
303	Endotracheal	51.5	163	8.39	7.7
307	Endotracheal	48.5	177	8.58	7.9
316	Endotracheal	50.9	168	8.55	7.9
Mean \pm SD		49.5 \pm 2.02	171 \pm 6.68	8.46 \pm 0.13	7.8 \pm 0.15
294	Oral	536	196	105	10.4
296	Oral	520	200	104	10.3
309	Oral	468	220	103	10.2
315	Oral	519	191	99.2	9.9
Mean \pm SD		511 \pm 29.5	202 \pm 12.7	103 \pm 2.54	10.2 \pm 0.22
290	Endotracheal	480	202	96.9	9.6
295	Endotracheal	489	197	96.4	9.6
301	Endotracheal	478	206	98.4	9.8
308	Endotracheal	477	203	96.9	9.6
Mean \pm SD		481 \pm 5.5	202 \pm 3.74	97.2 \pm 0.87	9.6 \pm 0.10

^aSD = Standard Deviation

WPC/1138-3

000032

APPENDIX III

Radioactive Balance for Individual Animals in DETA Material Balance Study

(1 Page)

000033

Radioactivity Balance for Individual Animals
used in DETA Material Balance Study

Animal Number	Dosage Level and Route of Administration	Percentage of Dosed Radioactivity					
		Urine	Feces	CO ₂	Cage Washings	Carcass	Total
291	50 mg/kg Oral	32.8	45.8	1.23	2.38	2.51	84.7
299		28.0	46.2	1.02	2.23	1.81	79.3
305		30.2	42.7	1.13	12.5	2.02	88.6
311		33.3	47.6	0.99	4.51	1.68	88.1
Mean		31.1	45.6	1.09	5.41	2.01	85.2
SD ^a		2.46	2.08	0.10	4.86	0.37	4.27
292	50 mg/kg Endotracheal	45.9	27.8	1.43	4.39	3.23	82.7
303		26.8	42.0	1.10	8.58	2.76	81.3
307		28.2	40.5	1.42	8.41	3.82	82.3
316		28.0	48.7	1.15	10.6	2.48	90.9
Mean		32.2	39.7	1.28	8.00	3.08	84.3
SD		9.15	8.74	0.18	2.60	0.59	4.43
294	500 mg/kg Oral	29.1	45.3	0.56	18.4	1.80	95.2
296		48.1	44.9	0.54	7.43	1.74	103
309		48.5	44.3	0.51	5.85	1.82	101
315		45.8	42.4	0.48	8.86	1.95	99.4
Mean		42.9	44.2	0.52	10.1	1.84	99.6
SD		9.26	1.30	0.04	5.63	0.10	3.26
290	500 mg/kg Endotracheal	43.6	52.1	0.48	5.75	1.84	104
295		42.4	41.7	0.50	16.2	1.75	103
301		26.6	52.6	0.74	20.5	2.44	103
308		48.5	35.5	0.54	14.0	2.40	101
Mean		40.3	45.5	0.57	14.1	2.12	103
SD		9.50	8.35	0.12	6.21	0.36	1.23

^aSD = Standard Deviation

WPC/1138-5

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APPENDIX IV

Percentages of Recovered Radioactivity Under Peaks of Urinary Chromatograms
for Individual Animals

(1 Page)

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Percentage of Recovered Radioactivity Under Peaks
of Urinary Chromatograms for Individual Animals

Animal Number	Dosage Group	Fraction			
		CV ₁	CV ₂	CV ₃	CV ₄
291	Oral, 50 mg/kg	11	1	16	55
299	Oral, 50 mg/kg	14	2	19	53
305	Oral, 50 mg/kg	20	2	26	33
311	Oral, 50 mg/kg	14	2	21	50
Mean \pm SD ^a		15 \pm 3.8	1.8 \pm 0.5	20 \pm 4.2	48 \pm 10.0
292	Endo, 50 mg/kg	18	2	17	37
303	Endo, 50 mg/kg	20	2	20	42
307	Endo, 50 mg/kg	23	2	20	35
316	Endo, 50 mg/kg	11	2	11	64
Mean \pm SD		18 \pm 5.1	2.0 \pm 0.0	17 \pm 4.2	44 \pm 13.3
294	Oral, 500 mg/kg	4	1	13	77
296	Oral, 500 mg/kg	4	1	13	72
309	Oral, 500 mg/kg	4	1	12	74
315	Oral, 500 mg/kg	3	1	13	72
Mean \pm SD		3.8 \pm 0.5	1.0 \pm 0.0	13 \pm 0.5	74 \pm 2.4
290	Endo, 500 mg/kg	5	0	14	72
295	Endo, 500 mg/kg	5	1	13	72
301	Endo, 500 mg/kg	5	1	15	68
308	Endo, 500 mg/kg	4	1	13	72
Mean \pm SD		4.8 \pm 0.5	0.8 \pm 0.5	14 \pm 1.0	71 \pm 2.0

^aSD = Standard Deviation

WPC/1138-3

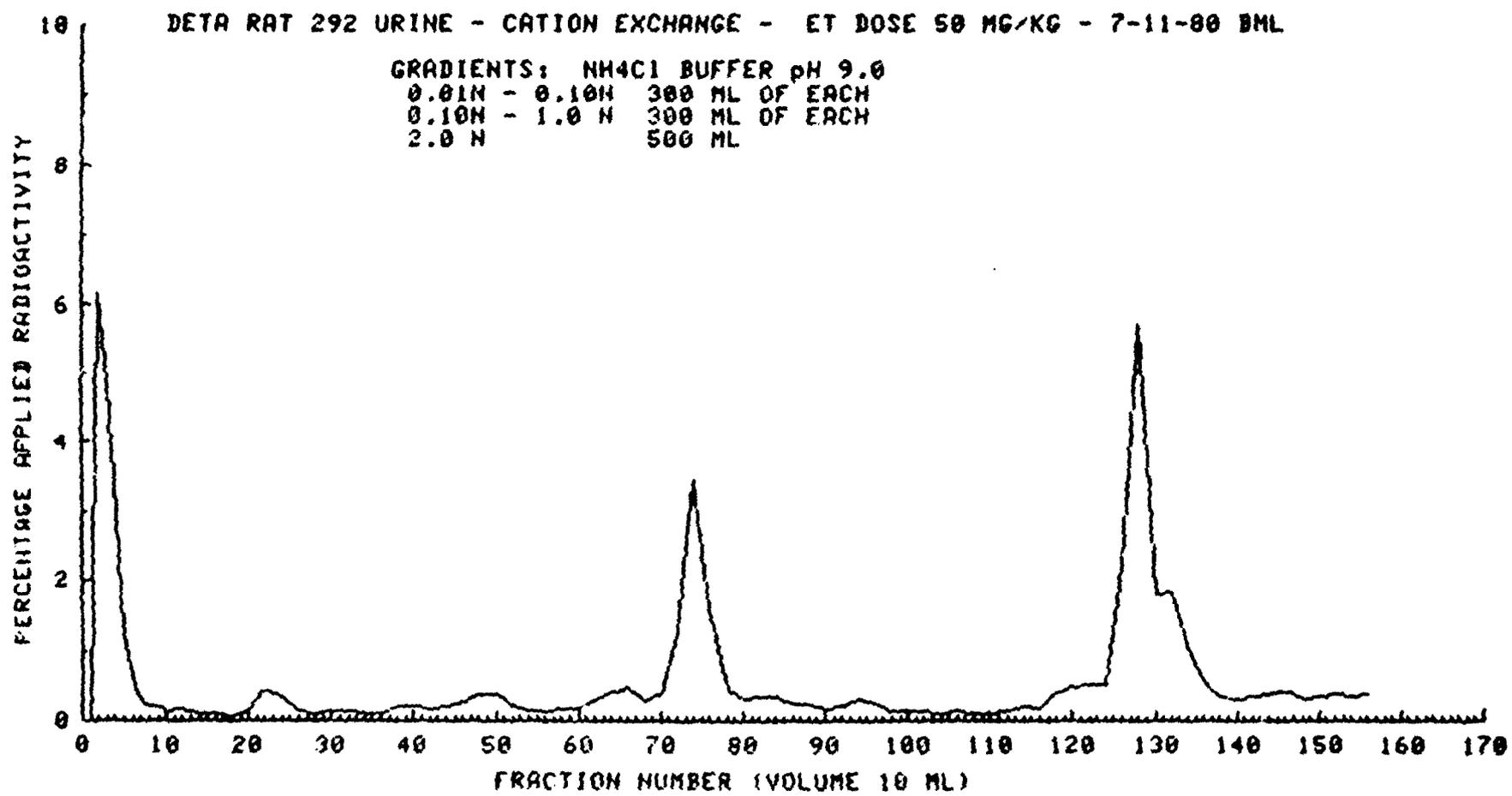
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APPENDIX V

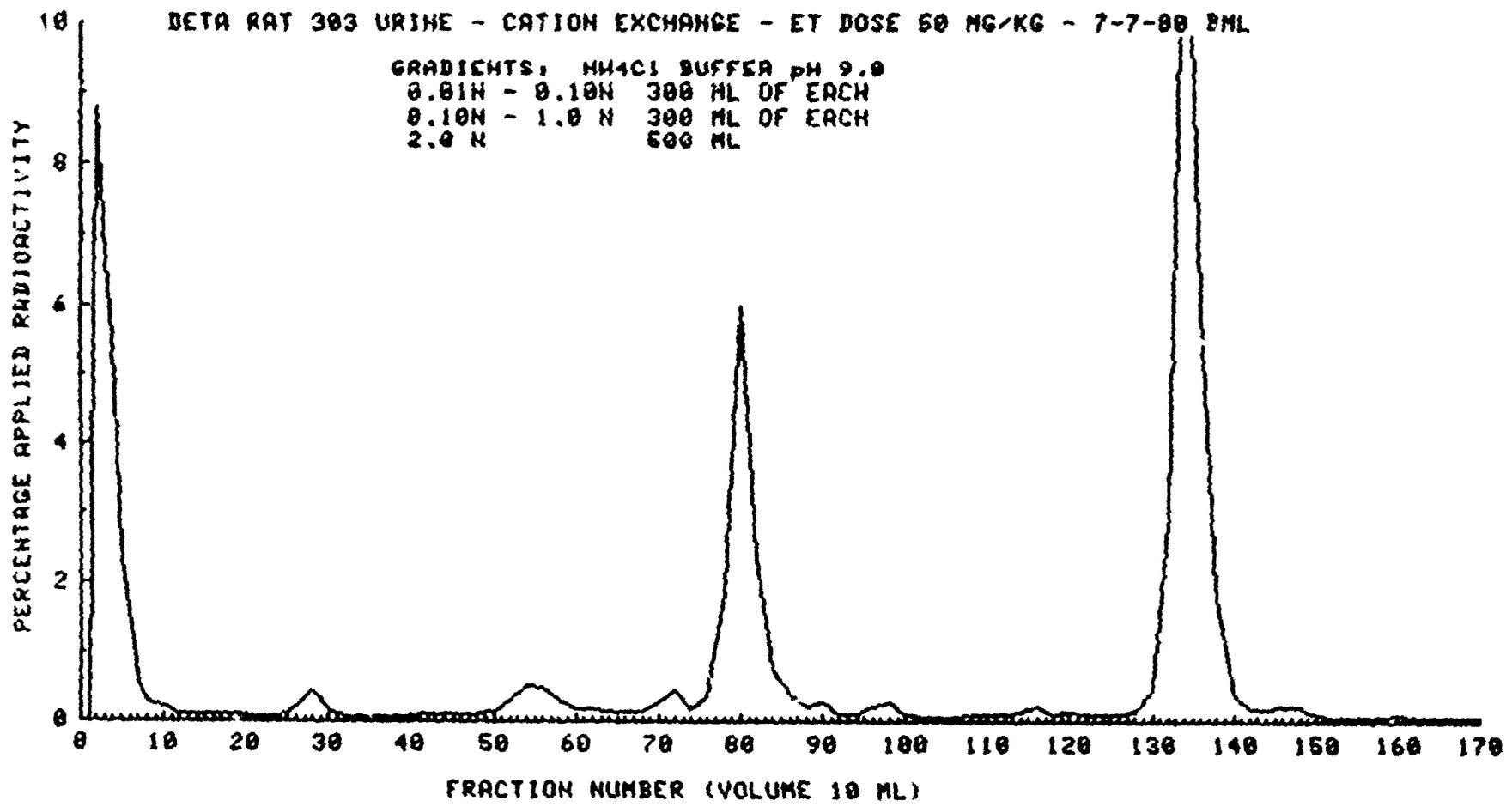
Chromatograms of Urinary Radioactivity for Individual Animals

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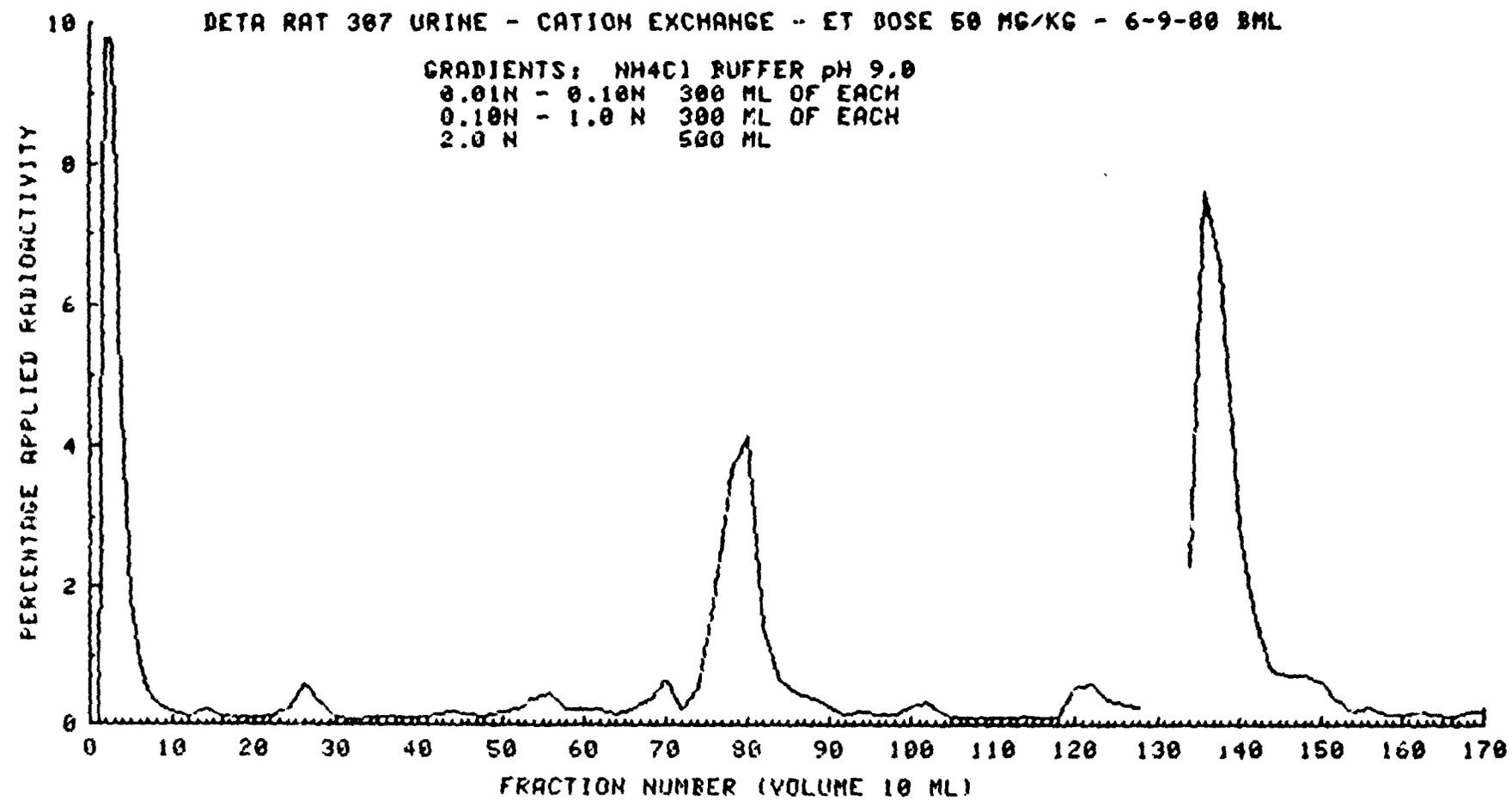
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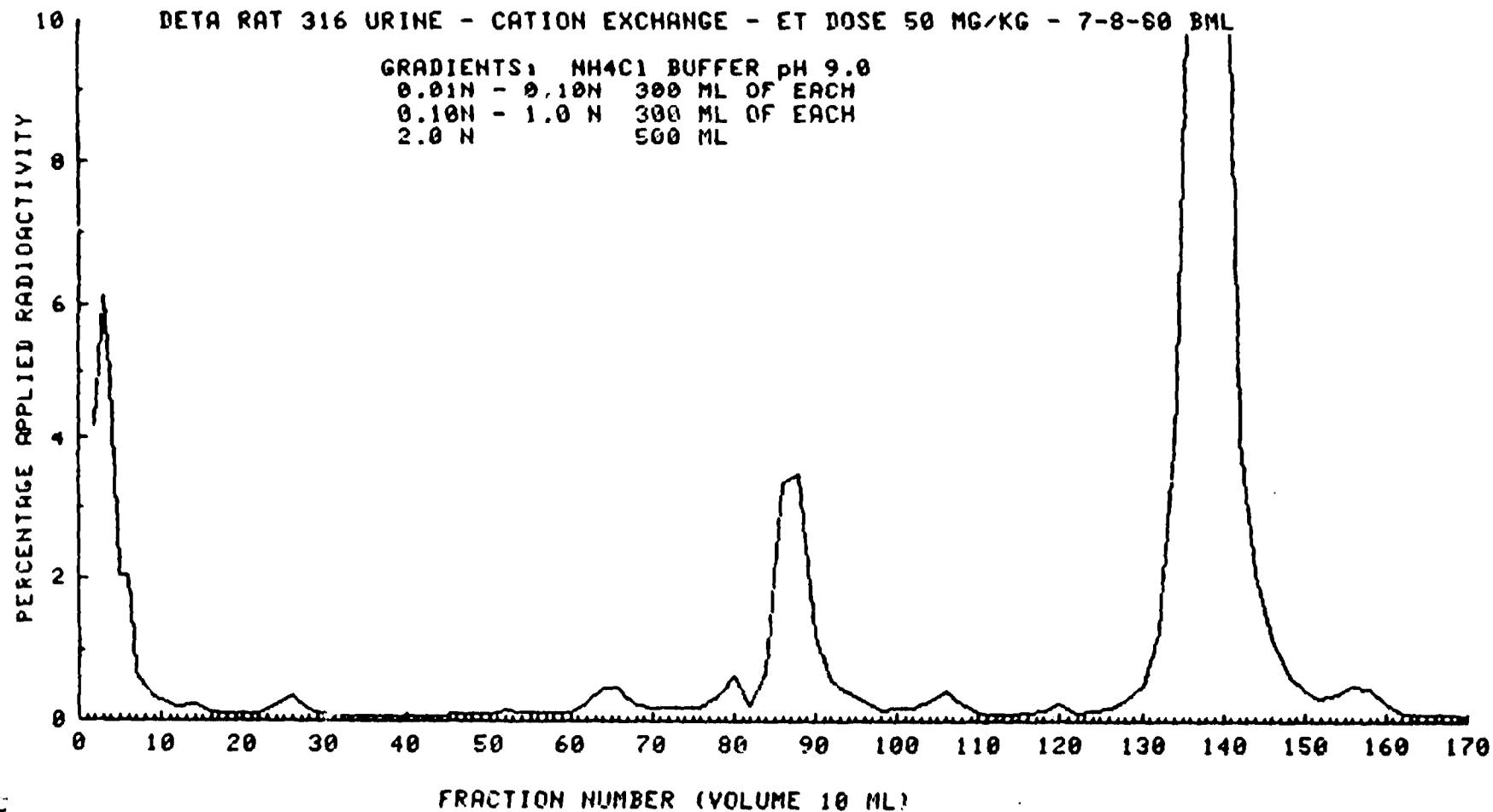
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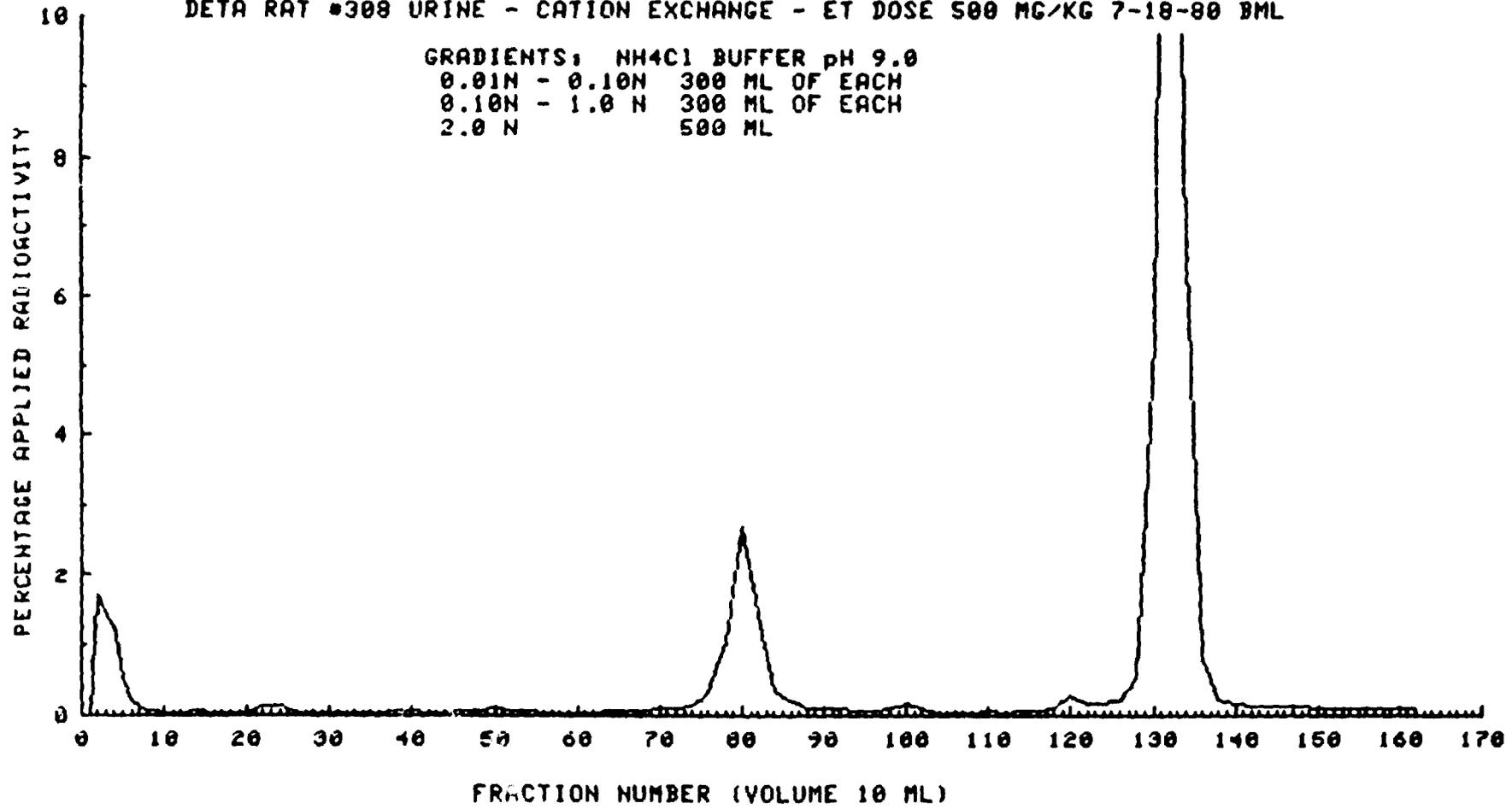
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DETA RAT #308 URINE - CATION EXCHANGE - ET DOSE 500 MG/KG 7-18-80 BML

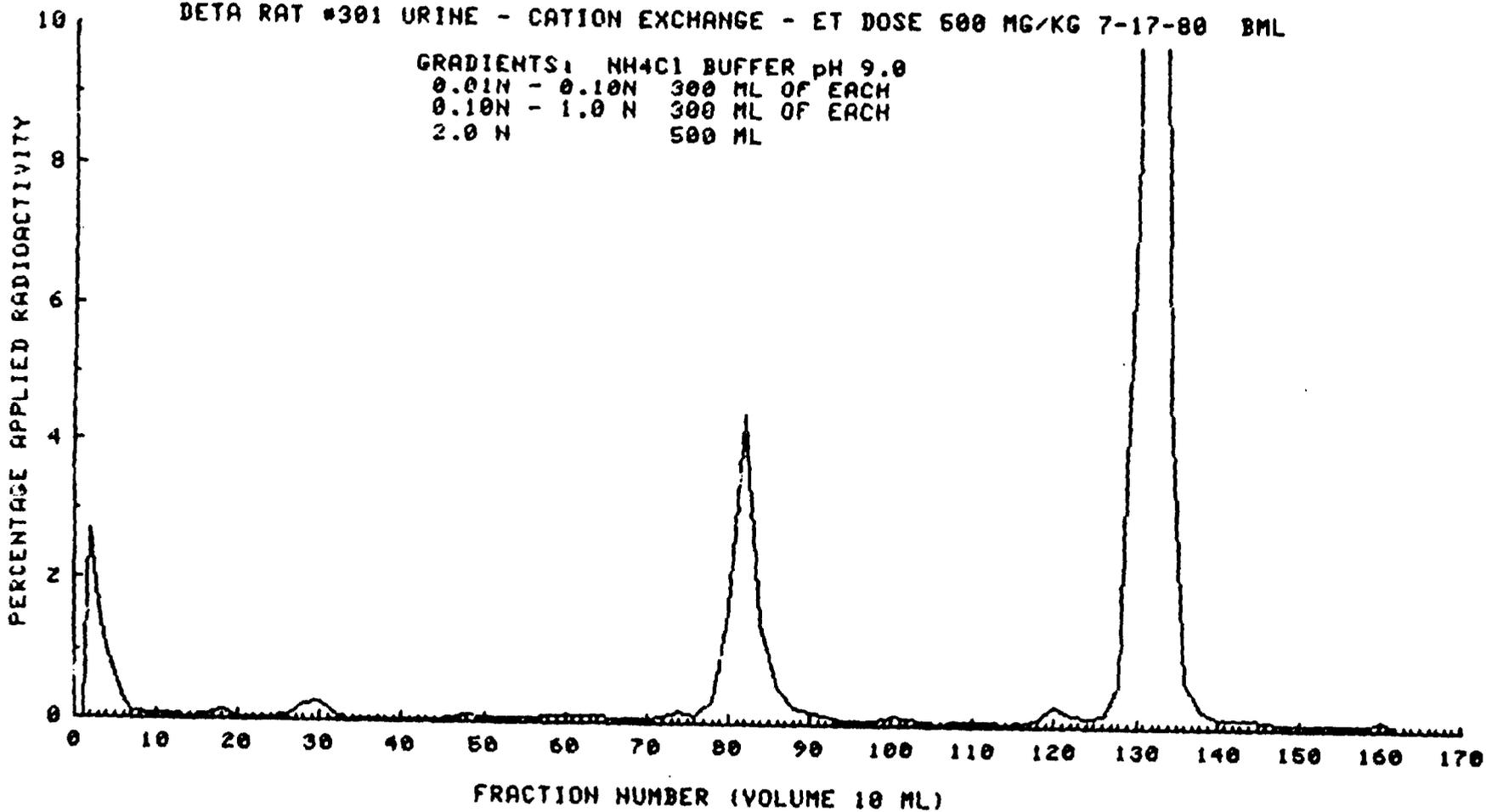
GRADIENTS: NH₄Cl BUFFER pH 9.0
0.01N - 0.10N 300 ML OF EACH
0.10N - 1.0 N 300 ML OF EACH
2.0 N 500 ML



