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Wacker Chemicals (USA), Inc.

50 Locust Avenue
New Canaan, CT 06840
Phone (203) 966-9999
TWX 643-0004
FAX 203-966-0004

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June 12, 1987

Document Control Officer (TS-790)
(Attn: Section 8(e) Coordinator)
Office of Toxic Substances
US Environmental Protection Agency
401 M Street, SW
Washington, DC 20460

RE: Section 8(e) Submission on Chloroacetone and
Chloroacetaldehyde
EPA Document Control # 8 EHQ-0387-0660

Dear Mrs. Loranger,

In reference to our telephone conversation on June 9,
1987 and June 10, 1987, please find enclosed the
publications:

- "Toxicity Profile of Chloroacetaldehyde", Journal of
Pharmaceutical Sciences, Vol. 61, No. 1, January 1972.

- "Dose Response Relationships for Mutations Induced in
E. coli by Some Model Compounds", Hereditas 101, 57, 1984.

If you have any further questions, please feel free to
contact me at any time.

Sincerely,

Dr. Hans Pommerening
Product Manager
WACKER CHEMICALS (USA), INC.

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Received from the Seater Chemistry Laboratory, Pomona College, Claremont, CA 91711
 ▲ To whom inquiries should be directed.

RESEARCH ARTICLES

Toxicity Profile of Chloroacetaldehyde

W. H. LAWRENCE, E. O. DILLINGHAM, J. E. TURNER, and J. AUTIAN^A

Abstract □ Chloroacetaldehyde, a probable metabolite of 2-chloroethanol (ethylene chlorohydrin), was studied in a number of *in vivo* and *in vitro* systems, in *in vitro* hemolysis tests, and in tissue cultures to obtain a toxicity profile of the compound. Acute toxicity tests were conducted in mice, rats, guinea pigs, and rabbits by one or more routes of administration. Tissue culture tests utilized both the agar-overlay and protein assay methods. Irritant activity was evaluated by intramuscular implantation, intradermal injection, and dermal and ophthalmic applications in the rabbit. Acute cardiovascular effects in rabbits were also determined. Sleeping-time tests in mice were conducted to assess the effect of chloroacetaldehyde pretreatment (inhalation and intraperitoneal) upon drug-metabolizing enzymes. The compound was tested in guinea pigs for its sensitizing potential. Cumulative (30 daily injections) and subacute (three injections per

week for 12 weeks) toxicity studies were conducted to evaluate subtle toxic effects (e.g., weight gain, hematology, and histopathology) as well as lethality. Chloroacetaldehyde is a very toxic and irritating compound in acute tests; in tests of longer duration, most of the parameters measured appeared to be normal in animals that survived its lethal activity. The acute toxic effects of chloroacetaldehyde are compared with those of 2-chloroethanol. The former is inherently more toxic and irritating, while the latter exhibits greater ease of quantitative penetration through the GI tract and the intact skin.

Keyphrases □ Chloroacetaldehyde—toxicity, compared to ethylene chlorohydrin □ Toxicity—chloroacetaldehyde, compared to ethylene chlorohydrin

Chloroacetaldehyde (ClCH₂CHO) is a liquid at room temperature. As the anhydrous material, it polymerizes on standing (1) and, in aqueous solutions in excess of 50%, forms a half-hydrate which precipitates as white crystals (2). It is intensely irritating to human eyes, skin, mucous membranes, and the respiratory tract (1), and its highly toxic nature is suggested by its "threshold limit value" (TLV) of 1 p.p.m., which should not be

allowed to fluctuate above this amount even for short periods of time (3). The uses of chloroacetaldehyde as well as its physical and chemical properties were presented previously (1, 2).