000042

DETA RAT #301 URINE - CATION EXCHANGE - ET DOSE 500 MG/KG 7-17-80 BML

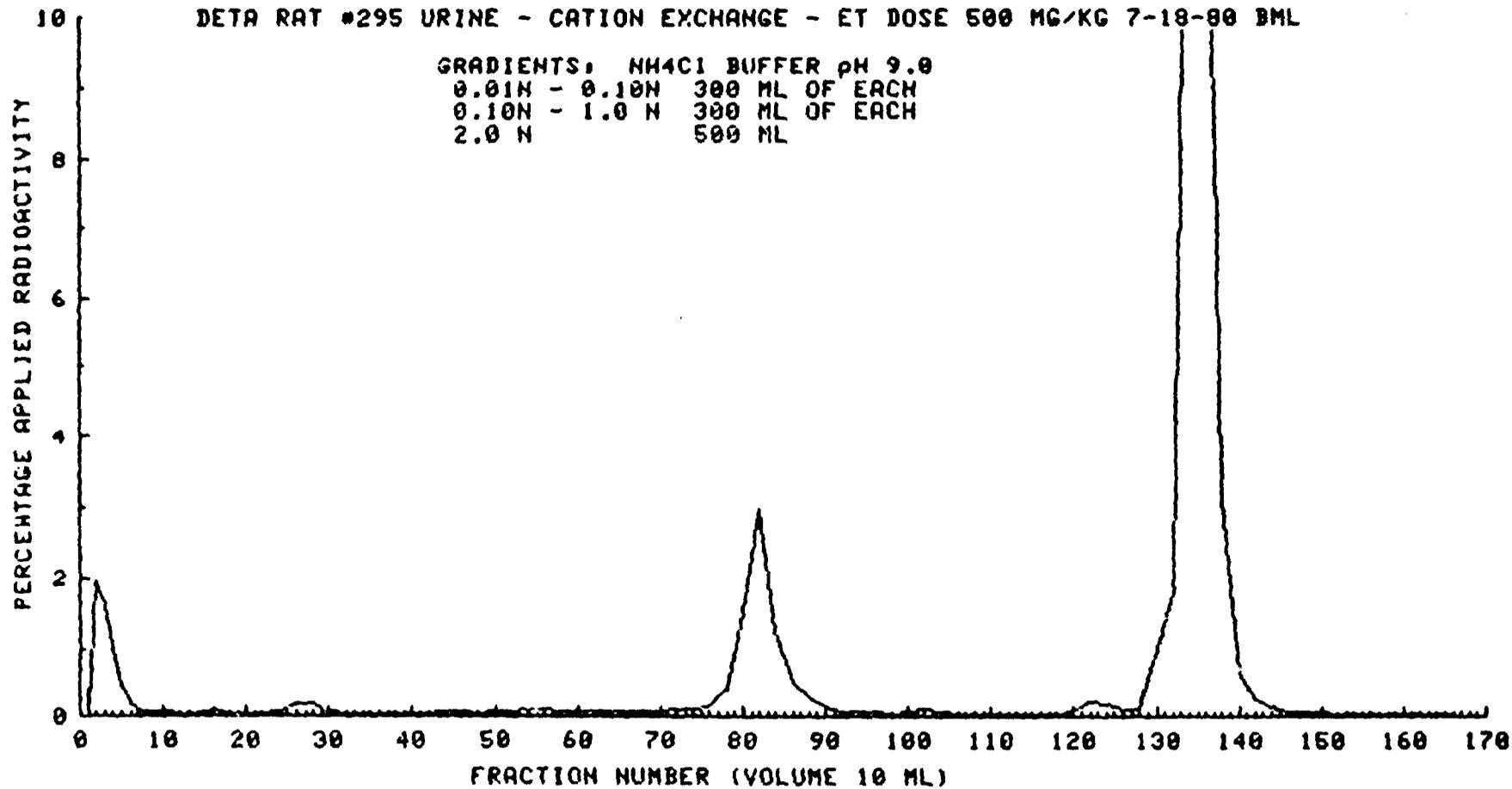
GRADIENTS: NH₄Cl BUFFER pH 9.0
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0.10N - 1.0 N 300 ML OF EACH
2.0 N 500 ML



000043

DETA RAT #295 URINE - CATION EXCHANGE - ET DOSE 500 MG/KG 7-18-80 BML

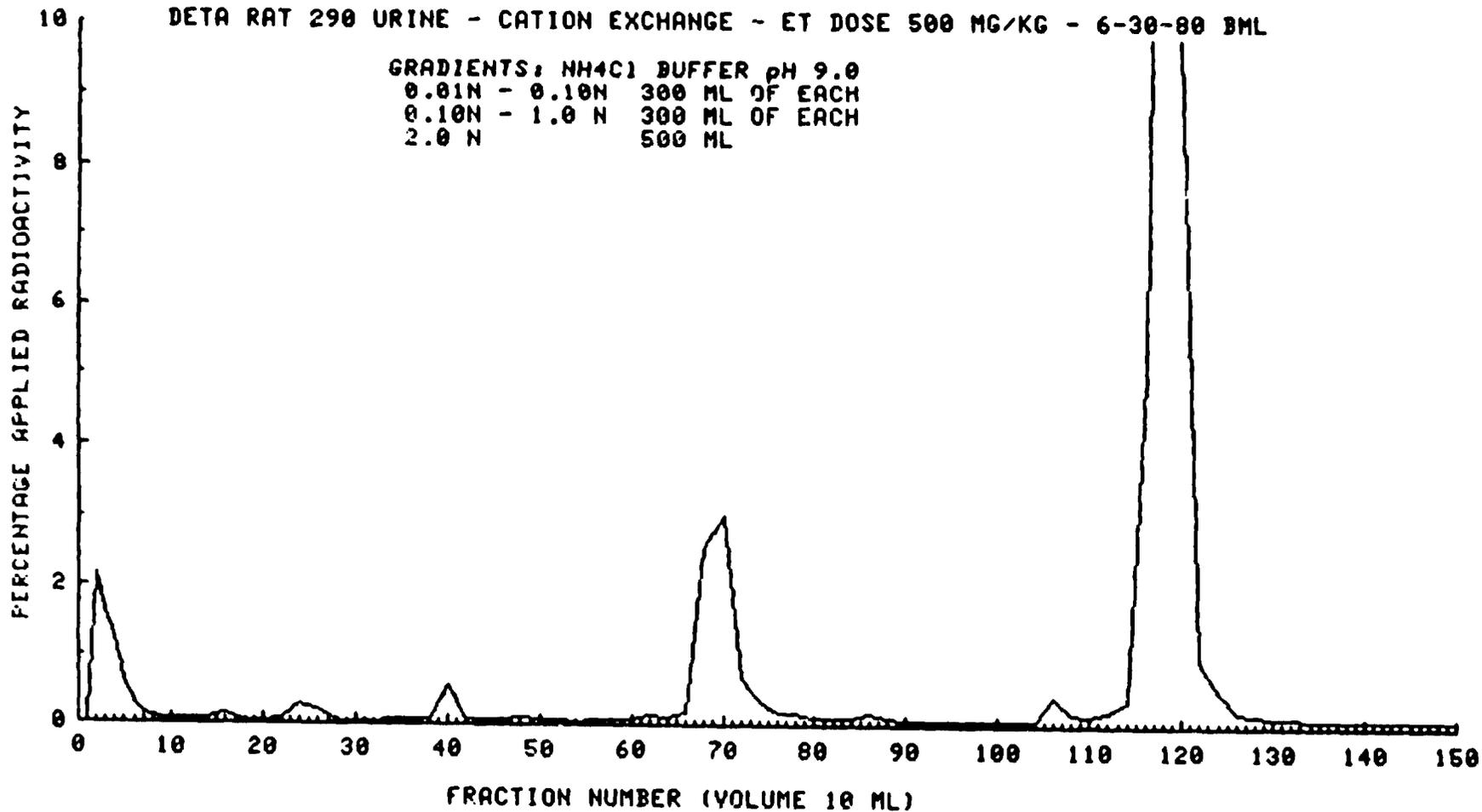
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0.10N - 1.0 N 300 ML OF EACH
2.0 N 500 ML

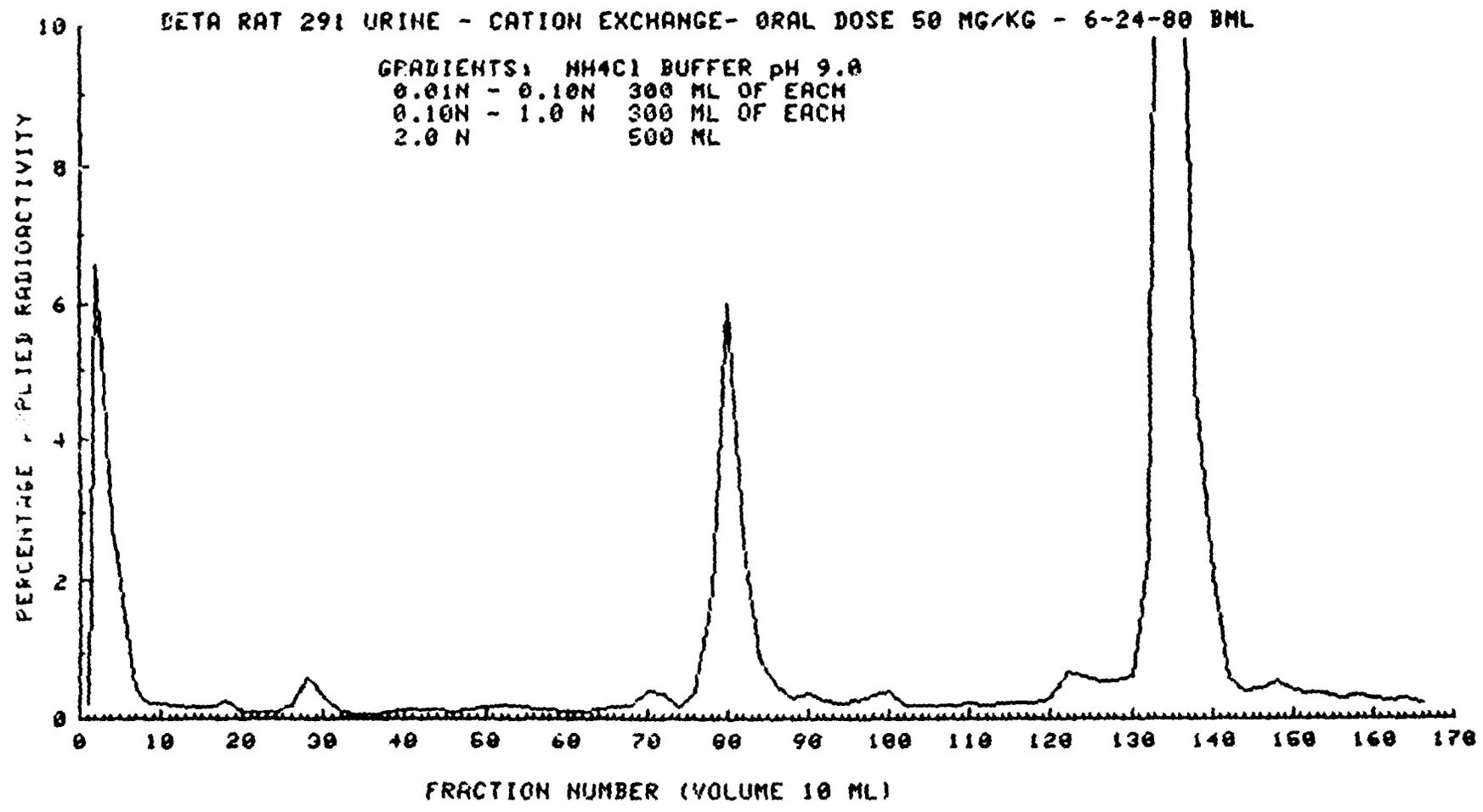


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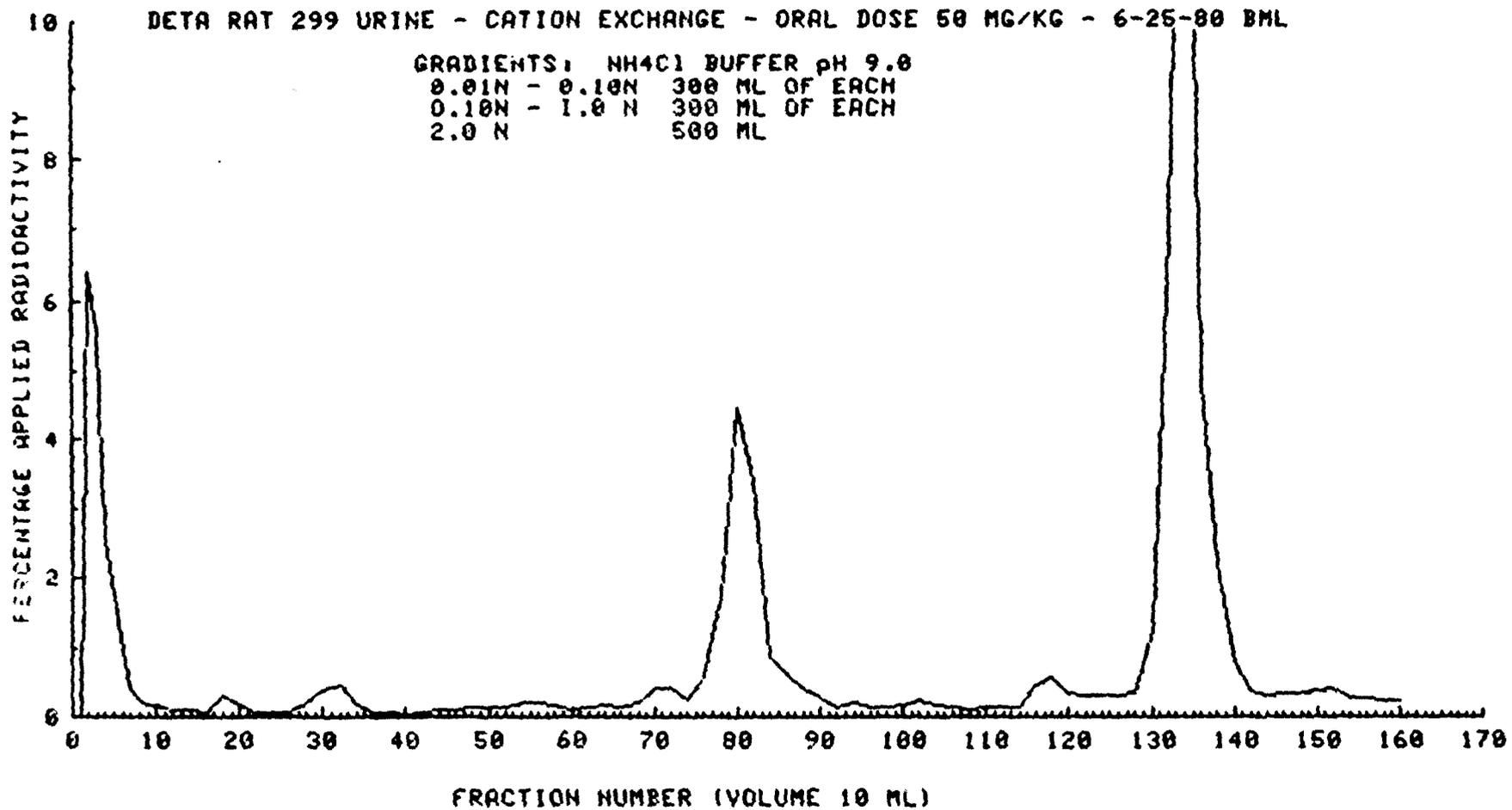
DETA RAT 290 URINE - CATION EXCHANGE - ET DOSE 500 MG/KG - 6-30-80 BML

GRADIENTS: NH₄Cl BUFFER pH 9.0
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0.10N - 1.0 N 300 ML OF EACH
2.0 N 500 ML





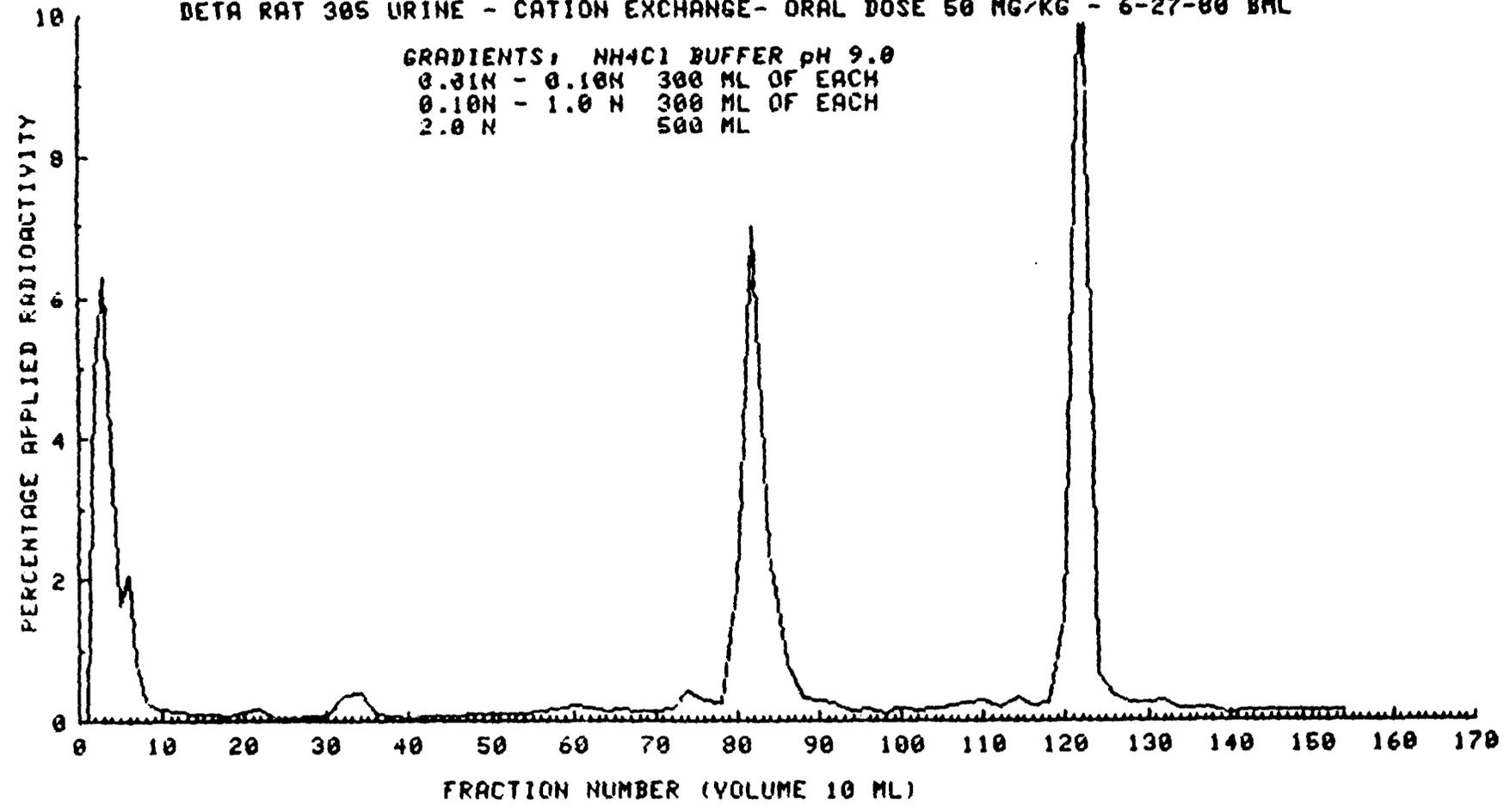
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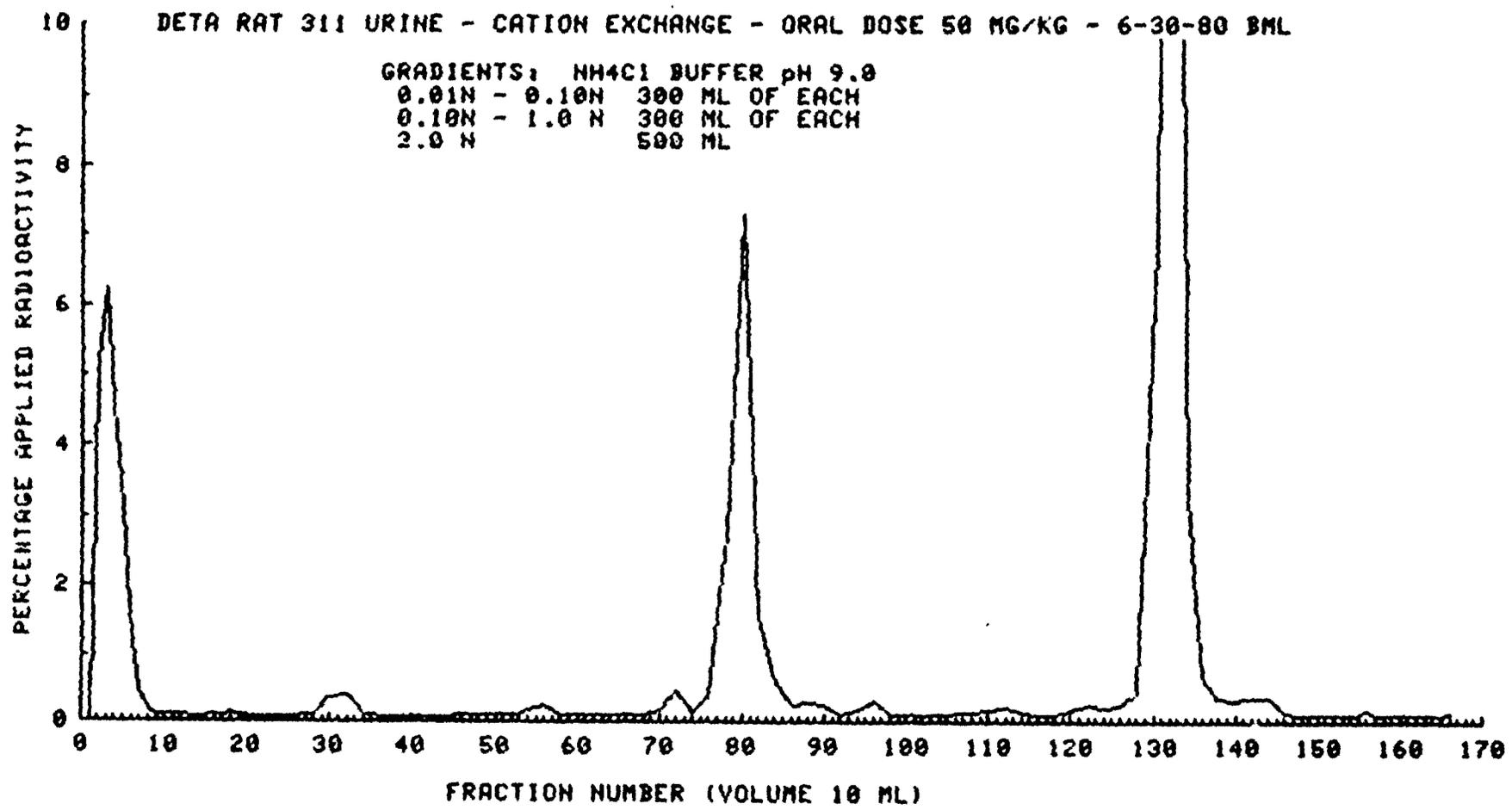
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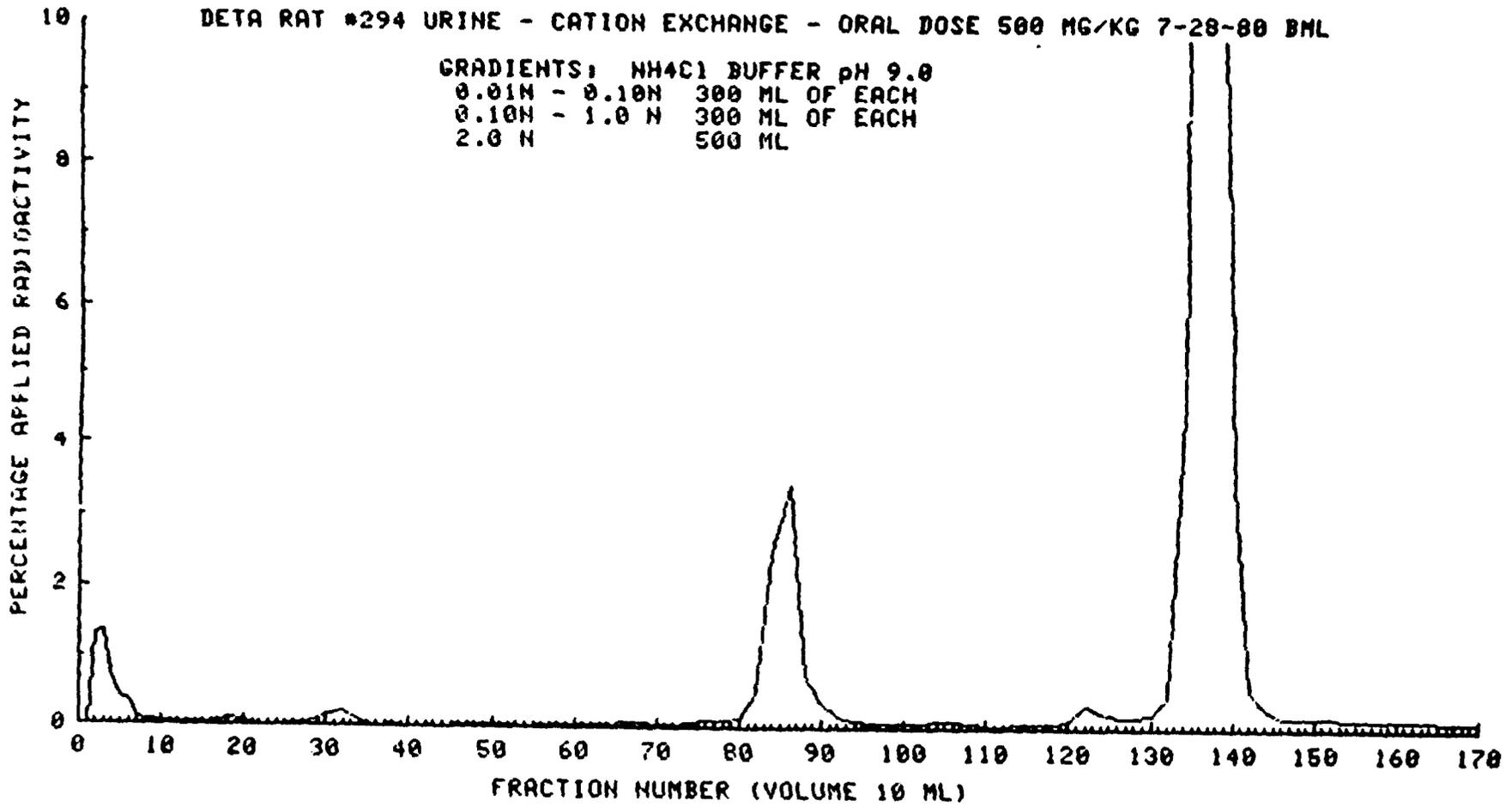
DETA RAT 305 URINE - CATION EXCHANGE- ORAL DOSE 50 MG/KG - 6-27-68 BML

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0.10N - 1.0 N 300 ML OF EACH
2.0 N 500 ML



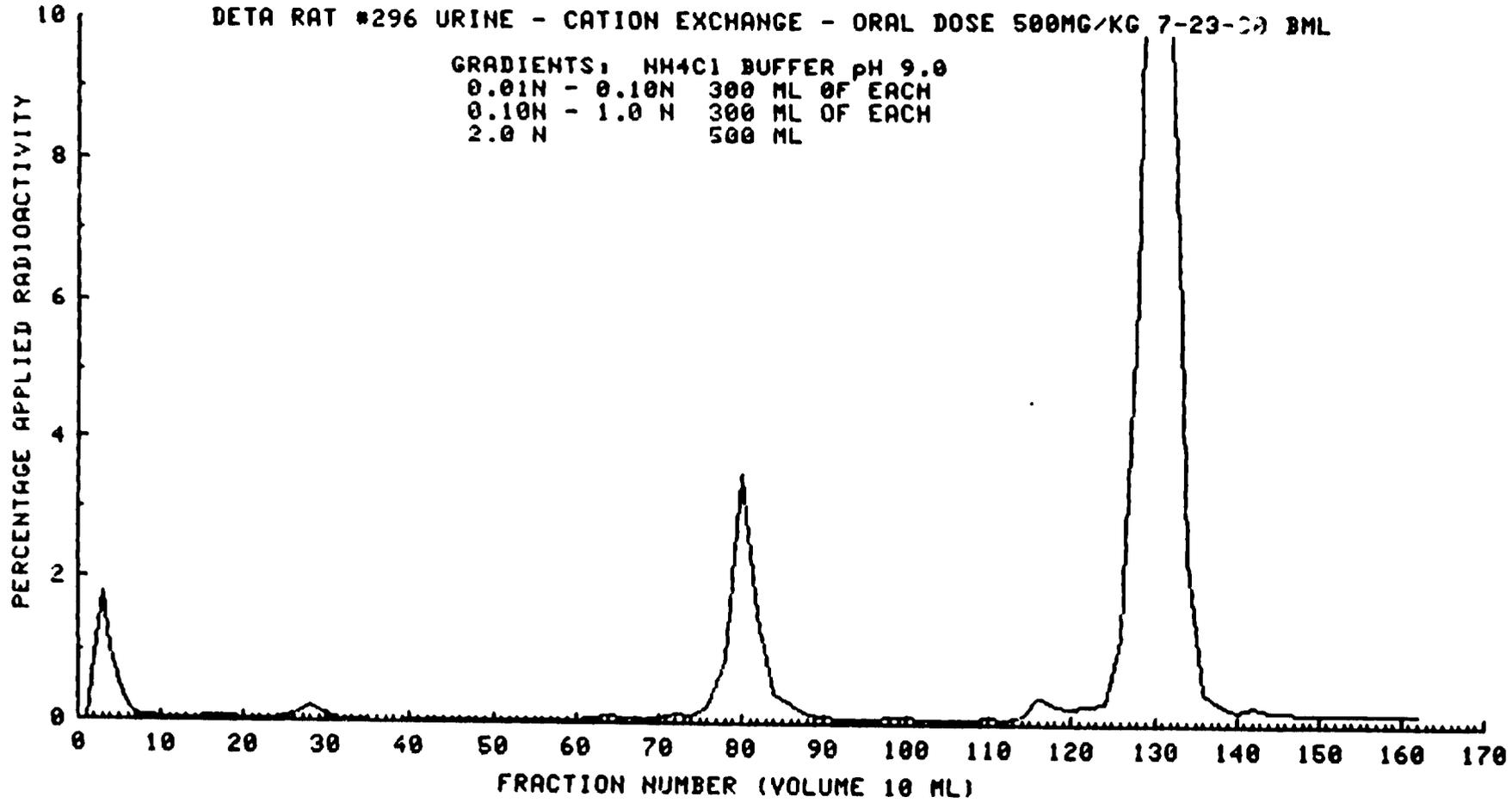
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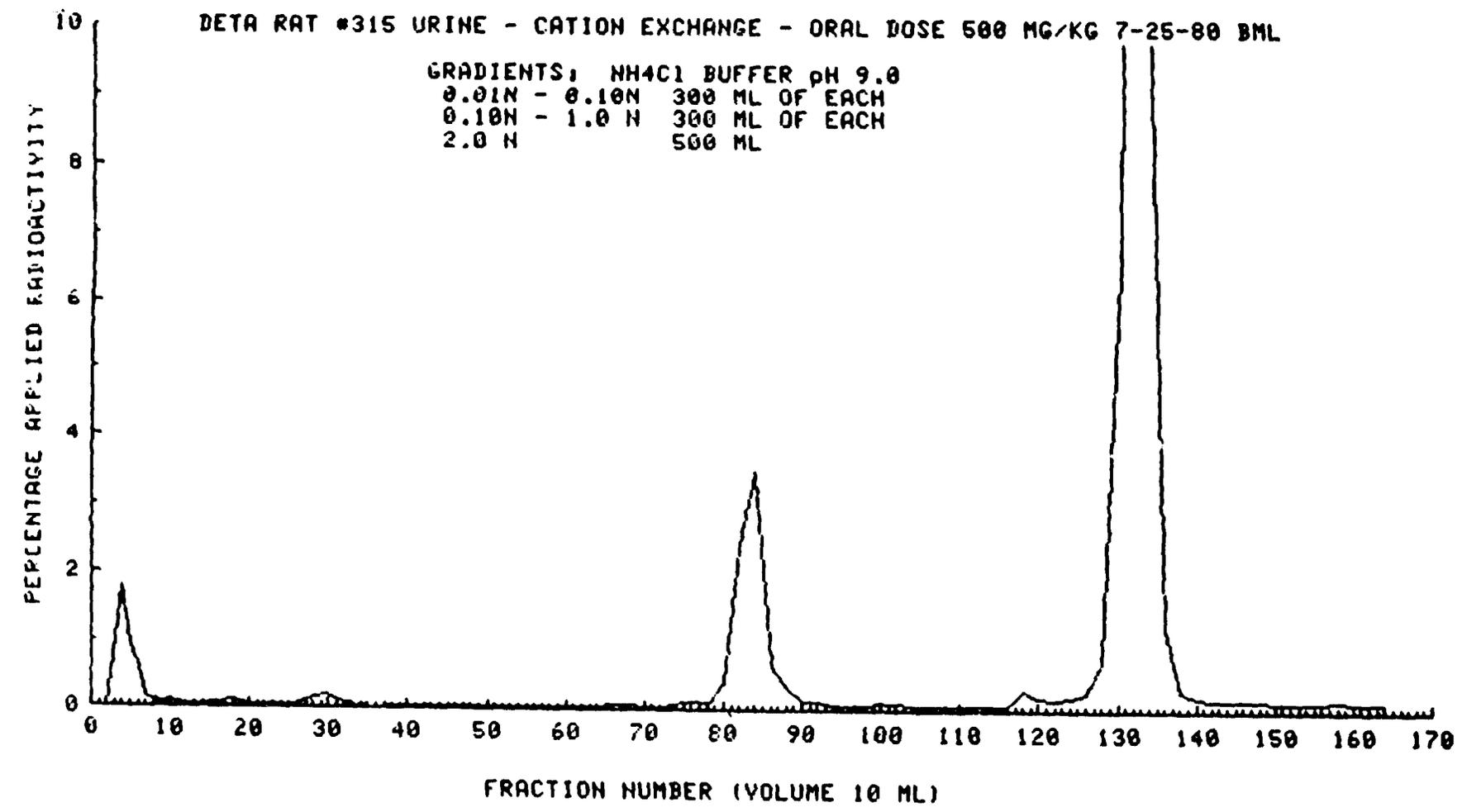




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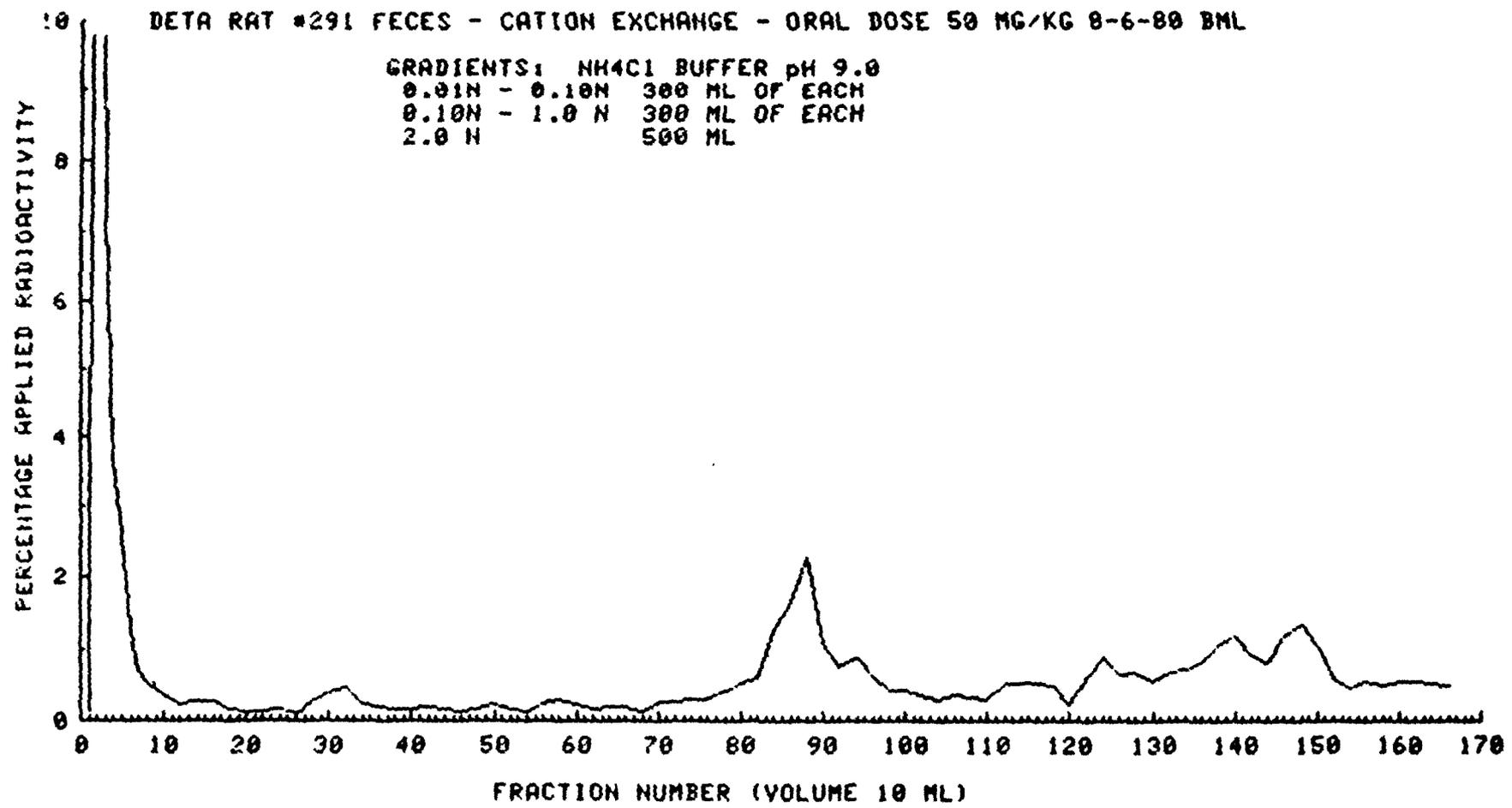
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APPENDIX VI

Chromatograms of Fecal Radioactivity from Selected Animals

(4 Page)

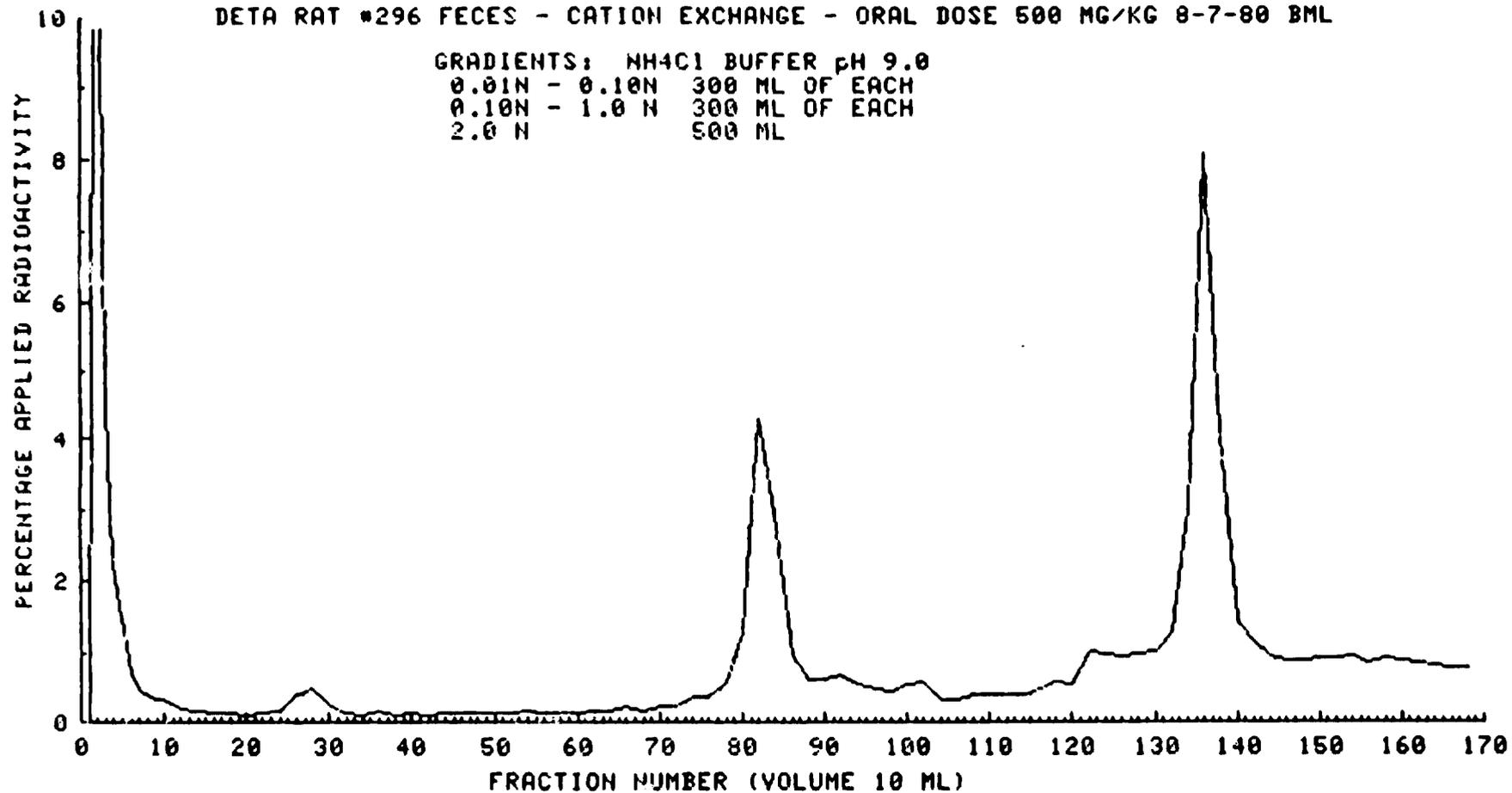
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1110054

DETA RAT #296 FECES - CATION EXCHANGE - ORAL DOSE 500 MG/KG 8-7-80 BML

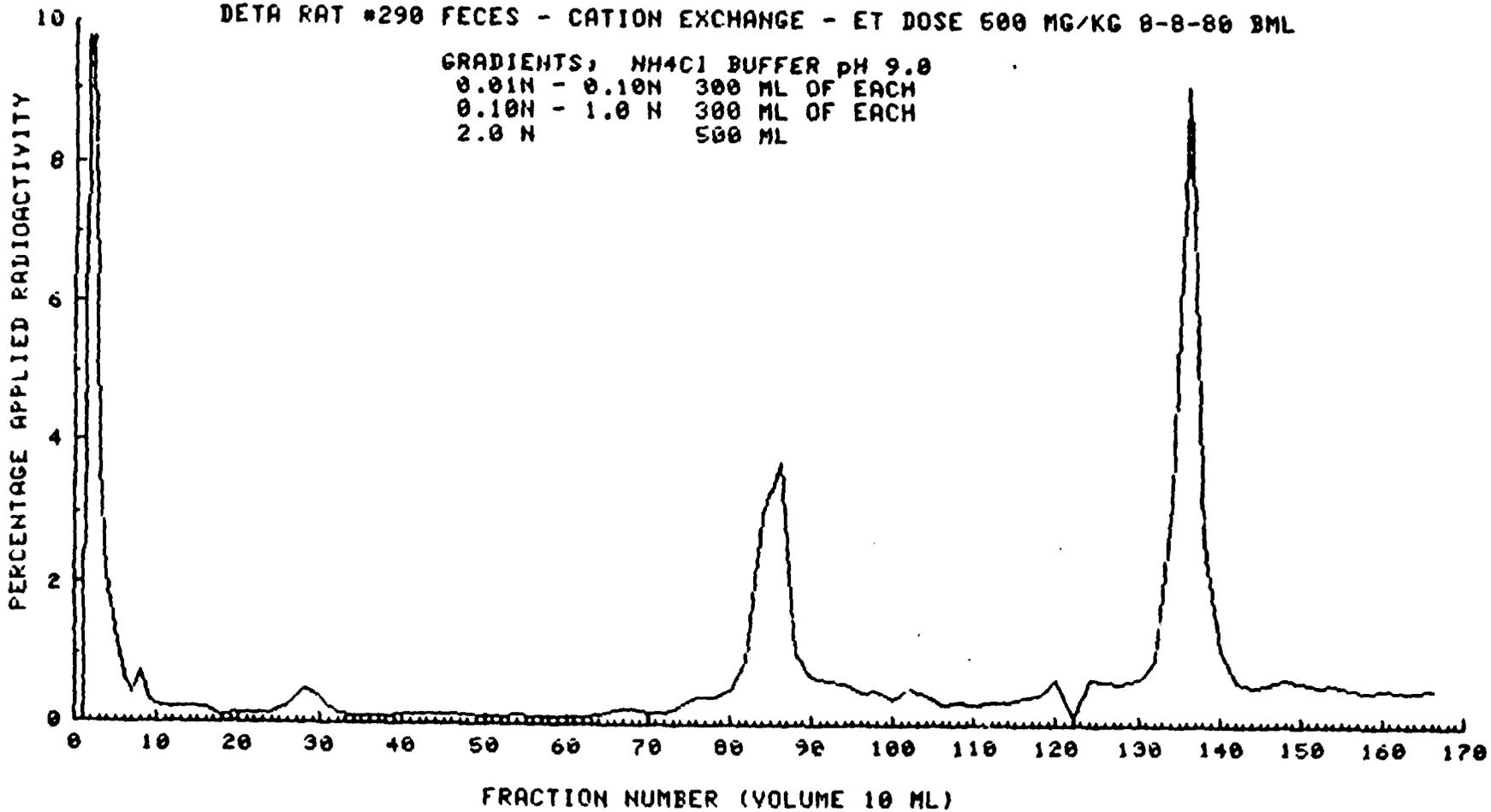
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0.10N - 1.0 N 300 ML OF EACH
2.0 N 500 ML



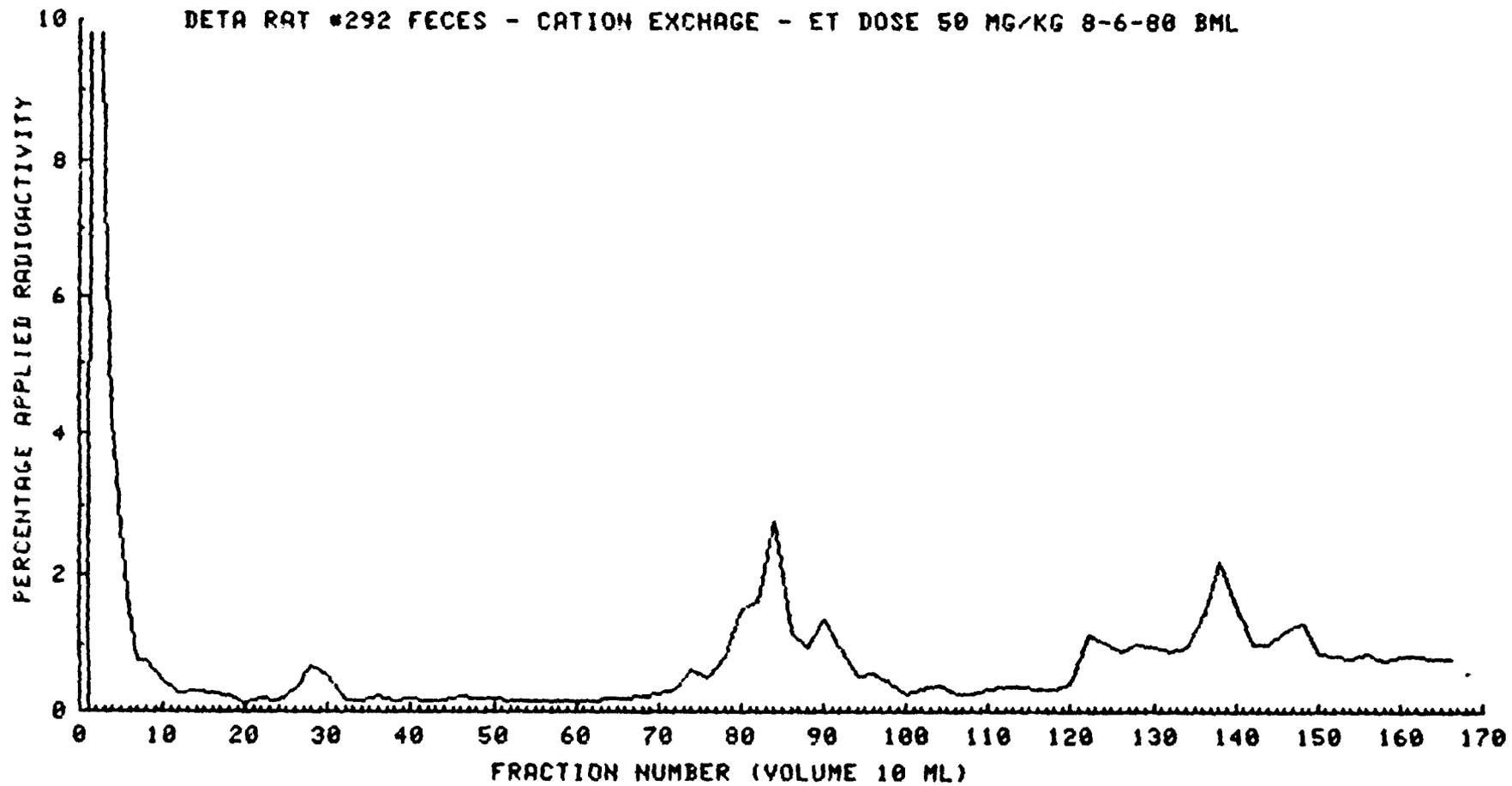
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DETA RAT #290 FECES - CATION EXCHANGE - ET DOSE 500 MG/KG 8-8-80 BML