Ethylene oxide sterilization of plastics, spices, and foods, in the presence of chlorides, produces 2-chloroethanol as a reaction product. Johnson (4), in studies conducted on rats, indicated that 2-chloroethanol was

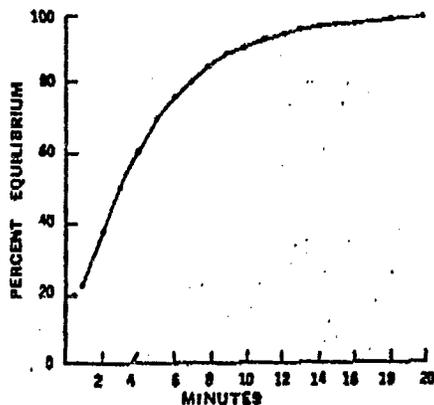


Figure 1—Percent equilibrium of chamber atmosphere to incoming air-vapor mixture (theoretical curve) (chamber = 8.75 l. and air-flow = 2 l./min.).

oxidized to chloroacetaldehyde via a major metabolic pathway. The latter then reacted with reduced glutathione at the cellular level and finally oxidized to S-carbomethylglutathione. He thus proposed this mechanism to account for the highly toxic nature of 2-chloroethanol, since simultaneous administration of ethanol (which reduces the rate of chloroacetaldehyde formation by competing with 2-chloroethanol for oxidative enzymes) retards reduced glutathione depletion.

Thus, under appropriate conditions, it might be possible to encounter chloroacetaldehyde toxicity problems as a consequence of the ethylene oxide sterilization process. Therefore, it was of interest to develop a toxicity profile for chloroacetaldehyde along the same lines as were previously used for ethylene chlorohydrin (2-chloroethanol).

EXPERIMENTAL

Materials—The chloroacetaldehyde used in these experiments was obtained as an aqueous solution containing either 30 or 45% chloroacetaldehyde. Tests were performed using 30% solutions, aqueous dilutions, or dilutions made isotonic with sodium chloride, as appropriate for the specific test.

LD₅₀ Determinations—Experiments were conducted on male ICR mice, Sprague-Dawley and Black Bethedr strains of rats, New Zealand albino rabbits, and guinea pigs of the Hartley strain. Graded doses of the compounds were administered to the animals by the specified route, and the animals were observed 1 week for mortalities. The LD₅₀ values and 95% confidence limits were calculated by Cornfield and Mantel's (5) modification of Karber's method or by the method of Weil (6), based upon the 7-day mortalities.

The test material was administered as a single dose by oral intubation, intraperitoneal injection, or topical (dermal) application. Dermal administration to rabbits was accomplished by: (a) placing the desired dose on a single Webril patch of sufficient size to absorb the liquid; (b) placing the patch in contact with a nonbraded area of the rabbit's skin, previously freed of hair by shaving with a safety razor and commercial shaving cream; and (c) securing the patch with a polyethylene overwrap. After 24 hr., the bandage was removed and the rabbit was observed an additional 6 days for signs of toxicity or mortality.

To facilitate comparison of results, all values are calculated in terms of pure chloroacetaldehyde.

Inhalation Toxicity—Groups of male ICR mice, five at a time, were placed in an all-glass inhalation chamber of 8.75-l. capacity. Filtered

Table I—Acute Toxicity of Chloroacetaldehyde

Test Animal	LD ₅₀ ^a , ml./kg.	95% Confidence Limits	Concentration in Water, %	Number of Animals per Dose
Intraperitoneal Administration				
Mice, male, ICR	0.00398	0.00475–0.00752	30	10
Rats, male, S-D	0.00602	0.00393–0.00906	30	4
Rats, male, BB	0.00827	0.00657–0.01040	30	3
Guinea pigs, male, Hartley, albino	0.00212	0.00068–0.00638	30	3
Rabbits, male, New Zealand, albino	0.00464	0.00357–0.00603	30	4
Intragastric Administration				
Mice, male, ICR	0.06918	0.05063–0.09407	0.5	4
Rats, male, S-D	0.07507	0.04700–0.11989	3.0	4
Rats, female, BB	0.08665	0.07067–0.10151	2.0	5
Dermal (Topical) Application				
Rabbits, male, New Zealand, albino	0.2243	0.1579–0.3186	30	4
Inhalation				
Mice, male, ICR	2.57 ^b	2.20–2.99 min.	30	10