GRADIENTS; NH₄Cl BUFFER pH 9.0
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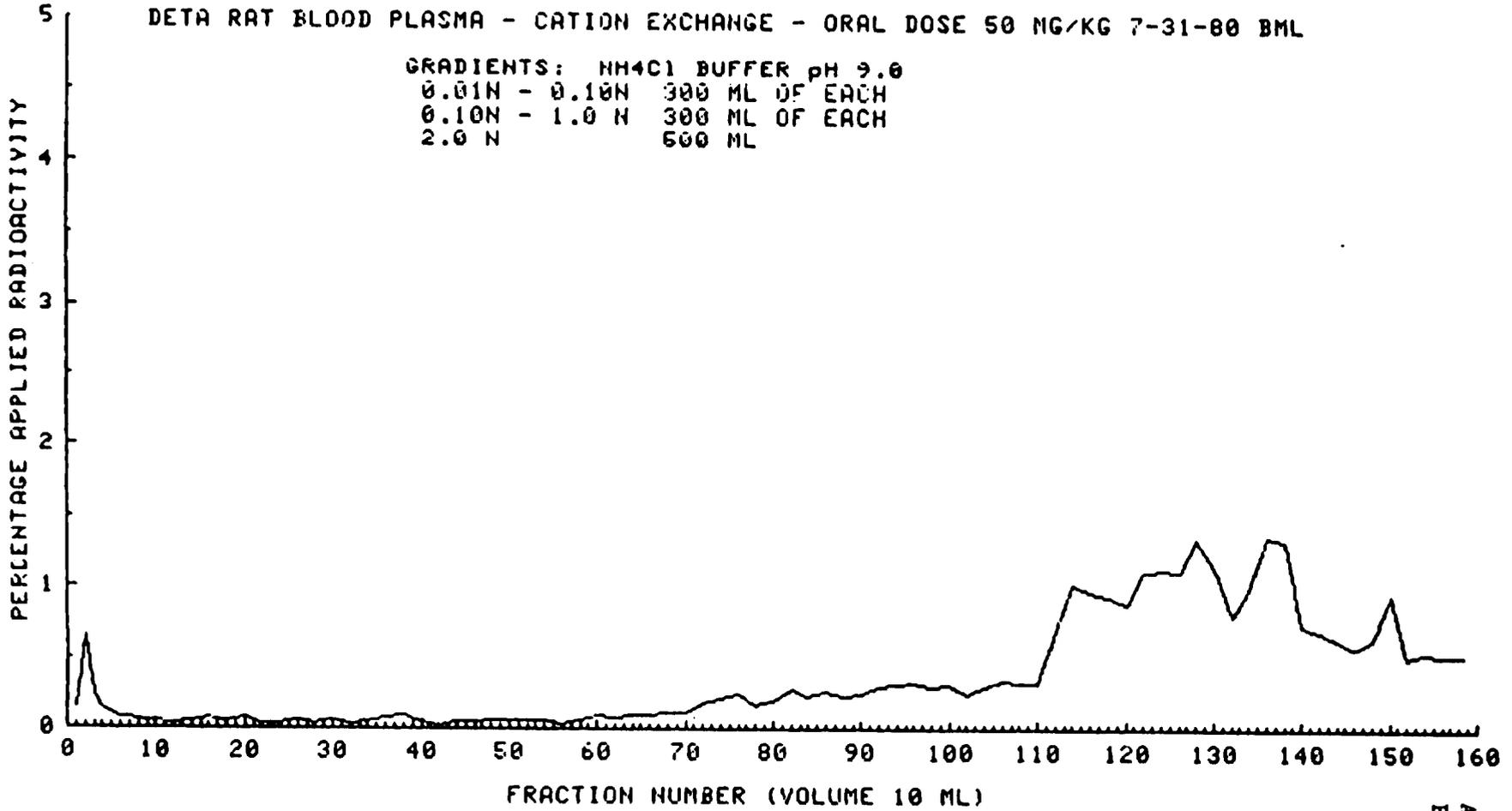
APPENDIX VII

Chromatogram of Radioactivity in Rat Plasma

(1 Page)

DETA RAT BLOOD PLASMA - CATION EXCHANGE - ORAL DOSE 50 NG/KG 7-31-80 BML

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0.10N - 1.0 N 300 ML OF EACH
2.0 N 500 ML



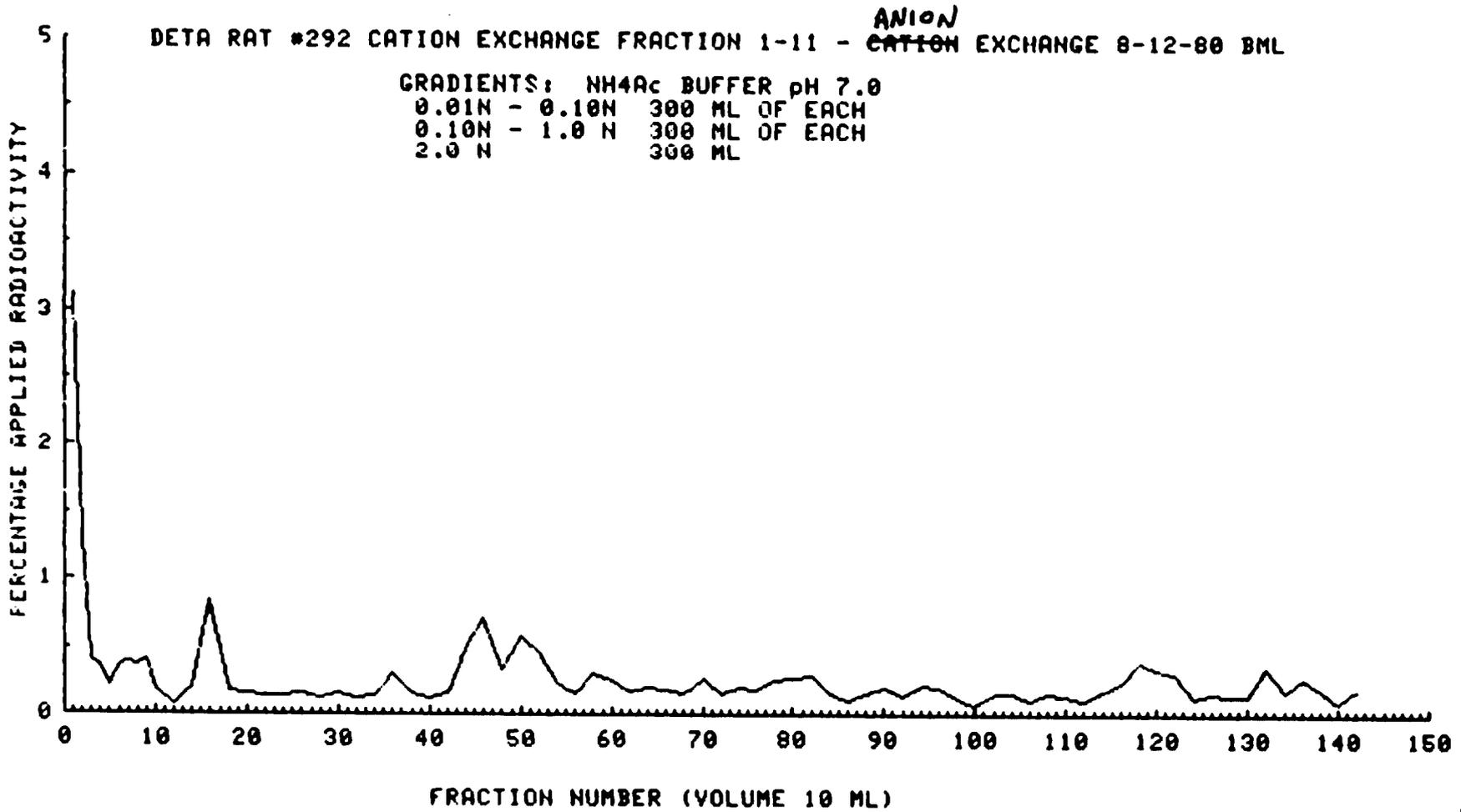
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APPENDIX VIII

ANION Exchange Chromatogram of "Neutral" (CV₁) Radioactivity

(1 Page)

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1900011

APPENDIX IX

Experimental Parameters for Individual Animals
used in DETA Pharmacokinetics Study

(1 Page)

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Experimental Parameters for Individual Animals
used in DETA Pharmacokinetic Study

<u>Animal Number</u>	<u>Dosage Route</u>	<u>Dosage Level (mg/kg)</u>	<u>Animal Weight (g)</u>	<u>Radioactivity Received (uCi)</u>
282	Oral	50.6	168	30.9
284	Oral	51.4	160	29.9
285	Oral	42.7	194	30.1
287	Oral	47.5	176	30.4
294	Oral	42.1	196	30.0
Mean \pm SD ^a		46.8 \pm 4.33	178 \pm 15.8	30.3 \pm 0.40
281	Endotracheal	46.0	183	30.6
286	Endotracheal	44.2	186	29.9
288	Endotracheal	46.4	184	31.0
290	Endotracheal	46.8	181	30.8
Mean \pm SD		45.8 \pm 1.15	183 \pm 2.1	30.6 \pm 0.48
273	Intravenous	54.2	188	48.4
277	Intravenous	55.1	187	48.6
283	Intravenous	54.5	189	48.6
293	Intravenous	58.4	178	49.2
Mean \pm SD		55.6 \pm 1.94	185 \pm 5.1	48.7 \pm 0.34

^aSD = Standard Deviation

WPC/1138-2

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APPENDIX X

Pharmacokinetic Parameters for Individual Rats

(1 Page)

Parameters for Individual Rats used in Pharmacokinetic Study

Animal Number	Dosage Route	Dosage Received (mg)	Area Under Curve (ug ml ⁻¹ hr ⁻¹)	Total Clearance (ml hr ⁻¹)	Terminal Half-Life (hr)
282	Oral	8.51	60.8	132	18.7
284	Oral	8.22	65.0	120	17.3
285	Oral	8.29	57.8	136	14.7
287	Oral	8.36	53.8	147	7.14
294	Oral	8.25	58.3	134	23.9
Mean ± SD ^a		8.33 ± 0.12	59.1 ± 4.13	134 ± 9.65	16.3 ± 6.14
281	Endotracheal	8.41	45.7	166	4.33
296	Endotracheal	8.22	60.8	122	16.1
288	Endotracheal	8.54	65.9	117	7.45
290	Endotracheal	8.47	51.7	147	8.15
Mean ± SD		8.41 ± 0.14	56.0 ± 9.05	138 ± 22.8	9.01 ± 5.01
273	Intravenous	10.2	84.9	120	12.8
277	Intravenous	10.3	65.5	157	13.9
283	Intravenous	10.3	49.5	208	2.19
293	Intravenous	10.4	49.0	212	9.90
Mean ± SD		10.3 ± 0.08	62.2 ± 16.9	174 ± 44.0	9.70 ± 5.28

^aSD = Standard Deviation

WPC/1138-2

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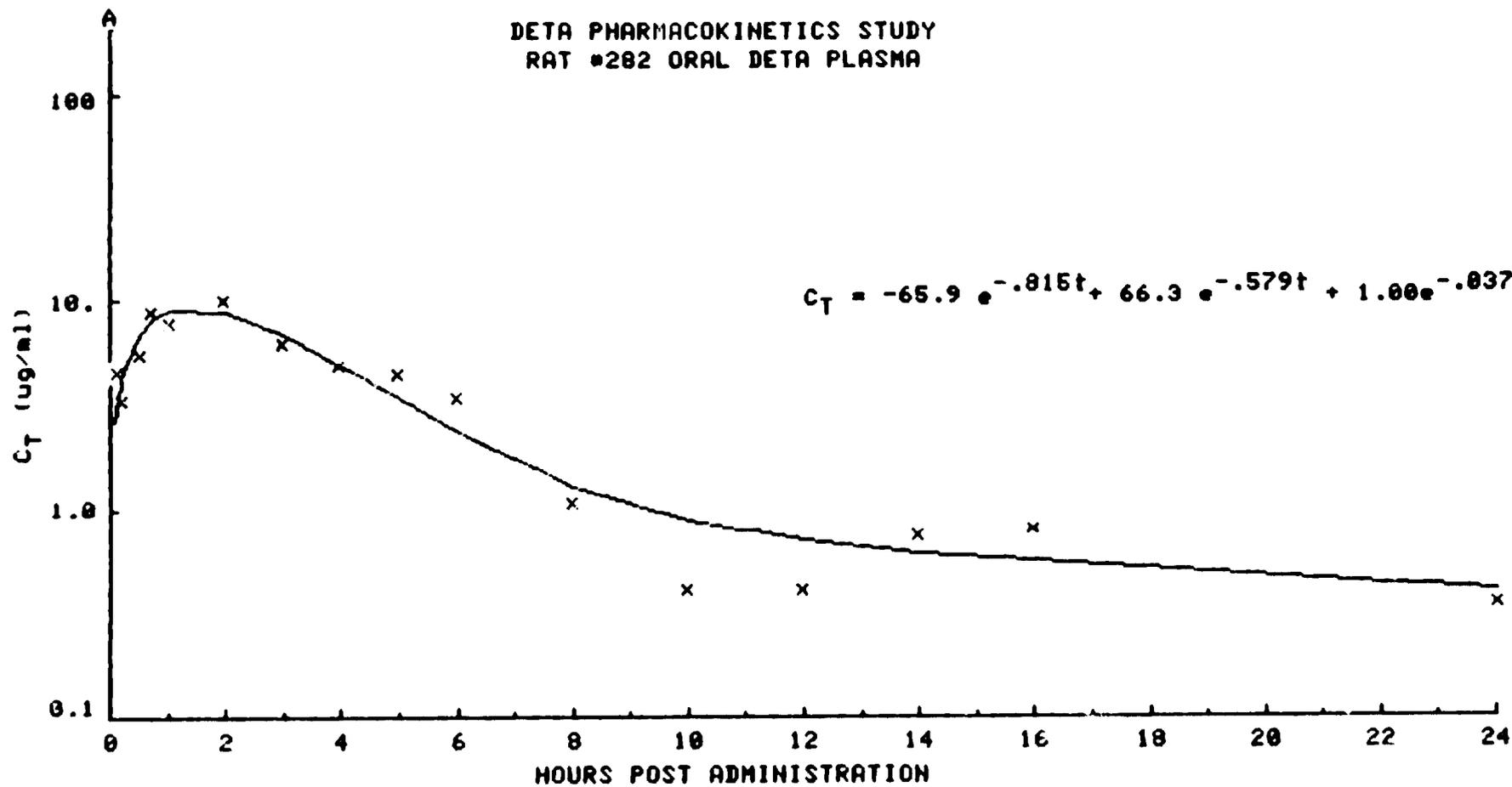
APPENDIX XI

Log-Linear Plots of Plasma Concentration vs. Time for Individual Animals

(13 Pages)

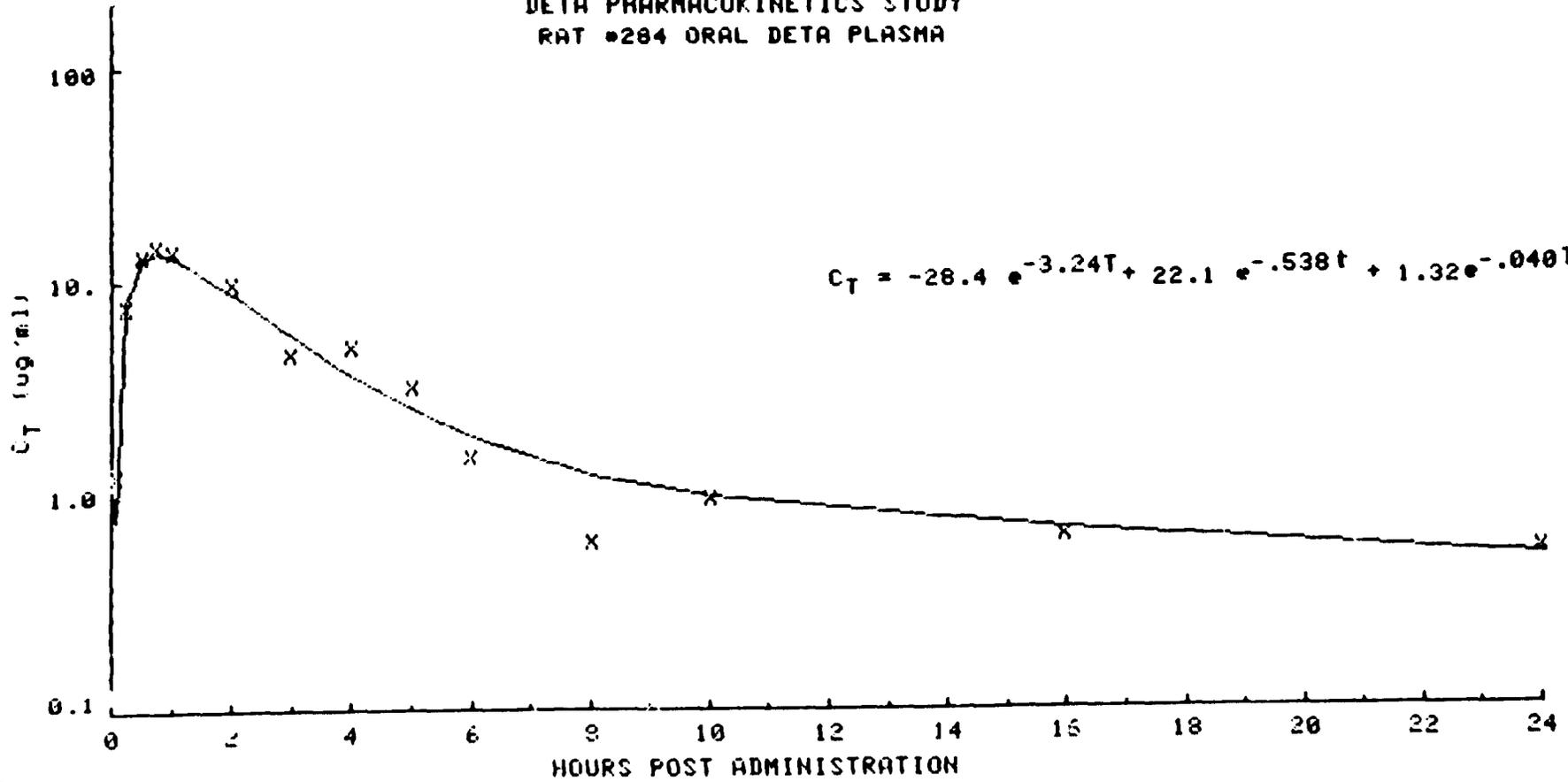
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DETA PHARMACOKINETICS STUDY
RAT #282 ORAL DETA PLASMA

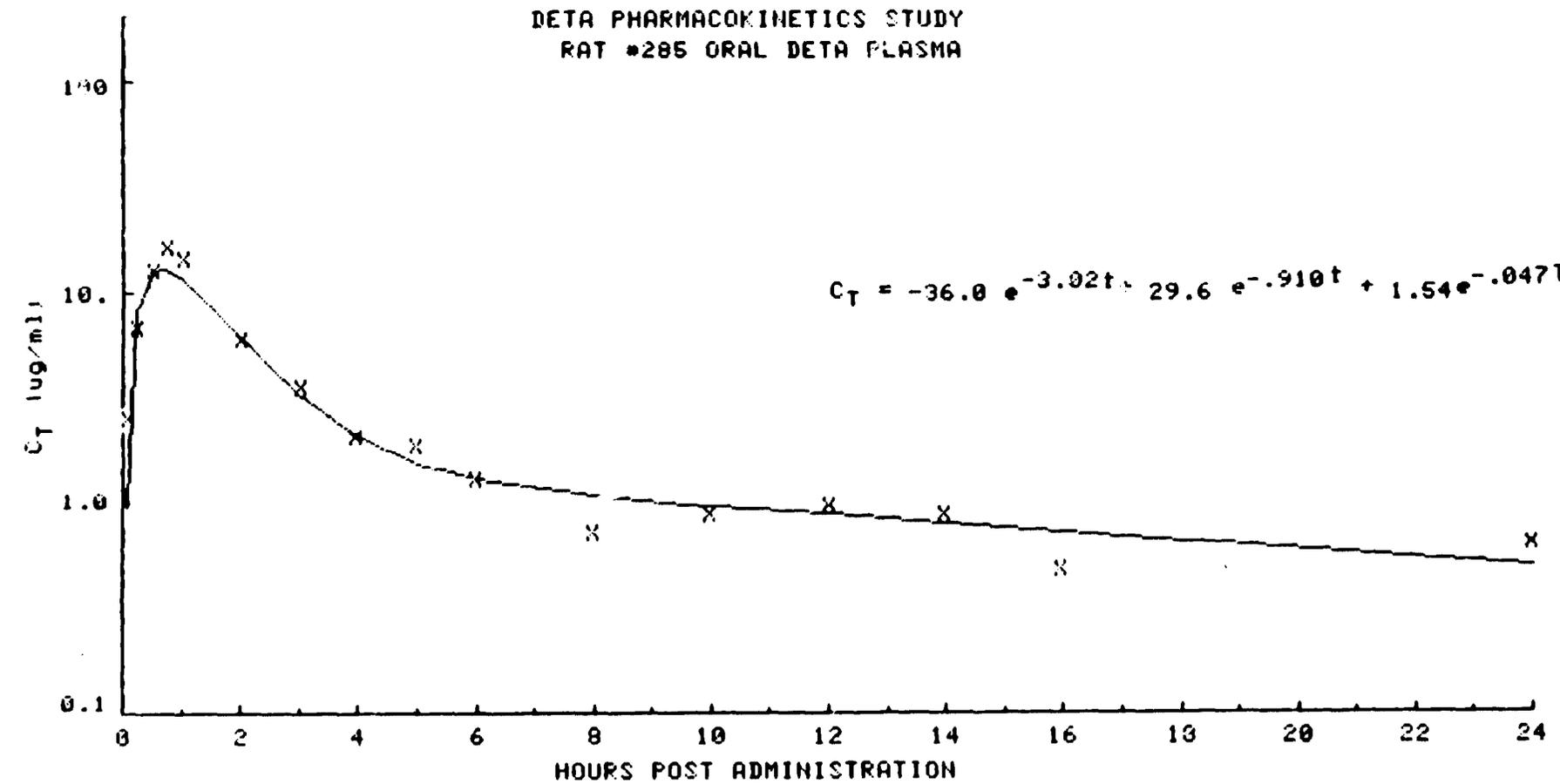


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RAT #284 ORAL DETA PLASMA

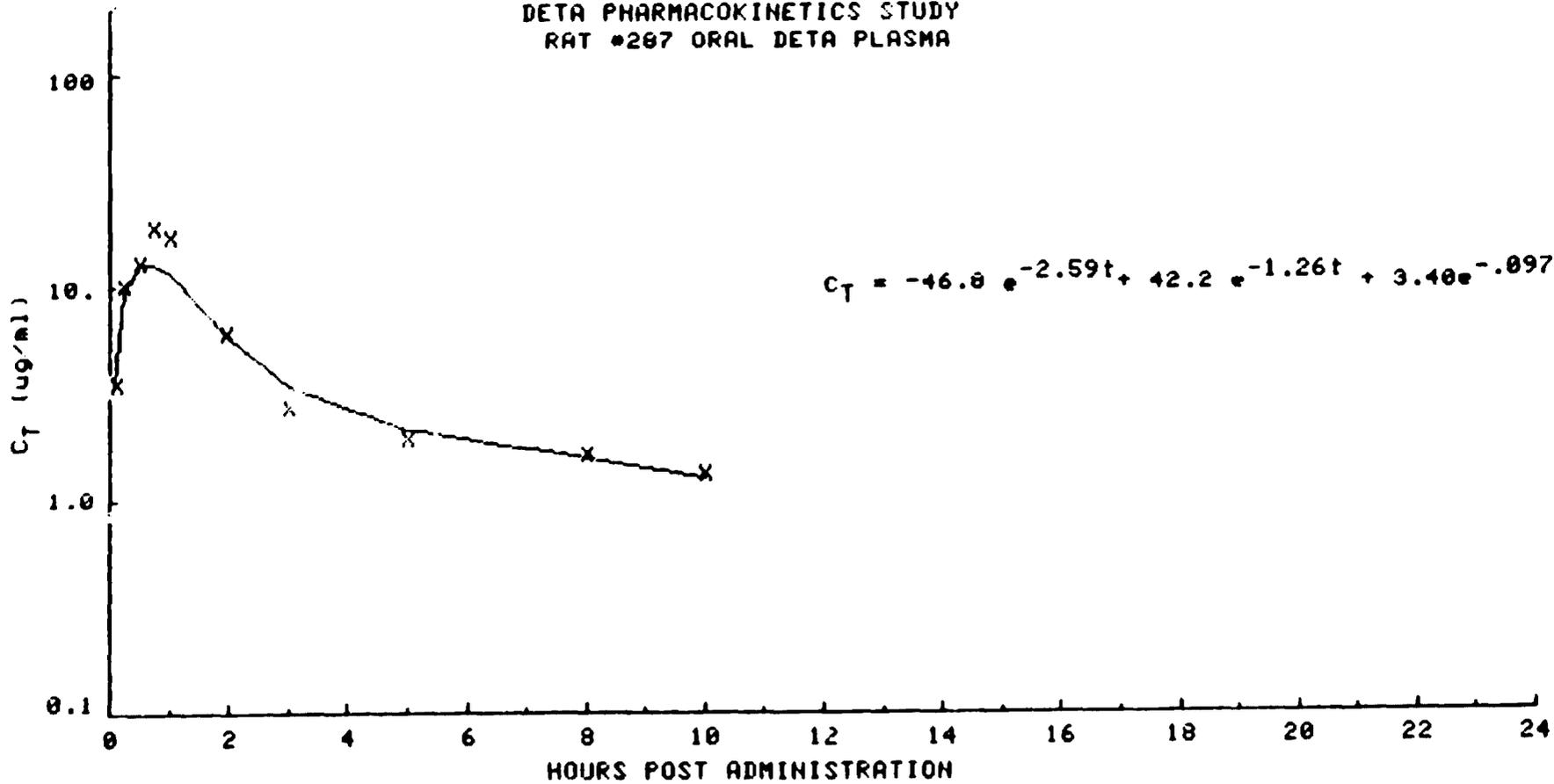


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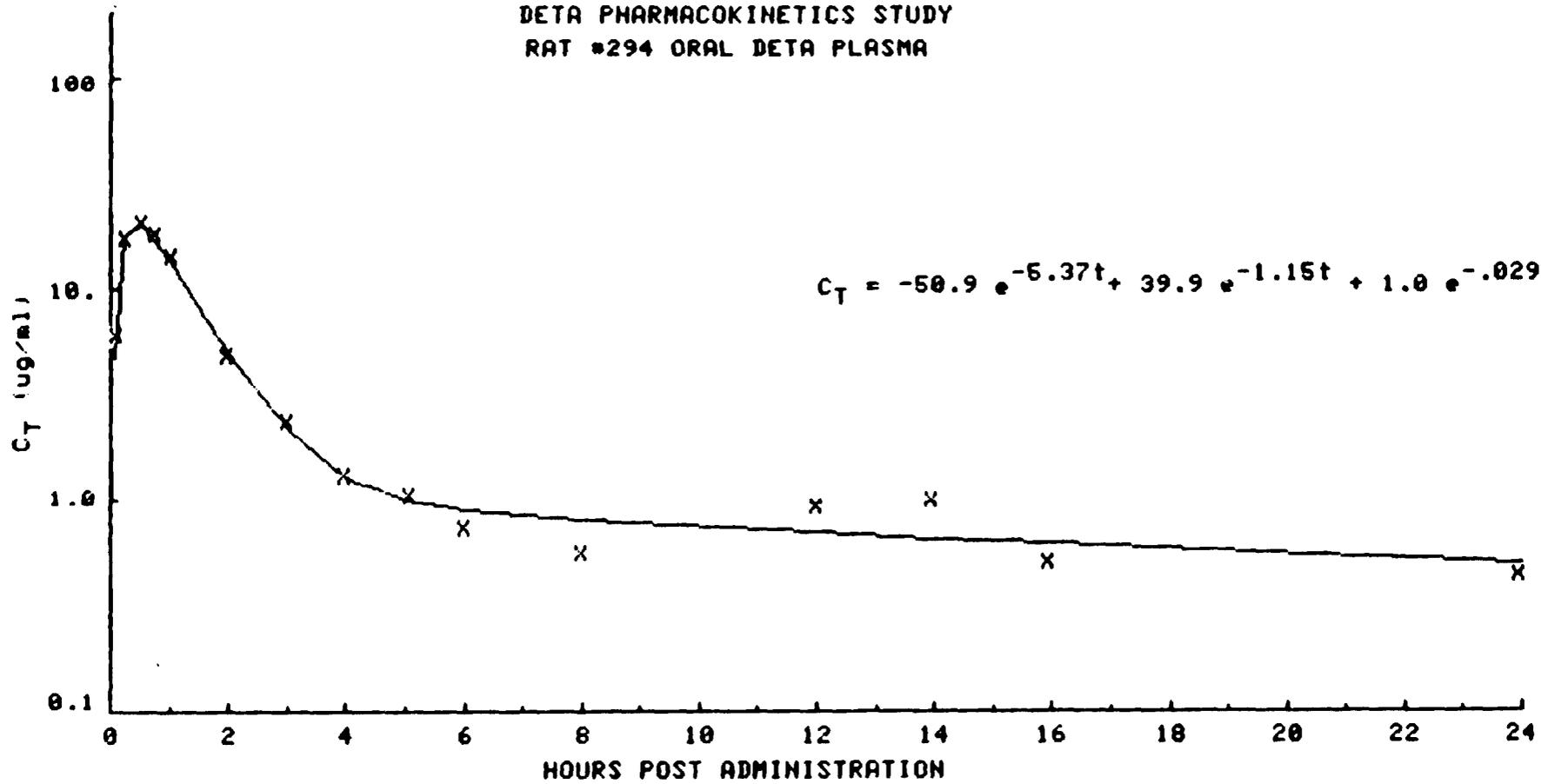
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DETA PHARMACOKINETICS STUDY
RAT #287 ORAL DETA PLASMA



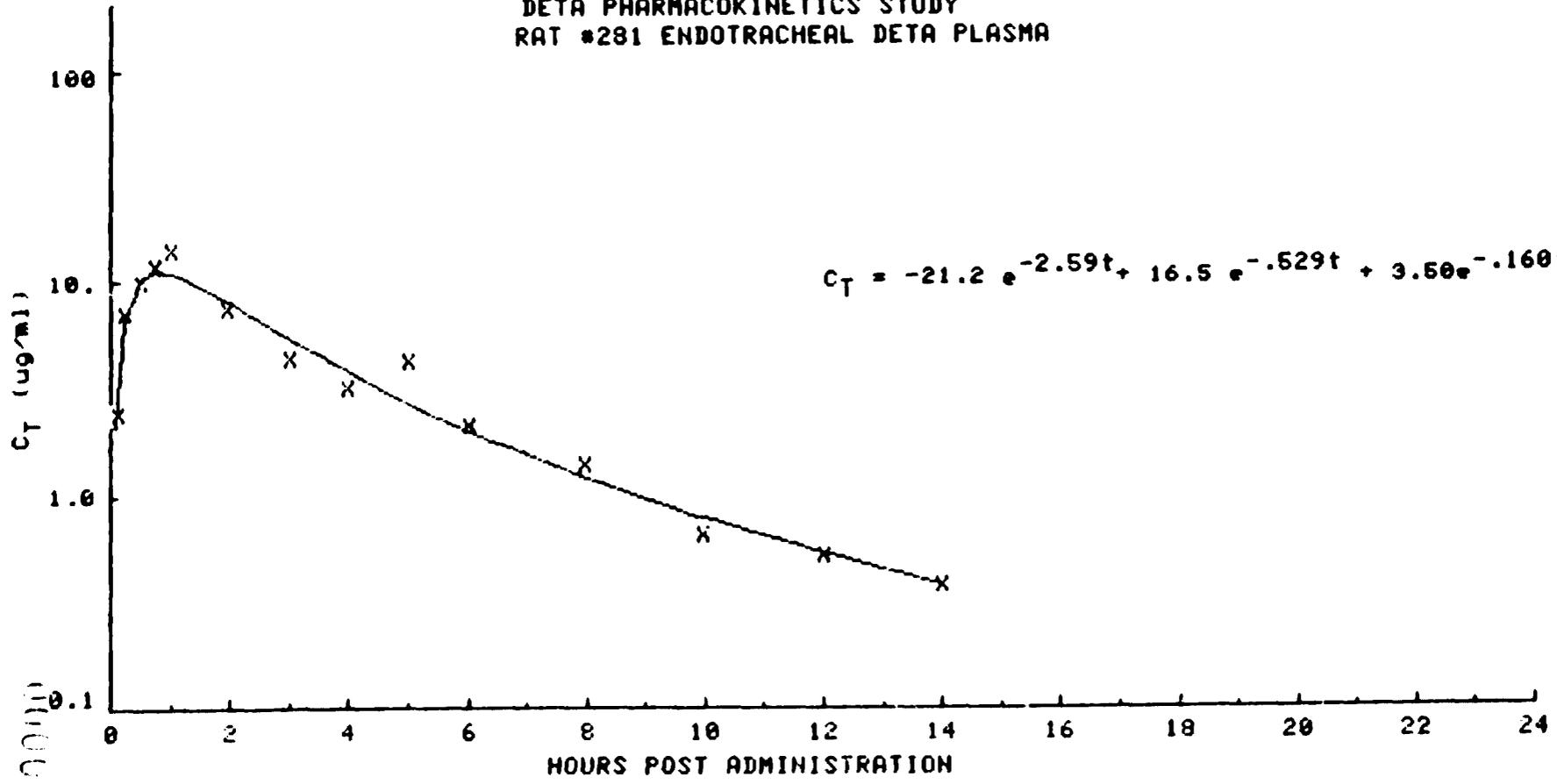
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RAT #294 ORAL DETA PLASMA



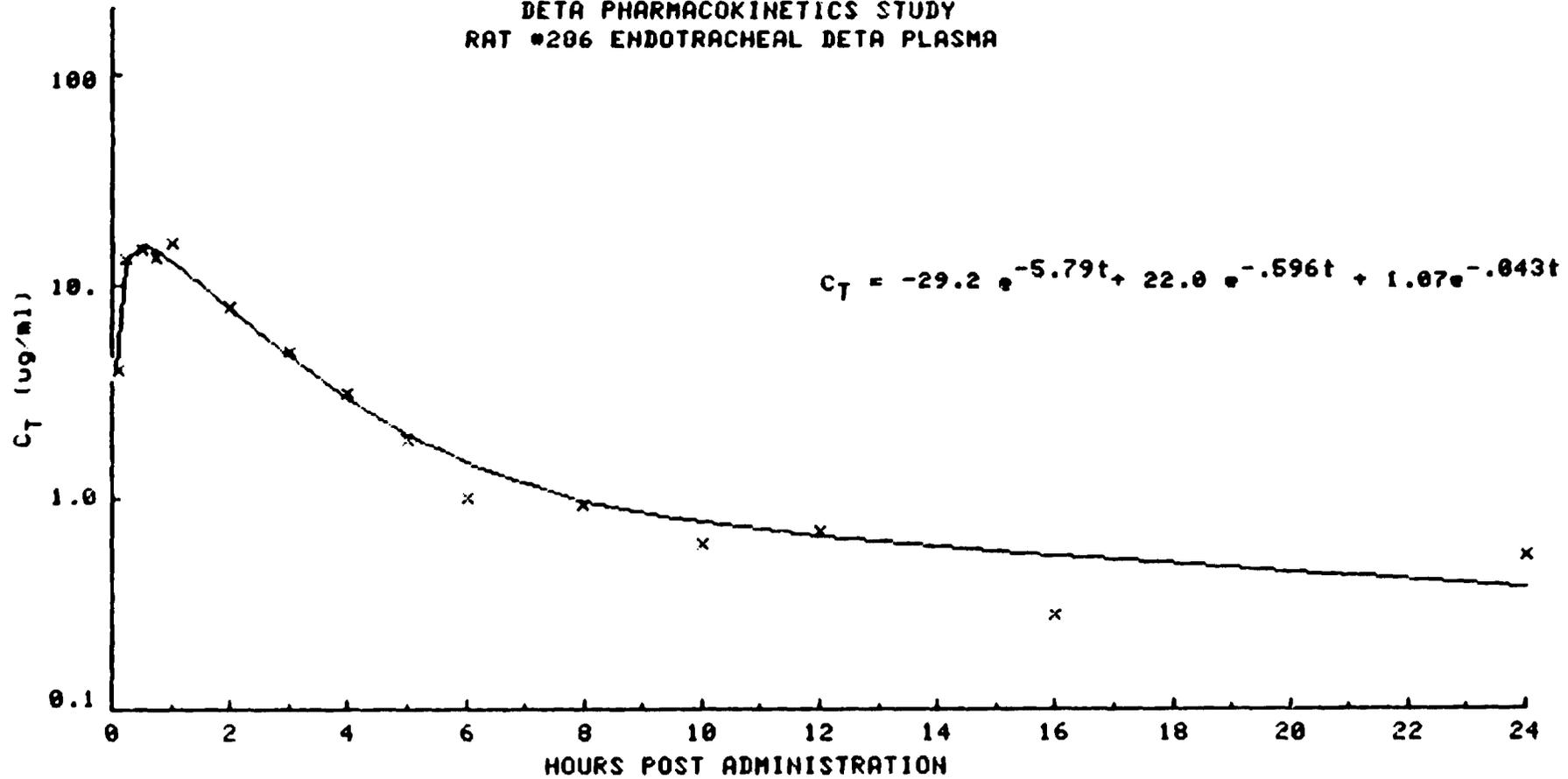
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DETA PHARMACOKINETICS STUDY
RAT #281 ENDOTRACHEAL DETA PLASMA



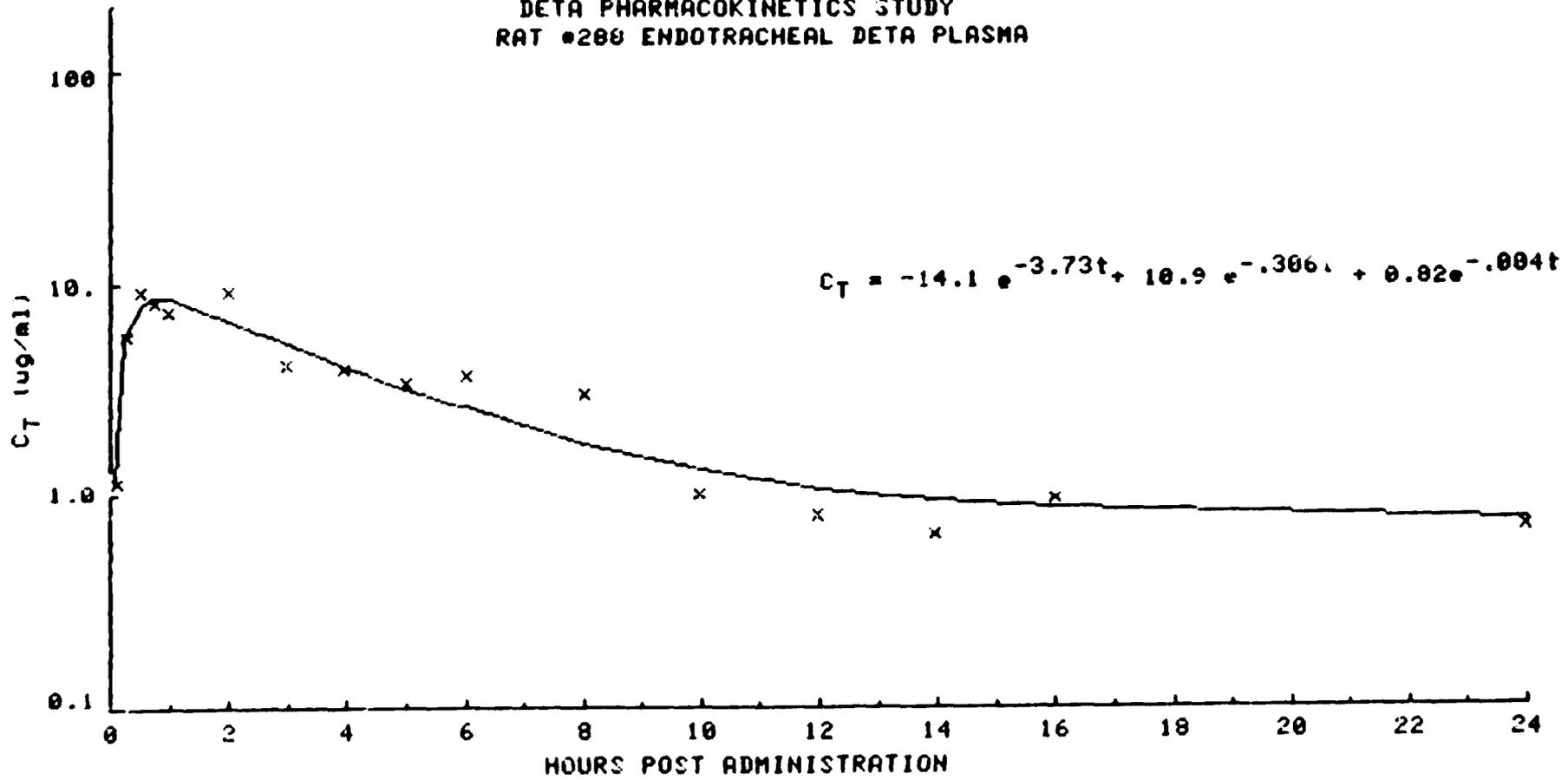
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RAT #206 ENDOTRACHEAL DETA PLASMA



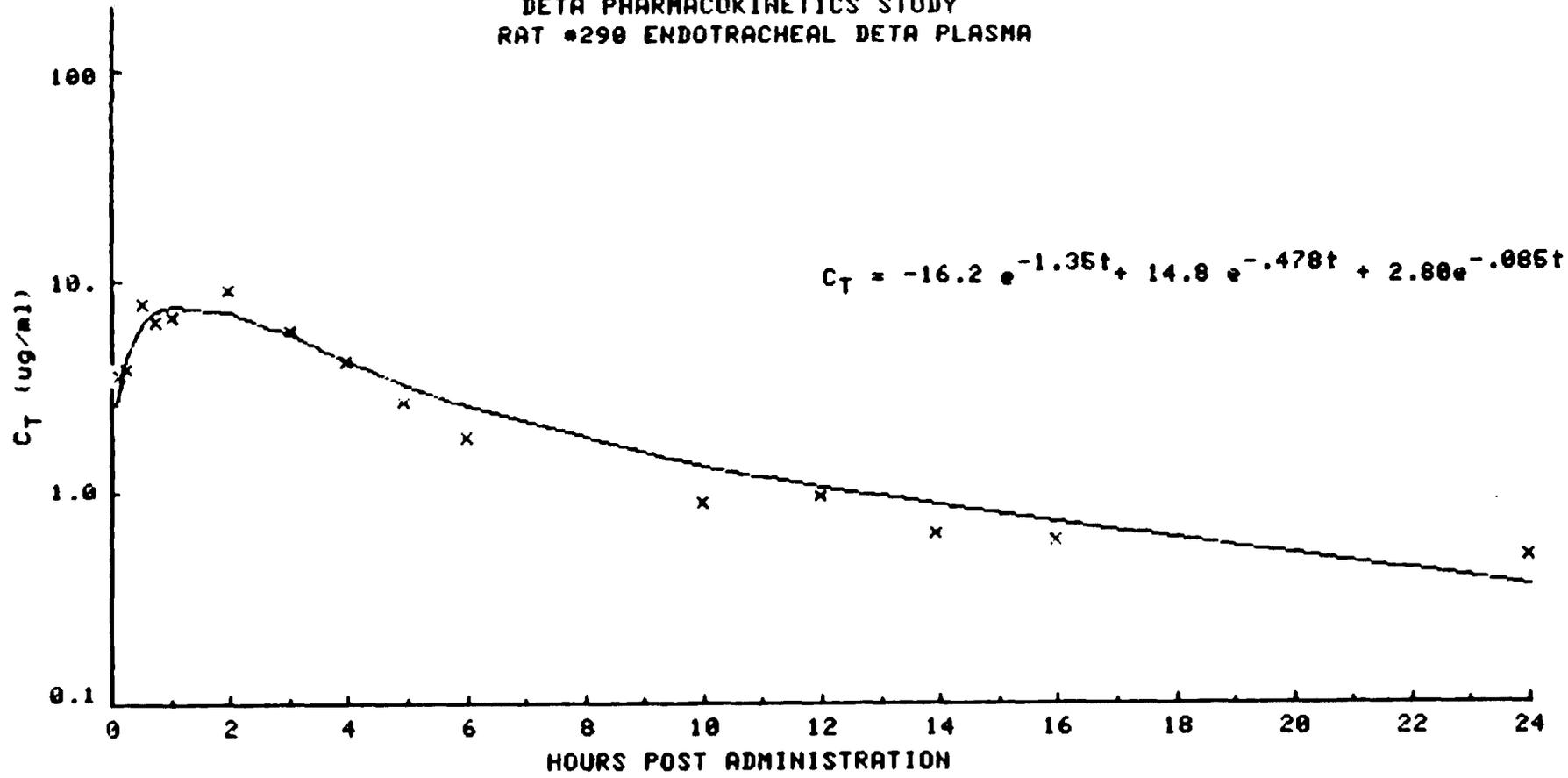
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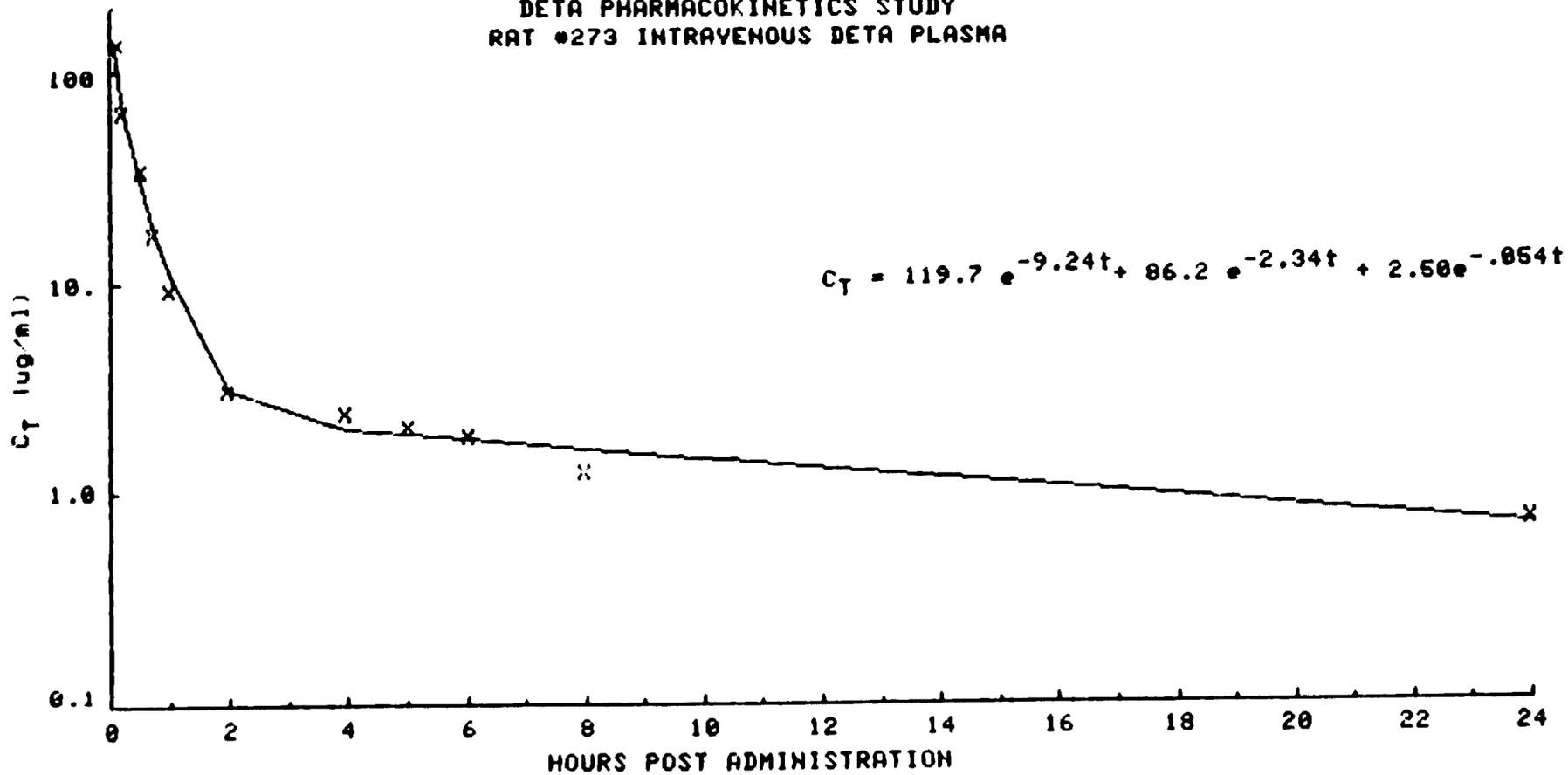


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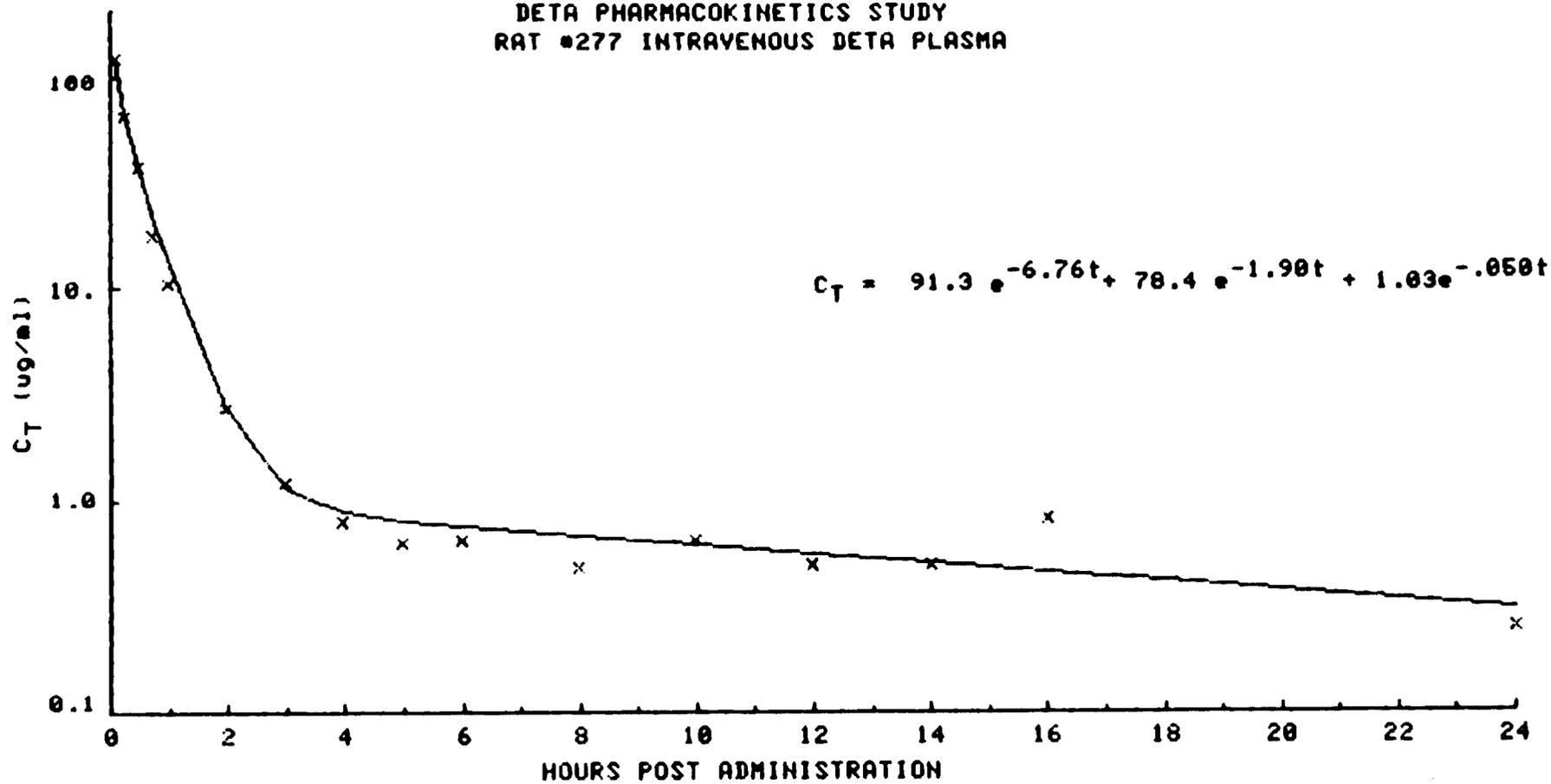


DETA PHARMACOKINETICS STUDY
RAT #273 INTRAVENOUS DETA PLASMA



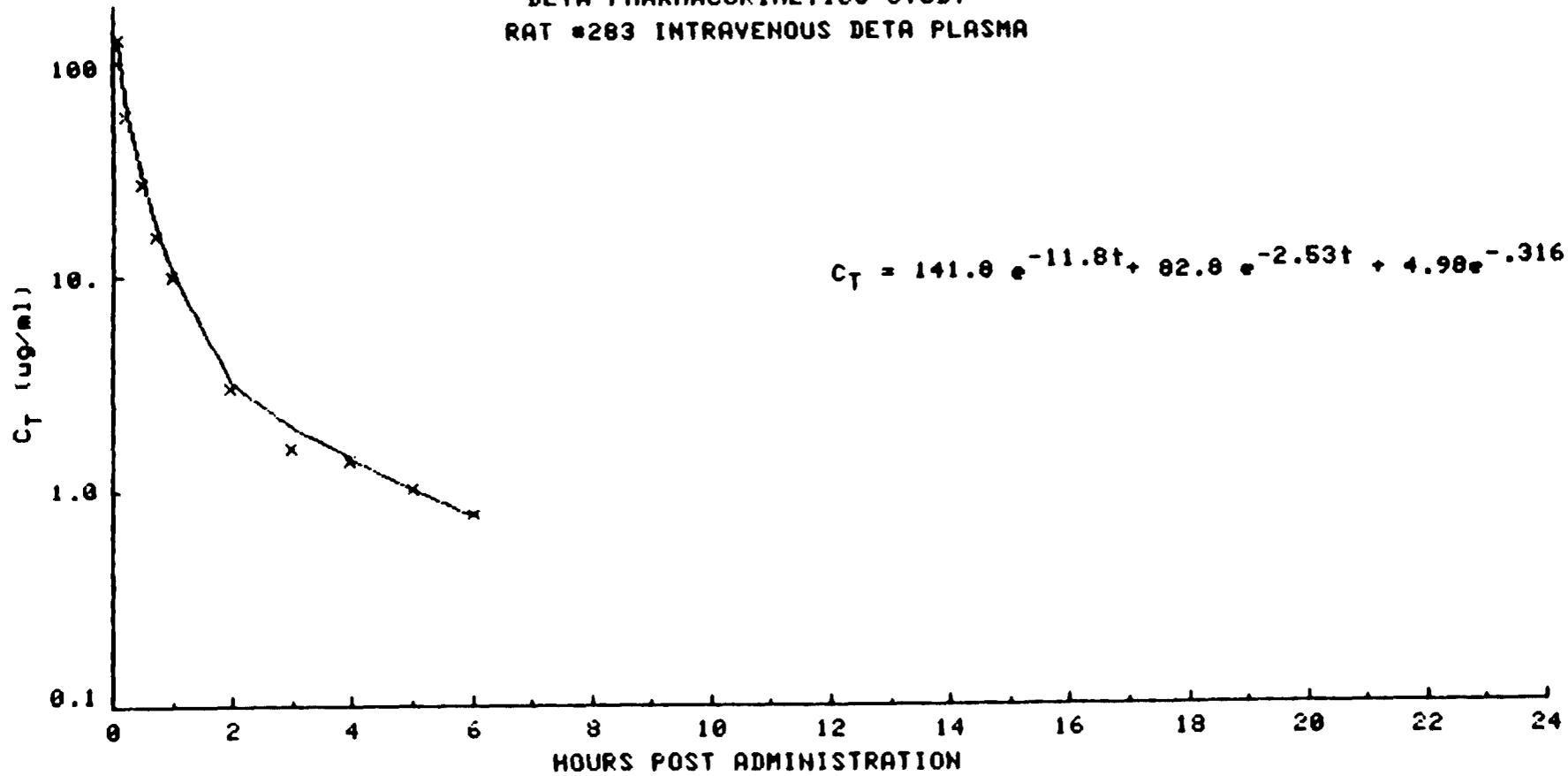
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RAT #277 INTRAVENOUS DETA PLASMA



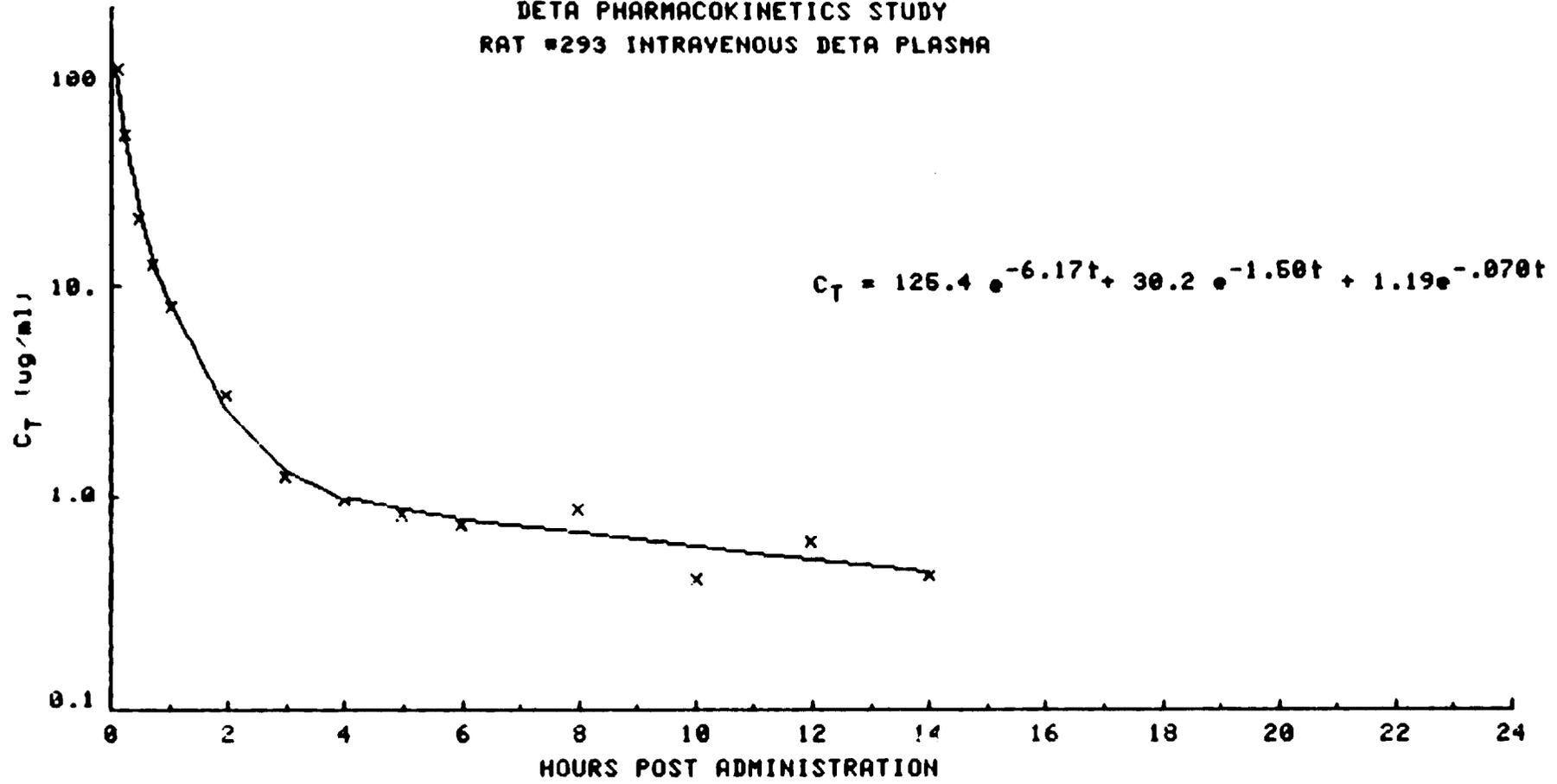
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DETA PHARMACOKINETICS STUDY
RAT #283 INTRAVENOUS DETA PLASMA



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DETA PHARMACOKINETICS STUDY
RAT #293 INTRAVENOUS DETA PLASMA



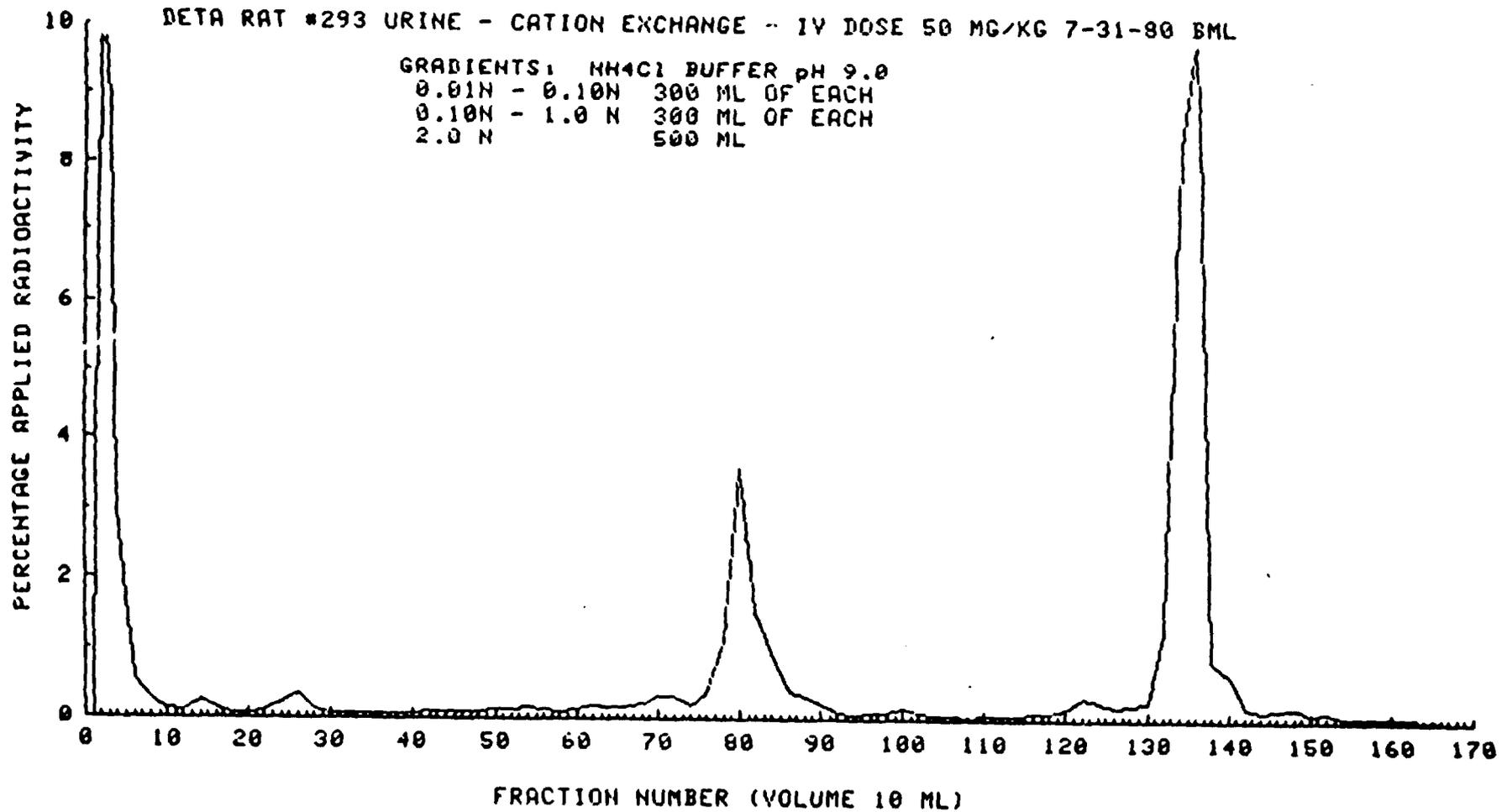
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APPENDIX XII

Chromatogram of Urinary Radioactivity from IV Dose Rat

(1 Page)

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Quality Assurance Unit Study Inspection Summary

Test Substance: Diethylenetriamine

Study: Pharmacokinetics and Metabolism Study in Rats

Study Director: Dr. T. R. Tyler

The Quality Assurance Unit has conducted the following inspections and reported the results to the Study Director and to Management on the dates indicated.

<u>Date</u>	<u>Inspection Type</u>	<u>Date QAU Report Issued</u>	
		<u>To Study Director</u>	<u>To Management</u>
5-5-80	Protocol	5-5-80	5-6-80
7-25-80	Ongoing	7-25-80	8-28-80
2-12 to 2-19-81	Final Data and Final Report	2-19-81	5-26-81

David L. Henry (in) *May 26, 1981*
 Quality Assurance Officer Date

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Doc Title	AMENDMENT 1 TO PHARMACOKINETICS AND METABOLISM OF DIETHYLENETRIAMINE IN THE RAT			23
Chemical Name (300 per name)	25	CAS No. (10)	24	
DIETHYLENETRIAMINE		111-4φ-φ		

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9/1/82



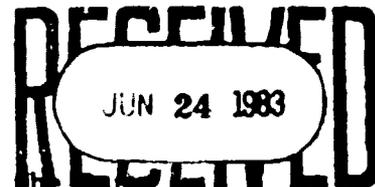
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Report 44-47, Amendment 1
19 Pages
August 13, 1981
Tel: (412) 327-1020



Amendment 1 to

Pharmacokinetics and Metabolism of Diethylenetriamine in the Rat

Sponsor: Union Carbide Corporation

1. Original Item Changed and Description of the Change:

Replace Table 2 with the accompanying table.

Reason for the change:

To correct typographical error made in total row for 500 mg/kg endotracheal column. This change makes Table 2 and Appendix III, Page 1, consistent.

2. Original Item Changed and Description of the Change:

Replace Table 6 with the accompanying table.

Reason for the change:

The necessity for this change results from a discrepancy in the terminal half-life values between the original Table 6 and those listed in Appendix X. The values listed in Appendix X have been verified and were generated from the ESTRIP program after appropriate selection of plasma concentration time data. The final selection of plasma concentration time data was such that the subsequent pharmacokinetic analysis would result in considerable improvement in F values and correlation coefficients.

The source of the values listed in the original Table 6 was attributed to the preliminary pharmacokinetic analysis by visual and graphical methods. Those data were subsequently refined using computer analysis (ESTRIP). Statistical analysis was conducted using values listed in Appendix X. The replacement of the original Table 6 with the accompanying revised table does not result in any change in interpretation of this report.



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Report 44-47, Amendment 1, Page 2
August 13, 1981

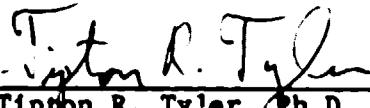
3. Original Item Changed and Description of the Change:

Replace Appendix XI, page 8 with the accompanying revised Figure.

Reason for the change:

The original Figure in Appendix XI, page 8 was generated by using all plasma concentrations time points collected for rat #288. Subsequently time points 2, 10, 12 and 14 hours were removed from consideration. The elimination of these data points overcame the unrealistic anomaly (Terminal $T_{1/2} = 173$ hours) which existed in the original pharmacokinetic analysis when all data points were used. The reason for this was probably larger analytical errors in the later time points. Replacing this figure allows for consistency between Appendix X and Appendix XI. This change in no way changes the interpretation of the report.

Reviewed and Approved by:



Tipton R. Tyler, Ph.D.
Study Director



Elton R. Homan, Ph.D.
Associate Director, Toxicology



Fred R. Frank, Ph.D.
Director

WPC/esk/1138-4
08-12-81

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Table 2
Radioactivity Balance in Animals Dosed with ¹⁴C Labeled DETA

	Oral		Endotracheal	
	50 mg/kg	500 mg/kg	50 mg/kg	500 mg/kg
<u>Percentage of Dosed Radioactivity</u>				
Urine	31	43	32	40
Feces	46	44	40	45
CO ₂	1.1	0.5	1.3	0.6
Cage Washings	5.4	10	8.0	14
Carcass	2.0	1.8	3.1	2.1
Total	85	100	84	103

WPC/1138-2

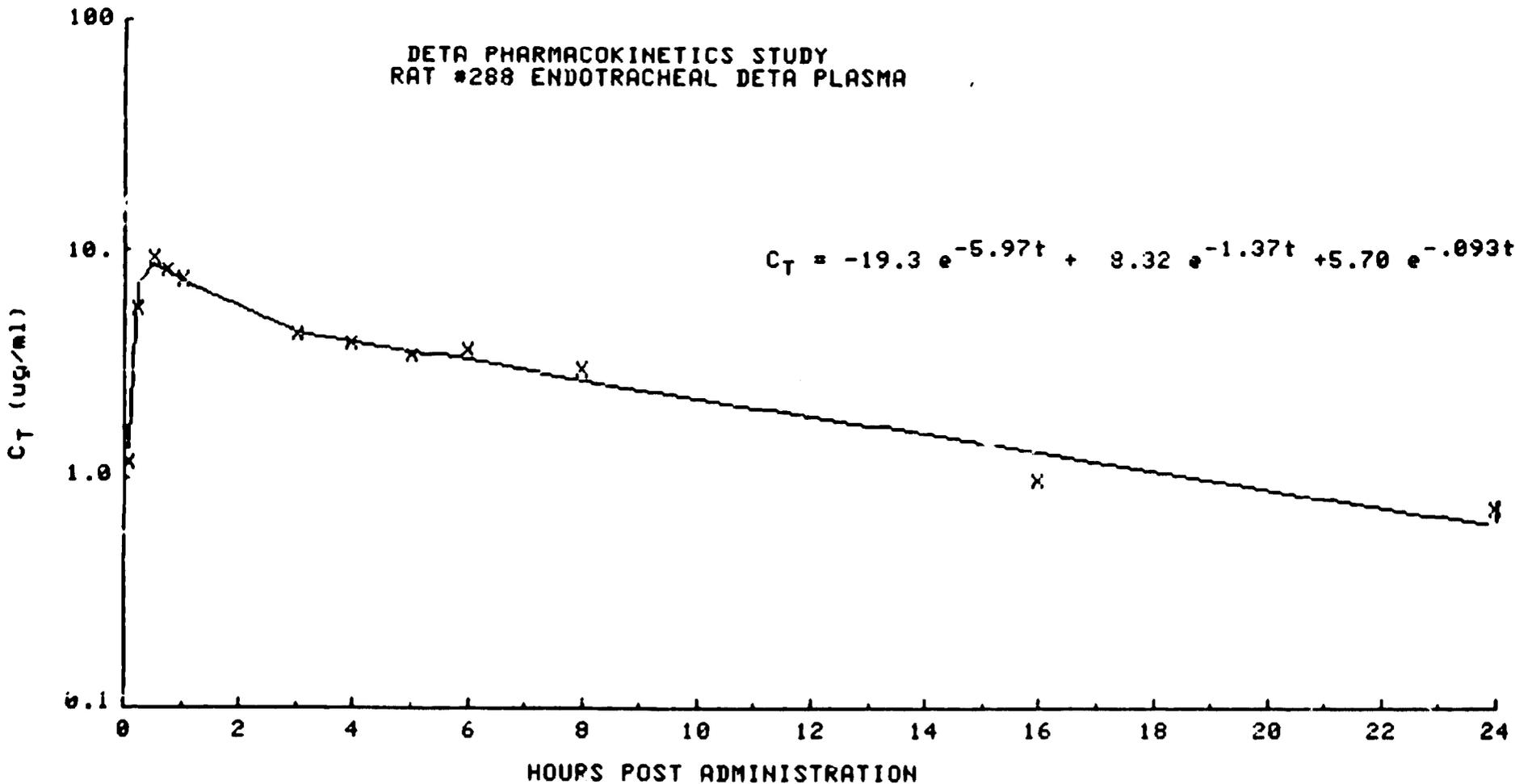
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Table 6
DETA Pharmacokinetic Parameters in Rats

<u>Parameter</u>	<u>Oral</u>	<u>Endotracheal</u>	<u>Intravenous</u>
Bioavailability	0.95	0.90	1
Total Clearance (ml hr ⁻¹)	134	138	174
Terminal Half-Life (hr)	16.3	9.01	9.70

WPC/1138-2

DETA PHARMACOKINETICS STUDY
RAT #288 ENDOTRACHEAL DETA PLASMA



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R. D. 4, Mellon Road, Export, Pennsylvania 15632

Telephone (412) 327-1020

Quality Assurance Unit Study Inspection Summary

Test Substance: Diethylenetriamine

Study: Pharmacokinetics and Metabolism in the Rat

Study Director: T. R. Tyler

The Quality Assurance Unit of BRRC conducted the inspections listed below and reported the results to the study director and to management on the dates indicated. It is the practice of this Quality Assurance Unit to report the results of each inspection to both the study director and management.

<u>Date</u>	<u>Inspection Type</u>	<u>Date QAU Report Issued</u>	
		<u>To Study Director</u>	<u>To Management</u>
5-5-80	Protocol	5-5-80	5-6-80
7-25-80	Ongoing	7-25-80	8-28-80
2-12 to 2-19-81	Final Data and Report	2-19-81	5-26-81
7-24 to 8-14-81	Report Amendment 1	8-14-81	8-14-81

Daniel G. Henry (JGC) 8-14-81
 Quality Assurance Officer Date

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Doc Title	DIETHYLENETRIAMINE-COMMERCIAL DERMAL CARCINOGENESIS STUDY IN MALE C3H/HEJ MICE			23
Chemical Name (300 per name)	25		CAS No. (10)	24
DIETHYLENETRIAMINE			111-40-0	

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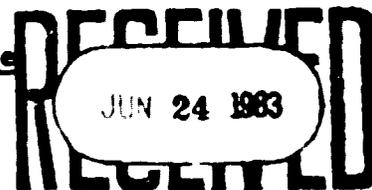
Project Report 45-82
7 Pages
Tel: (412) 327-1020
November 11, 1982

Diethylenetriamine-Commercial

Dermal Carcinogenesis Study in Male C3H/HeJ Mice

Authors: P. J. Guzzie and L. R. DePass

Sponsor: Union Carbide Corporation (UCC)
Ethylene Oxide Derivatives Division



Summary

The dermal carcinogenic potential of diethylenetriamine-commercial (DETA-C) was assessed by applying 25 ul of a 5% (v/v) solution in deionized water to the backs of 50 male C3H/HeJ mice. A negative control group was dosed with deionized water. Both applications were performed three times a week until the death of the animals. No treatment related skin tumors were observed in the groups treated with DETA-C or with deionized water. One mouse in the negative control group had a sebaceous adenoma of the skin of the thorax. A subcutaneous cavernous hemangioma was observed in a DETA-C-treated animal, but its appearance was not considered biologically important. No statistically significant difference in mortality rates was observed between the treated group and the deionized water control group. DETA-C was not locally carcinogenic when applied to the skin of C3H/HeJ mice.

Objective

The objective of this study was to determine the dermal neoplasm producing potential of DETA-C.

Samples

Two parent samples of DETA-C (CAS #111-40-0) were received from UCC, South Charleston, WV to be used for testing throughout this study. The first sample, reference no. 1-PRU-50 was received on February 23, 1979 as a 1 quart sample and was assigned BRRC No. 42-103. On August 21, 1980, an additional one pint sample was received as reference no. 1 PRU-50 and was designated as BRRC sample no. 43-257.

000002

Analytical data for the first sample were supplied by P. R. Umberger, Union Carbide Corp., South Charleston, WV. Preshipment analyses using a derivative method of gas chromatography, showed the major components of DETA-C to be 90.8% DETA, 8.9% N-(2, aminoethyl)piperazine, and 0.34% ethylenediamine.

The reports of analyses performed on the second sample prior to shipping and the subsequent analyses of the parent samples at 6, 18 and 24 months of dosing, have not yet been received from UCC, South Charleston, WV. The stability analyses of diluted DETA-C using NMR spectroscopy and titration analysis showed that DETA-C in water was stable for at least one month. Monthly samples of the diluted DETA-C, used for dosing, were sent to UCC, South Charleston, WV for titration and NMR analyses in order to determine whether concentrations used for dosing were accurate. Reports indicate slight concentration differences in the samples over the dosing period. Titration analysis showed values ranging from 3.82 to 5.55 wt % with a mean value of 5.15 ± 0.33 wt %.

The solvent for this study was water (CAS #7732-18-5) that was deionized in a Milli-Q reagent-grade water system (#Z02011574, Millipore Corp., Bedford, MA).

Experimental Methods

The mice used in this study were part of a large shipment to be used in various dermal carcinogenesis studies. C3H mice were used in this study because of their low incidence of spontaneous skin tumors. Deionized water was selected for use as the negative control substance because no skin tumors have ever been observed at BRRC in approximately 30 years of skin painting of C3H mice with water. A total of 1797 C3H/HaJ male mice were received (763 were received on January 31, 1979, and 1034 were received on February 27, 1979) from Jackson Laboratories, Bar Harbor, ME. The mice were housed individually in stainless steel wire suspension cages. Zeigler block feed (Zeigler Bros. Inc., Gardners, PA) and water (Municipal Water Authority of Westmoreland County, Greensburg, PA) from an automatic watering system were provided for the animals ad libitum. All mice were identified by a toe-clipping method according to preassigned, unique identification numbers.

Mice selected for randomization were within two standard deviations from the mean with respect to their individual weights. The mice were randomized into test groups, of which two groups of 50 mice per group were assigned to this study. The randomization was statistically verified by the Bartlett's test for homogeneity of variances and the analysis of variance (Sokal and Rohlf, 1979). The two groups were assigned to receive either DETA-C or deionized water, and appropriate cage cards were prepared. The body weights of the mice assigned to the DETA-C group weighed from 18.6 to 27.8 grams and the deionized water group weighed from 17.2 to 27.8 grams on the day of randomization. Ten mice from each group were randomly selected and predesignated for interim sacrifice after eighteen months of dosing. The remaining mice not randomized into these test groups were culled, used as quality control sentinels or assigned to other skin carcinogenesis studies.

As a preliminary assessment of toxicity, groups of five C3H/HeJ mice were dosed daily for 10 days with 25 ul of solutions of 5% or 10% (v/v) of DETA (BRRC sample no. 41-259) in deionized water. The preliminary test indicated that DETA-C should be applied as a 5% concentration. This dilution of DETA was relatively non-irritating and non-toxic to the mice in the preliminary study and, therefore, was used in the chronic study.

Testing was initiated on March 12, 1979 when the mice were approximately 46 to 77 days of age. On Tuesday or Thursday of each week, the fur was clipped from the back of each mouse. Mice were treated three times weekly, following a Monday, Wednesday, and Friday treatment schedule (holidays excepted). A 25 ul dose was applied using an Eppendorf automatic pipette, by spreading the aliquot up the back of each mouse with a clean disposable tip. Mice were observed daily for mortality and were carefully examined monthly for lesions of the skin. Necropsies were performed on all mice shortly after death or after sacrifice of culled and moribund animals. Necropsy included the careful examination of the skin and body cavities, and the recording of observations. All suspect tumors and the dorsal skin of all mice, with or without tumors, were fixed in 10% neutral buffered formalin (NBF). In addition, all livers, kidneys and lungs, unless autolyzed, were fixed in NBF for possible histopathologic examination. Sections were prepared from the dorsal skin of all mice and any suspect internal tumors. Histopathologic examinations were performed and reported (see Pathology Report, attached as Appendix I, for details).

Mortality incidences were assessed by the product-limit method (Kaplan and Meier, 1958). The Mantel-Cox and Breslow statistics were used for testing the equality of the survival curves (Mantel, 1966; Breslow, 1970).

Records and Raw Data

All records, raw data, specimens and final report will be stored in the BRRC archives upon completion of this report.

Deviation From the Protocol

The Study Director, L. G. Peterson, left this laboratory on August 13, 1981. L. R. DePass, Manager of Oral/Dermal Toxicology, assumed his duties as Study Director.

Results

The results of this study are summarized in Table I and a survival curve is presented as Figure I. In the group that received DETA-C as a 5% (v/v) dilution in deionized water the mean survival time was not statistically different from that of the deionized water control group (662 versus 626 days, respectively) by the Mantel-Cox method or the Breslow statistical method. No treatment-related papillomas or carcinomas were observed on the skin of any mice in either the TEPA-treated group or the deionized water control group.

A subcutaneous mass was observed in the dorsal cervical region of a DETA-C-treated mouse from the 18 month interim sacrifice and was diagnosed as a subcutaneous cavernous hemangioma. However, no biological importance is placed on the appearance of this lesion and it was not considered toxicologically significant because related tumors have been observed in historical controls. A sebaceous adenoma of the skin of the thorax was observed in a deionized water control mouse.

The last surviving mouse in the DETA-C-treated group died on May 31, 1981. The last mouse in the deionized water control group died on May 23, 1981.

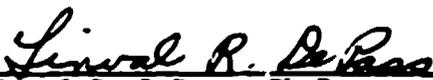
Conclusion

The results of the present study indicated that DETA-C was not carcinogenic to the skin of male C3H/HeJ mice when applied as a 5% (v/v) solution in deionized water until the death of the animals.

References

- Breslow, N. A generalized Kruskal-Wallis test for comparing k samples subject to unequal patterns of censorship. *Biometrika*. 57: 579-594, 1970.
- Kaplan, E. L. and P. Meier. Nonparametric estimation from incomplete observations. *J. Amer. Statist. Assoc.* 53: 457-481, 1958.
- Mantel, N. Evaluation of survival data and two new rank order statistics arising in its consideration. *Cancer Chemotherapy Reports*. 50: 163-170, 1966.
- Sokal, R. R. and F. J. Rohlf. Biometry. W. M. Freeman and Company, San Francisco, CA, 1969.

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Table I
Summary of Results of
Skin Carcinogenesis Test of DETA-C

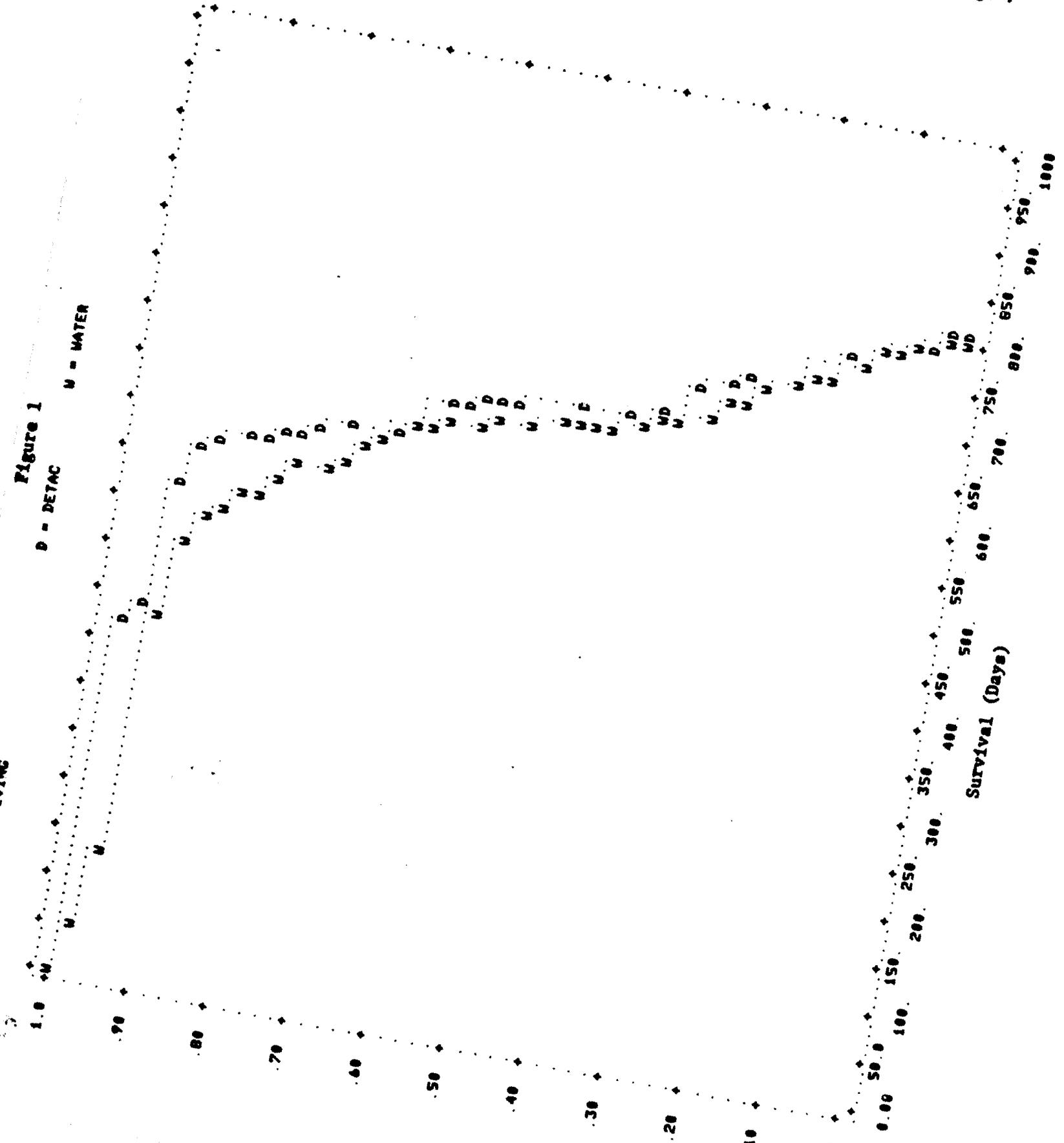
	DETA-C	Deionized Water
Concentration	5%	100%
Volume (ul/application)	25	25
Mean Survival Time (days)	662	626
Tumor Bearing Animals	1*	1**

* A subcutaneous cavernous hemangioma of the dorsal cervical region.
** A sebaceous adenoma of the skin of the thorax

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Figure 1
D - DETAC
M - WATER



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BUSHY RUN RESEARCH CENTER

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Quality Assurance Unit Study Inspection Summary

Test Substance: Diethylenetriamine - Commercial

Study: Dermal Carcinogenesis in Male C3H/HeJ Mice

Study Director: L. R. DePass, Ph.D.

The Quality Assurance Unit of BRRC conducted the inspections listed below and reported the results to the study director and to management on the dates indicated. It is the practice of this Quality Assurance Unit to report the results of each inspection to both the study director and management.

<u>Date</u>	<u>Inspection Type</u>	<u>Date QAU Report Issued</u>	
		<u>To Study Director</u>	<u>To Management</u>
3-9-79	Protocol	3-9-79	3-9-79
11-13 to 11-16-79	In Progress	11-16-79	1-28-79
2-13 to 3-3-80	In Progress	3-3-80	4-22-80
5-5 to 5-14-80	In Progress	5-14-80	5-21-80
10-6 to 10-9-80	In Progress	10-9-80	10-15-80
3-20 to 3-27-81	In Progress	3-27-81	4-1-81
5-14 to 5-25-82	Anatomic Pathology Data and Report	5-25-82	6-23-82
5-14 to 5-25-82	Final Data	5-25-82	5-26-82
8-17 to 8-18-82	Final Report	8-18-82	11-10-82

D.L.G:acc

Daniel L. Geary
 Daniel L. Geary, M.S. 11/10/82
 Manager, Quality Assurance Date

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**REPORT 45-82
APPENDIX I**

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ANATOMIC PATHOLOGY REPORT

DERMAL CARCINOGENESIS LIFETIME SKIN PAINTING IN MICE

OF

DIETHYLENTRIAMINE-COMMERCIAL (DETA-C)

Table of Contents

**Summary and Conclusions, Introduction, Methods, Results and Discussion,
References, Acknowledgements**

- I. Table of Gross Findings - 18-Month Interim Sacrifice**
- II. Table of Histologic Findings - 18-Month Interim Sacrifice**
- III. Table of Gross Findings - Lifespan Study**
- IV. Table of Histologic Findings - Lifespan Study**
- V. Individual Animal Pathology Records**
 - a. DETA-C - 18-Month Sacrifice**
 - b. DETA-C - Lifespan Study**
 - c. Deionized Water (negative control) - 18-Month Sacrifice**
 - d. Deionized Water (negative control) - Lifespan Study**

**NOTE: This Pathology Report begins on page 2 5 2 6 6 and ends on
page 2 5 2 7 6.**

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Summary and Conclusions

Male C3H/HeJ mice were randomized into groups of 50 and treated three times weekly (holidays excepted) for 18 months (8 mice) or throughout their lifetime (42 mice) with a dilution of DETA-C in deionized water which was applied to the clipped skin of their backs. A negative control group consisting of a randomized group of 50 mice was treated three times weekly for 18 months (9 mice) or throughout their lifetime (41 mice).

One subcutaneous nodule subsequently diagnosed as a cavernous hemangioma was found in a DETA-C-treated mouse sacrificed following 18 months of treatment. One nodule was also found in a negative control mouse at the end of 18 months which was histologically diagnosed as a sebaceous adenoma.

In the lifetime study no skin or subcutaneous neoplasms were found in the DETA-C-treated mice or in the negative control mice .

Other gross and microscopic findings in the 18-month interim sacrifice mice and the lifetime studies for the DETA-C and negative control mice were consistent with the spontaneous background incidence of inflammatory and neoplastic lesions in this strain used in lifetime studies.

Introduction

The purpose of this study was to determine the dermal neoplastic potential of DETA-C by applying it to the skin of male C3H/HeJ mice over the period of their lifetime and determining the gross and microscopic appearance of the resulting lesions.

Methods

Male C3H/HeJ mice, 4-6 weeks of age were obtained from Jackson Laboratories, Bar Harbor, ME. The mice were randomized into groups of 40 or 50 mice. Two groups of 50 male mice received either DETA-C or deionized water three times weekly, with the exception of holidays, applied with an Eppendorf pipette to the clipped skin of the back. Of the ten mice per group predesignated for the 18-month interim sacrifice, eight of the DETA-C-treated mice and nine of the deionized water-treated mice were sacrificed following 18 months of treatment. The remaining animals in these two groups were allowed to live out their lifespan with continued thrice weekly treatments. The predesignated animals which died prematurely prior to the 18-month sacrifice were also considered to have lived out their lifespan.

Following the sacrifice or death of each mouse a gross necropsy was performed. All body cavities were examined and all suspect internal tumors were fixed in 10% neutral buffered formalin (NBF) unless the degree of autolysis precluded saving the tissues for histologic examination. The dorsal skin of all mice, with or without skin tumors, was also fixed in 10% NBF for histologic examination unless the mouse was severely autolyzed. In addition, liver, kidneys and lungs of all animals were fixed in 10% NBF and those from mice sacrificed after 18 months were examined histologically.

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Tissues fixed in 10% NBF were carefully trimmed, embedded, sectioned and stained with hematoxylin and eosin for examination by a pathologist. All neoplastic and non-neoplastic lesions discovered during the histopathologic examination were recorded and tabulated.

Results and Discussion

The frequency of gross lesions found in the 18-month interim sacrificed mice is included in Table 1. One skin nodule was found in one of the eight DETA-C-treated mice as well as in one of the nine negative control mice treated with deionized water. Various other lesions were encountered in both the DETA-C-treated and the deionized water-treated mice in these sacrifice groups. These lesions are consistent with spontaneous lesions found in aging mice of this strain.

The histologic findings for the 18-month interim sacrifice are included in Table 2. The skin nodule found in the DETA-C-treated mouse was diagnosed as a cavernous hemangioma. No biological importance is placed on the appearance of this lesion. The nodule found grossly in the deionized water-treated negative control group was diagnosed as a sebaceous adenoma. Concomitant with the sebaceous adenoma was epidermal hyperplasia. The histologic lesions found in the other tissues examined are consistent with the spontaneous background lesions seen in this strain of mouse.

The frequency of gross findings for the mice included in the lifetime portion of this study are included in Table 3. No skin neoplasms or nodules were found in the DETA-C-treated mice or in the negative control mice.

The gross lesions encountered in the other organs were all considered part of the spontaneous background lesions frequently seen in mice of this strain used on lifetime studies.

The frequency of histologic findings for the mice included in the lifetime portion of this study are included in Table 4. No skin or subcutaneous neoplasms were found in the DETA-C-treated mice or in the deionized water-treated negative control mice. Lesions diagnosed in the other organs, including the neoplasms, were considered part of the spontaneous background lesions encountered in this strain of mice in lifetime studies.

The histologic changes observed in the adrenals of the DETA-C-treated and negative control mice reflect the gross observations, but it should be remembered that only adrenals with lesions observed at necropsy were examined histologically. Cortical hyperplasia/adenoma appeared in nearly all adrenals examined. Dunn (1970) considered the distinction between hyperplasia and neoplasia in mouse adrenals to be very difficult and somewhat arbitrary.

Lesions seen in the male urogenital system are primarily related to the Proteus mirabilis infection which is frequently encountered in this strain from this vendor (Maronpot and Peterson, 1981).

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Liver neoplasms are frequently encountered in this strain and therefore no significance can be attributed to their appearance in the treated or control mice.

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Edward H. Fowler, DVM, Ph.D.
Pathologist

8/12/82
Date

References

- Dunn, T. B. Normal and Pathologic Anatomy of the Adrenal Gland of the Mouse, Including Neoplasms. J. Natl. Cancer Inst. 44: 1323-1389, 1970.
- Maronpot, R. R. and L. G. Peterson. Spontaneous Proteus Nephritis Among Male C3H/HeJ Mice. Lab. Anim. Sci. 31: 697-700, 1981.

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06-09-82

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28271

TABLE 1

Diethylenetriamine-Commercial (DETA-C): Dermal Carcinogenesis Study

Frequency of Gross Findings Among C3H/HeJ Male Mice

18-Month Interim Sacrifices

<u>ORGANS/Findings</u>	<u>Diethylene- triamine- Commercial</u>	<u>Deionized Water (Negative Control)</u>
TOTAL NUMBER EXAMINED	8	9
SKIN/SUBCUTIS, NGL	7/8*	8/9
/Modules/Mass	1/8	1/9
ADRENALS, NGL	1/8	2/9
/Color change	7/8	7/9
/Enlarged/Modular	2/8	0/9
LUNGS, NGL	7/8	5/9
/Color change	1/8	4/9
LIVER, NGL	5/8	6/9
/Color change	1/8	1/9
/Enlarged/Modular	1/8	1/9
/Mass(es)	2/8	2/9
GALL BLADDER, NGL	7/8	9/9
/Enlarged	1/8	0/9

NGL = No gross lesions.

*Numerator equals number of mice with specified finding.

Denominator equals number of mice for which specified organ was examined.

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TABLE 2

Diethylenetriamine-Commercial (DETA-C): Dermal Carcinogenesis Study

Frequency of Histologic Findings Among C3H/HeJ Male Mice

18-Month Interim Sacrifice

<u>ORGANS/Findings</u>	<u>Diethylene- triamine- Commercial</u>	<u>Deionized Water (Negative Control)</u>
<u>SKIN/SUBCUTIS, NSL</u>	7/8*	8/9
/Epidermal hyperplasia/hyperplasia	0/8	1/9
/Sebaceous adenoma	0/8	1/9
/Hemangioma, cavernous	1/8	0/9
<u>ADRENALS, NSL</u>	0/7	0/7
/Brown degeneration	0/7	1/7
/Cortical hyperplasia/adenoma	7/7	7/7
/Cortical carcinoma	1/7	0/7
<u>LUNGS, NSL</u>	8/8	2/2
<u>KIDNEYS, NSL</u>	2/8	-
/Tubular cysts	1/8	-
/Tubular hyperplasia	5/8	-
<u>LIVER, NSL</u>	5/8	1/4
/Congestion/Sinusoidal distention	1/8	0/4
/Vascular thrombosis/ectasia	1/8	1/4
/Triaditis	0/8	1/4
/Hepatocellular hyperplasia	0/8	1/4
/Hepatocellular carcinoma	3/8	2/4

NSL - No significant lesions.

*Numerator equals number of mice with specified finding.

Denominator equals number of mice for which specified organ was examined.

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TABLE 3

Diethylenetriamine-Commercial (DETA-C): Dermal Carcinogenesis Study

Frequency of Gross Findings Among C3H/HeJ Male Mice

Lifespan Study

<u>ORGANS/Findings</u>	<u>Diethylene- triamine- Commercial</u>	<u>Deionized Water (Negative Control)</u>
TOTAL NUMBER EXAMINED GROSSLY	42	41
SKIN/SUBCUTIS, NGL	35/42^a	38/41
/Edema	3/42	3/41
/Surface alteration	3/42	0/41
ADRENALS, NGL	10/42	13/41
/Color change	33/42	25/41
/Enlarged/Modular surface	3/42	1/41
/Autolysis	0/42	3/41
HEART, NGL	40/42	34/41
/Color change	1/42	0/41
/Enlarged	1/42	5/41
/Autolysis	0/42	2/41
SPLEEN, NGL	41/42	36/41
/Color change	0/42	1/41
/Enlarged/Firm	1/42	2/41
/Autolysis	0/42	3/41
MESENTERIC LYMPH NODES, NGL	42/42	37/41
/Color change	0/42	1/41
/Enlarged	0/42	1/41
/Autolysis	0/42	3/41
LUNGS, NGL	32/42	30/41
/Autolysis	1/42	2/41
/Color change	8/42	9/41
/Module/Mass	1/42	2/41
SEMINAL VESICLES, NGL	39/42	38/41
/Color change	3/42	0/41
/Enlarged/Mass	3/42	0/41
/Autolysis	0/42	3/41
SCROTAL SAC, NGL	41/42	38/41
/Mass	1/42	0/41
/Autolysis	0/42	3/41
KIDNEYS, NGL	37/42	32/41
/Autolysis	1/42	3/41
/Irregular shape	1/42	0/41
/Indentations, surface	0/42	1/41
/Color change	4/42	5/41
/Masses	0/42	1/41
URINARY BLADDER, NGL	39/42	37/41
/Constriction	0/42	1/41
/Distended	3/42	0/41
/Color change	1/42	1/41
/Autolysis	0/42	3/41

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TABLE 3
(Continued)

Diethylenetriamine-Commercial (DETA-C): Dermal Carcinogenesis Study

Frequency of Gross Findings Among C3H/HeJ Male Mice

Lifespan Study

<u>ORGANS/Findings</u>	<u>Diethylene- triamine- Commercial</u>	<u>Deionized Water (Negative Control)</u>
<u>LIVER, NGL</u>	16/42	14/41
/Autolysis	3/42	3/41
/Color change	5/42	2/42
/Enlarged/Firm/Thickened	1/42	1/41
/Node/Masses	21/42	22/41
<u>GALL BLADDER, NGL</u>	39/42	38/41
/Enlarged	3/42	0/41
/Autolysis	0/42	3/41
<u>THORACIC CAVITY, NGL</u>	39/42	37/41
/Fluid filled	3/42	1/41
/Autolysis	0/42	3/41
<u>ABDOMINAL CAVITY, NGL</u>	39/42	38/41
/Fluid filled	3/42	0/41
/Autolysis	0/42	3/41
<u>TAIL, NGL</u>	41/42	41/41
/Macrotic	1/42	0/41

NGL - No gross lesions.

*Numerator equals number of mice with specified finding.

Denominator equals number of mice for which specified organ was examined.

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TABLE 4

Diethylenetriamine-Commercial (DETA-C): Dermal Carcinogenesis Study

Frequency of Histologic Findings Among C3H/HeJ Male Mice

Lifespan Study

<u>ORGANS/Findings</u>	<u>Diethylene- triamine- Commercial</u>	<u>Deionized Water (Negative Control)</u>
SKIN/SUBCUTIS, NSL	41/42*	41/41
/Epidermitis	1/42	0/41
ADRENALS, NSL	1/34	0/25
/Autolysis	2/34	0/25
/Congestion	0/34	1/25
/Brown degeneration	7/34	9/25
/Cortical cyst	1/34	0/25
/Cortical hyperplasia/adenoma	33/34	25/25
HEART, NSL	1/2	4/5
/Autolysis	0/2	1/5
-/Thrombosis	1/2	0/5
SPLEEN, NSL	0/1	0/2
/Extramedullary hematopoiesis	1/1	1/2
/Lymphoid hyperplasia	0/1	1/2
MESENTERIC LYMPH NODES, NSL	-	0/1
/Congestion	-	1/1
LUNGS, NSL	2/9	2/9
/Autolysis	0/9	1/9
/Congestion	4/9	4/9
/Alveolar histiocytosis	2/9	1/9
/Pulmonary adenoma	0/9	1/9
/Pulmonary adenocarcinoma	1/9	1/9
SEMINAL VESICLES, NSL	3/3	-
SCROTAL SAC, NSL	0/1	-
/Angiosarcoma	1/1	-
KIDNEYS, NSL	1/5	1/5
/Congestion	1/5	0/5
/Hydronephrosis	0/5	1/5
/Interstitial nephritis	1/5	0/5
/Pyelonephritis	1/5	2/5
/Mineralization	1/5	0/5
/Tubular proteinosis	0/5	1/5
/Tubular hyperplasia	0/5	1/5
/Tubular adenoma	1/5	0/5
URINARY BLADDER, NSL	2/3	-
/Autolysis	1/3	-
/Cystitis	1/3	-
LIVER, NSL	2/27	0/25
/Autolysis	3/27	1/25
/Congestion/Sinusoidal distention	2/27	2/25
/Fibrosis	1/27	0/25
/Macrosis/Infarction	1/27	1/25

(Continued)

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**TABLE 4
(Continued)**

Methylenetriamine-Commercial (DETA-C): Dermal Carcinogenesis Study

Frequency of Histologic Findings Among C3H/HeJ Yale Mice

Lifespan Study

<u>ORGANS/Findings</u>	<u>Methylene- tri- amine- Commercial</u>	<u>Deionized Water (Negative Control)</u>
LIVER (Continued)		
/Hepatocellular necrosis	0/27	3/25
/Hepatocellular degeneration	0/27	1/25
/Hepatocellular adenoma	1/27	2/25
/Hepatocellular carcinoma	21/27	21/25
GALL BLADDER, NSL	1/1	-
TAIL, NSL	0/1	-
/Necrosis	1/1	-

NSL - No significant lesions.

***Numerator equals number of mice with specified finding.**

Denominator equals number of mice for which specified organ was examined.

**WPC/rkk/0280A-4
06-09-82**

Pathology Report page number 28277 through 28376 of this report contained individual pathology data sheets for each animal. Those sheets have not been included in this report in order to decrease costs of reproduction and distribution of reports. A copy of the report containing the individual data sheets was distributed to the Project Initiator and the original is on file in the Busby Run Research Center Archives.

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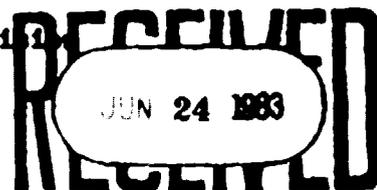
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Project Report 45-89
7 Pages
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Diethylenetriamine - High PurityDermal Carcinogenesis Study in Male C3H/HeJ Mice

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Sponsor: Union Carbide Corporation
Ethylene Oxide Derivatives Division

Summary

The dermal carcinogenic potential of diethylenetriamine-high purity (DETA-HP) was assessed by applying 25 ul of a 5% (v/v) solution in deionized water to the backs of 50 male C3H/HeJ mice. A negative control group was dosed with deionized water. Both applications were performed three times a week until the death of the animals. No tumors were observed in the group treated with DETA-HP. A sebaceous adenoma was diagnosed on the skin of the thorax of an animal in the deionized water control group. No significant difference in mortality rates was observed between the treated group and the deionized water control group. DETA-HP was not carcinogenic to the skin of C3H/HeJ mice under the conditions of this study.

Objective

The objective of this study was to determine the dermal carcinogenic potential of DETA-HP.

Samples

Two parent samples of DETA-HP (CAS #111-40-0) were received from UCC, South Charleston, WV to be used throughout the study. The first sample, reference no. 99 CAG-37-B was received on February 23, 1979 as a 4 ounce quantity and was assigned BRRC Sample No. 42-102. On August 21, 1980, a one pint sample was received as reference no. SPRU-35 and was designated as BRRC Sample No. 43-258.

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Analytical data for this first sample were supplied by P. R. Umberger, Union Carbide Corporation, South Charleston, WV. Freshment analyses using a derivative method of gas chromatography, showed DETA-HP to be a mixture consisting primarily of 96.77% DETA, 1.78% N-(2-aminoethyl)piperazine, 0.70% diethanolamine, 0.49% ethylenediamine, and 0.25% triethanolamine.

The reports of analyses performed on the second sample prior to shipping and the subsequent analyses of the parent samples at 6, 18 and 24 months of dosing, have not yet been received from UCC, South Charleston, WV. The stability analyses of diluted DETA-HP using NMR spectroscopy and titration analysis showed that the sample in water was stable for at least one month. Monthly samples of diluted DETA-HP used for dosing have been sent to UCC, Charleston, WV for titration and NMR analyses to determine whether the concentrations used for dosing were accurate. Reports received for these analyses indicate slight dilution differences in the samples over the dosing period. Titration analysis showed values ranging from 4.32 to 5.54 wt % with a mean value of 5.07 ± 0.26 wt %.

The solvent for this study was water (CAS #7732-18-5) that was deionized in a Milli-Q reagent-grade water system (#Z02011574, Millipore Corp., Bedford, MA).

Experimental Methods

The mice used in this study were part of a large shipment to be used in various dermal carcinogenesis studies. C3H mice were used in this study because of their low incidence of spontaneous skin tumors. Deionized water was selected for use as the negative control substance because no skin tumors have ever been observed at BRRC in approximately 30 years of skin painting of C3H mice with water. A total of 1797 C3H/HaJ male mice were received (763 were received on January 31, 1979, and 1034 were received on February 27, 1979) from Jackson Laboratories, Bar Harbor, ME. The mice were housed individually in stainless steel wire suspension cages. Zeigler block feed (Zeigler Bros. Inc., Gardners, PA) and water (Municipal Water Authority of Westmoreland County, Greensburg, PA) from an automatic watering system were provided for the animals ad libitum. All mice were identified by a toe-clipping method according to preassigned, unique identification numbers.

Mice selected for randomization were within two standard deviations from the mean with respect to their individual weights. The mice were randomized into test groups, of which two groups of 50 mice per group were assigned to this study. The randomization was statistically verified by the Bartlett's test for homogeneity of variances and the analysis of variance (Sokal and Rohlf, 1979). The two groups were assigned to received either DETA-HP or deionized water, and appropriate cage cards were prepared. The body weights of the mice assigned to the DETA-HP group ranged from 17.6 to 26.8 grams and those of the deionized water group ranged from 17.2 to 27.8 grams on the day of randomization. Ten mice from each group were randomly selected and predesignated for interim sacrifice after eighteen months of dosing. The remaining mice not randomized into these test groups were culled, used as quality control sentinels or assigned to other skin carcinogenesis studies.

In a preliminary study to determine the chronic dosing concentration, groups of 5 C3H/HeJ mice were dosed daily for 10 days with 25 ul of solutions of 5% or 10% DETA (BRRC Sample No. 41-259) in deionized water. The preliminary test indicated that 5% DETA-HP was relatively non-irritating and non-toxic to the mice in the preliminary study and, therefore, it was the concentration used in the chronic study.

Testing was initiated on March 12, 1979 when the mice were approximately 46 to 77 days of age. On Tuesday or Thursday of each week, the fur was clipped from the back of each mouse. Mice were treated three times weekly, following a Monday, Wednesday, and Friday treatment schedule (holidays excluded). A 25 ul dose was applied using an Eppendorf automatic pipette, by spreading the aliquot up the back of each mouse with a clean disposable tip. Mice were observed daily for mortality and were carefully examined monthly for lesions of the skin. Necropsies were performed on all mice shortly after death or after sacrifice of culled and moribund animals. Necropsy included the careful examination of the skin and body cavities, and the recording of observations. All suspect tumors and the dorsal skin of all mice, with or without tumors, were fixed in 10% neutral buffered formaline (NBF). In addition, all livers, kidneys, and lungs were fixed in NBF for possible histopathologic examination. Sections were prepared from the dorsal skin of all mice and any suspect internal tumors. Histopathologic examinations were performed and reported (see Pathology Report, attached as Appendix I, for details).

Mortality incidences were assessed by the product-limit method (Kaplan and Meier, 1958). The Mantel-Cox and Breslow statistics were used for testing the equality of the survival curves (Mantel, 1966; Breslow, 1970).

Records and Raw Data

All records, raw data, specimens and the final report will be stored in the BRRC archives upon completion of this report.

Deviation From the Protocol

The Study Director, L. G. Peterson, left this laboratory on August 13, 1981. L. R. DePass, Manager of Oral/Dermal Toxicology, assumed his duties as Study Director.

Results

The results of this study are summarized in Table I and a survival curve is presented as Figure I. In the group that received DETA-HP as a 5% (v/v) dilution in deionized water the mean survival time was not statistically different from that of the deionized water control group (587 versus 626 days, respectively). No papillomas or carcinomas were observed on the skin of any mice in either the DETA-HP-treated group or the deionized water control group. A sebaceous adenoma on the skin of the thorax was observed on a mouse in the deionized water control group.

The last mouse in the DETA-HP-treated group died on June 26, 1981. The last mouse in the deionized water control group died on May 23, 1981.

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Conclusion

The results of the present study indicate that DETA-HP was not carcinogenic to the skin of male C3H/HeJ mice when applied as a 5% (v/v) solution in deionized water until the death of the animals.

References

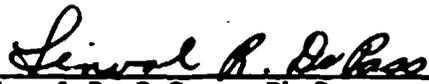
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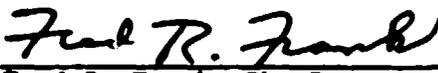
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Sokal, R. R. and F. J. Rohlf. Biometry. W. M. Freeman and Company, San Francisco, CA 1969.

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Table 1

Summary of Results of Skin Carcinogenesis Test of DETA-HP

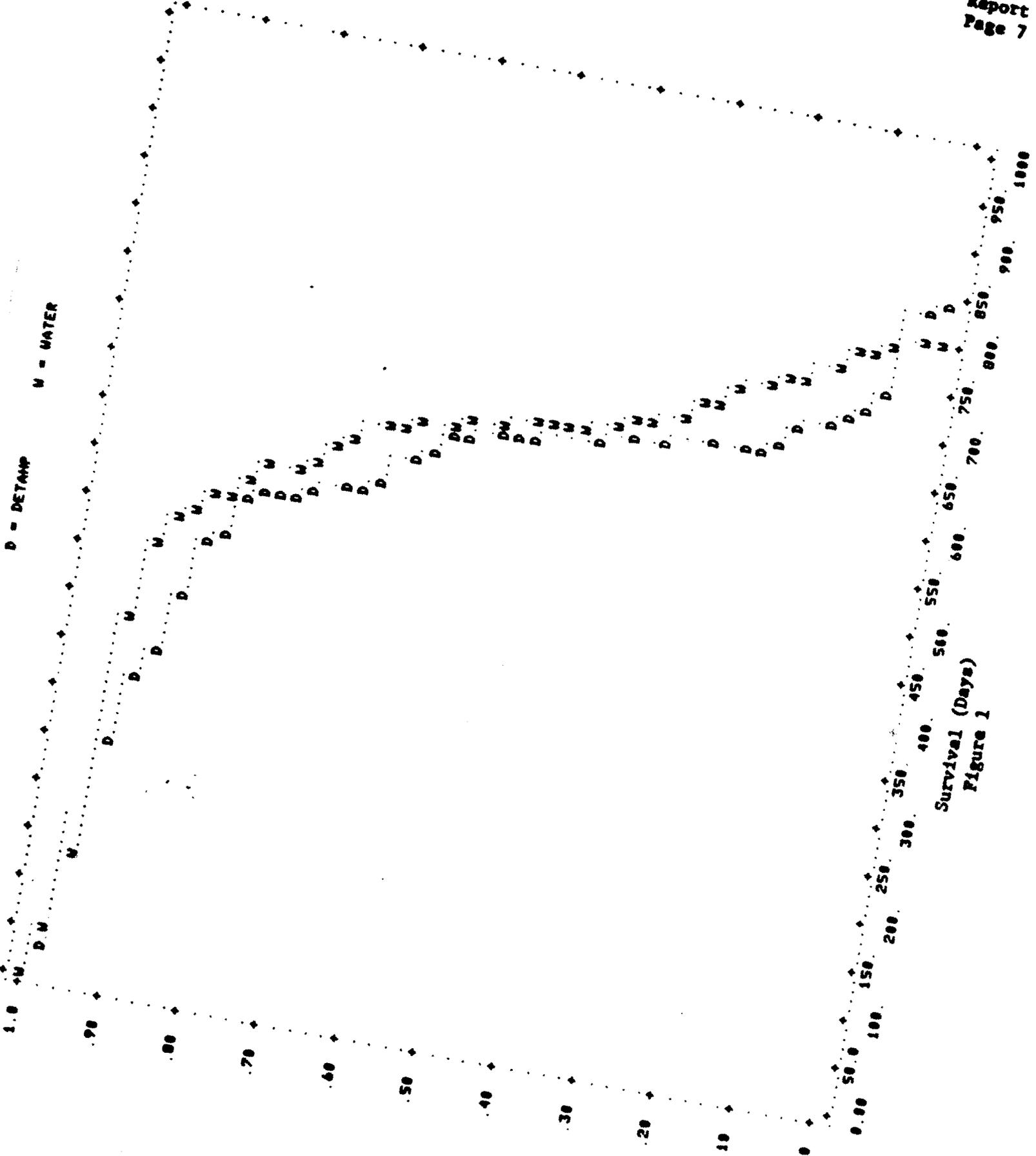
	DETA-HP	Deionized Water
Concentration	5%	100%
Volume (ul/application)	25	25
Mean Survival Time (days)	587	626
Tumor Bearing Animals	0	1*

*A sebaceous adenoma on the skin of the thorax.

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SURVIVING



Survival (Days)
Figure 1

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Quality Assurance Unit Study Inspection Summary

Test Substance: Diethylenetriamine - High Purity

Study: Dermal Carcinogenesis in Male C3H/HeJ Mice

Study Director: L. R. DePass, Ph.D.

The Quality Assurance Unit of BRRC conducted the inspections listed below and reported the results to the study director and to management on the dates indicated. It is the practice of this Quality Assurance Unit to report the results of each inspection to both the study director and management.

<u>Date</u>	<u>Inspection Type</u>	<u>Date QAU Report Issued</u>	
		<u>To Study Director</u>	<u>To Management</u>
3-9-79	Protocol	3-9-79	3-9-79
11-13 to 11-16-79	In Progress	11-16-79	11-28-79
2-13 to 3-3-80	In Progress	3-3-80	4-22-80
5-5 to 5-14-80	In Progress	5-14-80	5-21-80
10-6 to 10-9-80	In Progress	10-9-80	10-15-80
3-20 to 3-27-81	In Progress	3-27-81	4-1-81
5-21 to 5-26-82	Final Data	5-26-82	5-26-82
5-21 to 5-25-82	Anatomic Pathology Data and Report	5-25-82	6-24-82
10-7 to 10-11-82	Final Report	10-11-82	11-10-82

DLC:acc

Daniel L. Geary
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 Manager, Quality Assurance
 11/10/82
 Date

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28769 ANATOMIC PATHOLOGY REPORT

DERMAL CARCINOGENESIS LIFETIME SKIN PAINTING IN MICE

OF

Diethylenetriamine-High Purity (DETA-HP)

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- IV. Table of Histologic Findings - Lifespan Study**
- V. Individual Animal Pathology Records**
 - a. DETA-HP - 18-Month Sacrifice**
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**NOTE: This Pathology Report begins on page 2 8 7 6 9 and ends on
page 2 8 8 7 9.**

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Summary and Conclusions

Male C3H/HeJ mice were randomized into a group of 50 and treated three times weekly (holidays excepted) for 18 months (7 mice) or throughout their lifetime (43 mice) with a dilution of DETA-HP in deionized water which was applied to the clipped skin of their backs. A negative control group of similar size was treated three times weekly for 18 months (9 mice) or throughout their lifetime (41 mice).

No skin or subcutaneous tumors were found in the DETA-HP-treated mice sacrificed following 18 months of treatment. However, one nodule was found in a negative control mouse at the end of 18 months which was histologically diagnosed as a sebaceous adenoma.

In the lifetime study no skin or subcutaneous neoplasms were found in the DETA-HP-treated mice or in the negative control mice.

Other gross and microscopic findings in the 18-month interim sacrifice mice and the lifetime studies for the DETA-HP and negative control mice were consistent with the spontaneous background incidence of inflammatory and neoplastic lesions in this strain used in lifetime studies.

Introduction

The purpose of this study was to determine the dermal neoplastic potential of DETA-HP by applying it to the skin of male C3H/HeJ mice over the period of their lifetime and determining the gross and microscopic appearance of the resulting lesions.

Methods

Male C3H/HeJ mice, 4-6 weeks of age were obtained from Jackson Laboratories, Bar Harbor, ME. The mice were randomized into groups of 50 mice. Two groups of 50 male mice received either DETA-HP or deionized water three times weekly, with the exception of holidays, applied with an Eppendorf pipette to the clipped skin of the back. Of the ten mice per group predesignated for the 18-month interim sacrifice, seven of the DETA-HP-treated mice and nine of the deionized water-treated mice lived to term and were sacrificed following 18 months of treatment. The remaining animals in these two groups were allowed to live out their lifespan with continued thrice weekly treatments. The predesignated animals which died prematurely prior to the 18-month sacrifice were also considered to have lived out their lifespan.

Following the sacrifice or death of each mouse a gross necropsy was performed. All body cavities were examined and all suspect internal tumors were fixed in 10% neutral buffered formalin (NBF) unless the degree of autolysis precluded saving the tissues for histologic examination. The dorsal skin of all mice, with or without skin tumors, was also fixed in 10% NBF for histologic examination unless the mouse was severely autolyzed. In addition, liver, kidneys and lungs of all animals were fixed in 10% NBF and those from mice sacrificed after 18 months were examined histologically.

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Tissues fixed in 10% NBF were carefully trimmed, embedded, sectioned and stained with hematoxylin and eosin for examination by a pathologist. All neoplastic and non-neoplastic lesions discovered during the histopathologic examination were recorded and tabulated.

Results and Discussion

The frequency of gross lesions found in the 18-month interim sacrificed mice is included in Table 1. One skin nodule was found among the nine negative control mice treated with deionized water. Various other lesions were encountered in both the DETA-HP-treated and the deionized water-treated mice in these sacrifice groups. These lesions are consistent with spontaneous lesions found in aging mice of this strain.

The histologic findings for the 18-month interim sacrifice are included in Table 2. The skin nodule found grossly in the deionized water-treated negative control group was diagnosed as a sebaceous adenoma. Concomitant with the sebaceous adenoma was epidermal hyperplasia. The histologic lesions found in the other tissues examined are consistent with the spontaneous background lesions seen in this strain of mouse.

The frequency of gross findings for the mice included in the lifetime portion of this study is included in Table 3. No skin neoplasms or nodules were found in the DETA-HP mice or the negative control mice.

The gross lesions encountered in the other organs were all considered part of the spontaneous background lesions frequently seen in mice of this strain used on lifetime studies.

The frequency of histologic findings for the mice included in the lifetime portion of this study is included in Table 4. No skin or subcutaneous neoplasms were found in the DETA-HP-treated mice or in the deionized water-treated negative control mice. Lesions diagnosed in the other organs including the neoplasms were considered part of the spontaneous background lesions encountered in this strain of mice in lifetime studies.

The histologic changes observed in the adrenals of the DETA-HP-treated and negative control mice reflect the gross observations, but it should be remembered that only adrenals with lesions observed at necropsy were examined histologically. Cortical hyperplasia/adenoma appeared in all adrenals examined. Dunn (1970) considered the distinction between hyperplasia and neoplasia in mouse adrenals to be very difficult and somewhat arbitrary.

Lesions seen in the male urogenital system are primarily related to the Proteus mirabilis infection which is frequently encountered in this strain from this vendor (Maronpot and Peterson, 1981).

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Liver neoplasms are frequently encountered in this strain and therefore no significance can be attributed to their appearance in the treated or control mice.


Edward H. Fowler, DVM, Ph.D. 8/19/82
Pathologist Date

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- Maronpot, R. R. and L. G. Peterson. Spontaneous Proteus Nephritis Among Male C3H/HeJ Mice. Lab. Anim. Sci. 31: 697-700, 1981.

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TABLE 1

Diethylenetriamine-High Purity (DETA-HP): Dermal Carcinogenesis Study

Frequency of Gross Findings Among C3H/HeJ Male Mice

18-Month Interim Sacrifice

<u>ORGANS/Findings</u>	<u>Diethylene- triamine High Purity</u>	<u>Deionized Water (Negative Control)</u>
TOTAL NUMBER EXAMINED GROSSLY	7	9
SKIN/SUBCUTIS, NGL	7/7*	8/9
/Module(s)	0/7	1/9
ADRENALS, NGL	4/7	2/9
/Color change	3/7	7/9
/Enlarged	1/7	0/9
LUNGS, NGL	6/7	5/9
/Color change	1/7	4/9
KIDNEYS, NGL	6/7	9/9
/Color change	1/7	0/9
/Enlarged	1/7	0/9
LIVER, NGL	3/7	6/9
/Color change	1/7	1/9
/Enlarged	0/7	1/9
/Mass(es)	3/7	2/9
GALLBLADDER, NGL	6/7	9/9
/Enlarged	1/7	0/9

NGL = No gross lesions.

*Numerator equals number of mice with specified finding.

Denominator equals number of mice for which specified organ was examined.

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TABLE 2

Diethylenetriamine-High Purity (DETA-HP): Dermal Carcinogenesis Study

Frequency of Histologic Findings Among C3H/HeJ Male Mice

18-Month Interim Sacrifice

<u>ORGANS/Findings</u>	<u>Diethylene- triamine High Purity</u>	<u>Deionized Water (Negative Control)</u>
<u>SKIN/SUBCUTIS, NSL</u>	7/7 ^a	8/9
/Epidermal hyperplasia/hyperplasia	0/7	1/9
/Sebaceous adenoma	0/7	1/9
<u>ADRENALS, NSL</u>	0/3	0/7
/Brown degeneration	0/3	1/7
/Cortical hyperplasia/adenoma	3/3	7/7
<u>LUNGS, NSL</u>	7/7	2/2
<u>KIDNEYS, NSL</u>	5/7	-
/Tubular hyperplasia	2/7	-
<u>LIVER, NSL</u>	3/7	1/4
/Triaditis	0/7	1/4
/Vascular thrombosis/ectasia	0/7	1/4
/Hepatocellular hyperplasia	0/7	1/4
/Hepatocellular adenoma	1/7	0/4
/Hepatocellular carcinoma	4/7	2/4
<u>GALLBLADDER, NSL</u>	1/1	-

NSL = No significant lesions.

^aNumerator equals number of mice with specified finding.

Denominator equals number mice for which specified organ was examined.

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Diethylenetriamine-High Purity (DETA-HP): Dermal Carcinogenesis StudyFrequency of Gross Findings Among C3H/HeJ Male Mice

Lifespan Study

<u>ORGANS/Findings</u>	<u>Diethylene- triamine High Purity</u>	<u>Deionized Water (Negative Control)</u>
TOTAL NUMBER EXAMINED GROSSLY	43	41
SKIN/SUBCUTIS, NGL	38/43*	38/41
/Edema	1/43	3/41
/Surface alteration	3/43	0/41
/Color change	1/43	0/41
THYROIDS, NGL	42/43	38/41
/Enlarged	1/43	0/41
/Autolysis	0/43	3/41
ADRENALS, NGL	20/43	13/41
/Color change	23/43	25/41
/Enlarged	1/43	1/41
/Modular/Mass	1/43	0/41
/Autolysis	1/43	3/41
HEART, NGL	42/43	34/41
/Color change	1/43	0/41
/Enlarged	0/43	5/41
/Autolysis	1/43	2/41
THYMUS, NGL	41/43	38/41
/Color change	1/43	0/41
/Enlarged/Mass	2/43	0/41
/Autolysis	0/43	3/41
SPLEEN, NGL	38/43	36/41
/Color change	1/43	1/41
/Enlarged/Firm	5/43	2/41
/Autolysis	1/43	3/41
MESENTERIC LYMPH NODES, NGL	41/43	37/41
/Color change	2/43	1/41
/Enlarged	2/43	1/41
/Autolysis	1/43	3/41
LUNGS, NGL	31/43	30/41
/Color change	11/43	9/41
/Module/Mass	1/43	2/41
/Autolysis	1/43	2/41
PROSTATE, NGL	42/43	38/41
/Color change	1/43	0/41
/Autolysis	0/43	3/41
SEMINAL VESICLES, NGL	42/43	38/41
/Color change	1/43	0/41
/Enlarged/Firm/Mass	1/43	0/41
/Autolysis	0/43	3/41
KIDNEYS, NGL	37/43	32/41
/Indentations	0/43	1/41
/Perforation	1/43	0/41
/Color change	5/43	5/41
/Mass(es)	0/43	1/41
/Autolysis	2/43	3/41

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TABLE 3
(Continued)

Diethylenetriamine-High Purity (DETA-HP): Dermal Carcinogenesis Study

Frequency of Gross Findings Among C3H/HeJ Male Mice

Lifespan Study

<u>ORGANS/Findings</u>	<u>Diethylene- triamine High Purity</u>	<u>Deionized Water (Negative Control)</u>
URINARY BLADDER, NGL	41/43	37/41
/Constriction	0/43	1/41
/Distended	2/43	0/41
/Color change	1/43	1/41
/Autolysis	1/43	3/41
STOMACH, NGL	42/43	38/41
/Color change	1/43	0/41
/Autolysis	0/43	3/41
LIVER, NGL	19/43	14/41
/Autolysis	3/43	3/41
/Color change	8/43	2/41
/Enlarged/Firm/Thickened	3/43	1/41
/Module/Mass	15/43	22/41
PANCREAS, NGL	43/43	38/41
/Autolysis	0/43	3/41
THORACIC CAVITY, NGL	41/43	37/41
/Fluid filled	2/43	1/41
/Autolysis	1/43	3/41
ABDOMINAL CAVITY, NGL	40/43	38/41
/Fluid filled	3/43	0/41
/Autolysis	0/43	3/41

NGL = No gross lesions.

*Numerator equals number of mice with specified finding.

Denominator equals number of mice for which specified organ was examined.

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TABLE 4

Diethylenetriamine-High Purity (DETA-HP): Dermal Carcinogenesis Study

Frequency of Histologic Findings Among C3H/HeJ Male Mice

Lifespan Study

<u>ORGANS/Findings</u>	<u>Diethylene- triamine High Purity</u>	<u>Deionized Water (Negative Control)</u>
SKIN/SUBCUTIS, NSL	34/43*	41/41
/Ulcerative dermatitis	3/43	0/41
/Hyperkeratosis	2/43	0/41
/Keratin necrosis	4/43	0/41
THYROIDS, NSL	1/1	-
ADRENALS, NSL	0/24	0/25
/Congestion	0/24	1/25
/Brown degeneration	0/24	9/25
/Cortical hyperplasia/adenoma	24/24	25/25
HEART, NSL	0/1	4/5
/Autolysis	0/1	1/5
/Calcification	1/1	0/5
SPLEEN, NSL	0/5	0/2
/Autolysis	1/5	0/2
/Extramedullary hematopoiesis	5/5	1/2
/Lymphoid hyperplasia	0/5	1/2
MESENTERIC LYMPH NODES, NSL	0/1	0/1
/Autolysis	1/1	0/1
/Congestion/Hemorrhage	1/1	1/1
BRONCHIAL LYMPH NODES, NSL	0/1	-
/Sims histiocytosis	1/1	-
PANCREATIC LYMPH NODES, NSL	0/1	-
/Autolysis	1/1	-
MEDIASTINUM, NSL	0/1	-
/Abscesses	1/1	-
LUNGS, NSL	0/10	2/9
/Autolysis	0/10	1/9
/Congestion	6/10	4/9
/Alveolar histiocytosis	1/10	1/9
/Pulmonary adenoma	2/10	1/9
/Pulmonary adenocarcinoma	0/10	1/9
/Hepatocellular carcinoma, metastatic	1/10	0/9
PROSTATE, NSL	0/1	-
/Hemorrhage	1/1	-
/Inflammation	0/1	-
/Abscesses	0/1	-
/Prostatitis	0/1	-
SEMINAL VESICLES, NSL	1/1	-
/Inflammation	0/1	-
/Seminal vesiculitis	0/1	-

(Continued)

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TABLE 4
(Continued)

Diethylenetriamine-High Purity (DETA-HP): Dermal Carcinogenesis Study

Frequency of Histologic Findings Among C3H/HeJ Male Mice

Lifespan Study

<u>ORGANS/Findings</u>	<u>Diethylene- triamine High Purity</u>	<u>Deionized Water (Negative Control)</u>
KIDNEYS, NSL	2/5	1/5
/Autolysis	1/5	0/5
/Congestion	1/5	0/5
/Hydronephrosis	0/5	1/5
/Pyelonephritis	0/5	2/5
/Tubular cysts	1/5	0/5
/Tubular mineralization	1/5	0/5
/Tubular proteinosis	1/5	1/5
/Tubular hyperplasia	0/5	1/5
URINARY BLADDER, NSL	2/2	-
STOMACH, NSL	0/1	-
/Squamous cell carcinoma	1/1	-
LIVER, NSL	3/24	0/25
/Autolysis	3/24	1/25
/Congestion/Sinusoidal distention	2/24	2/25
/Peliosis hepatis	2/24	0/25
/Necrosis/Infarction	0/24	1/25
/Hepatocellular necrosis	3/24	3/25
/Hepatocellular dysplasia	1/24	0/25
/Hepatocellular degeneration	0/24	1/25
/Hepatocellular atrophy/fibrosis	1/24	0/25
/Hepatocellular adenoma	0/24	2/25
/Hepatocellular carcinoma	15/24	21/25
PANCREAS, NSL	1/1	-

NSL = No significant lesions.

*Numerator equals number of mice with specified finding.

Denominator equals number of mice for which specified organ was examined.

WPC/ask/0279A-2

03-12-82

Pathology Report page number 28780 through 28879 of this report contained individual pathology data sheets for each animal. Those sheets have not been included in this report in order to decrease costs of reproduction and distribution of reports. A copy of the report containing the individual data sheets was distributed to the Project Initiator and the original is on file in the Bushy Run Research Center Archives.

11/11/82

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