^a All values are expressed in terms of 100% chloroacetaldehyde. ^b LT₅₀ (lethal time—50%) = 2.57 min., at which time the chamber had attained approximately 45% of equilibrium with the incoming air-vapor mixture.

air was bubbled through a 30% aqueous solution of chloroacetaldehyde at a rate of 2 l./min. and passed into the inhalation chamber. Mice were in the air-vapor environment for a specified period, ranging from 1.44 to 4.40 min., after which the chamber was immediately opened (in a fume hood) and mice were promptly removed. The procedure was repeated for each experimental group to provide 10 mice at each exposure time. All surviving mice were placed in cages and observed 7 days for mortalities. From the 7-day mortality pattern, the time of exposure required to kill 50% of the animals (LT₅₀) and 95% confidence limits were calculated by the method of Weil (6). Under these conditions, the time required for the chamber atmosphere to reach 80% equilibrium with the incoming air-vapor mixture is 7.04 min., as calculated by the method of Silver (7). A plot of the theoretical relationship between time and degree of equilibrium of the chamber atmosphere to the incoming air-vapor mixture is shown in Fig. 1.

Tissue Culture—The cytotoxic activity of chloroacetaldehyde to mouse fibroblast cells (L-cells) in culture was determined by employing graded concentrations in the agar-overlay and protein assay methods. Both of these procedures were described in detail in a previous paper (8).

Dermal, Intradermal, and Ophthalmic Irritations—Albino New Zealand rabbits were employed in the evaluation of the irritancy of chloroacetaldehyde by these routes. Procedures employed and methods for evaluation of results were described in detail in a previous paper (8). However, due to the greater irritant activity of chloroacetaldehyde, more dilute solutions were employed.

Intramuscular Implantation—Small strips (approximately 0.5–1.0 mm. X 1 cm.) of a nonreactive polyvinyl chloride material were placed in a 30% solution of chloroacetaldehyde and allowed to remain for 24 hr. The samples were removed, blotted lightly to remove excess liquid, and implanted into the paravertebral muscle of the rabbit. Similar samples of the same material which had not been soaked in chloroacetaldehyde solution were implanted as a negative control, and samples of another polyvinyl chloride material (known to produce a positive response) were implanted as a positive control. Seven days later, the rabbits were sacrificed and the implanted materials were located and evaluated for tissue response. The responses were graded as follows: 0 = nonreactive, ± = slight or questionable response, 1+ = mild reaction, 2+ = moderate reaction, and 3+ = marked reaction.

Hemolysis Test—To determine the hemolytic threshold and concentrations which produce 50% hemolysis, a series of dilutions of chloroacetaldehyde in normal saline was prepared. Normal saline (without any toxicant) was employed as a negative control, and a 1% solution of sodium carbonate in saline served as the positive

¹ K & K Laboratories, Plainview, N. Y.

Table II—Chloroacetaldehyde Irritation Tests

	Concentration, % ^a	Irritant Response ^b
Intradermal, rabbits	TIC-4	0.0193% ^c
Dermal, rabbits	7.5	3+
	3.75	2+
	1.875	1+
	0.9375	+
Ophthalmic, rabbits	0.4688	0
	0.25	0
	0.125	0
	0.0625	0
	0.03125	0
	0.0156	0
	0.0078	0

^a All solutions, except those used for the dermal test, were made isotonic with sodium chloride. ^b Scored on a 0 to 3+ scale; see Reference 8 for definitions. ^c TIC-4 = threshold irritation concentration of Luduena and Hoppe (11); equivalent to 1+ response.

control (100% hemolysis). Ten milliliters of saline, sodium carbonate saline, or chloroacetaldehyde in saline was placed into a 16 X 150-mm. test tube and kept in a water bath at 37° for 30 min. to provide temperature equilibration. Then 0.2 ml. of oxalated, whole rabbit blood was added to each tube and incubated for 60 min. at the same temperature. Each tube was centrifuged for 10 min. at 1000Xg, and the supernatant was carefully removed with a pipet and placed into spectrophotometric cells; the absorbance of the sample at 540 nm. was then recorded.

The percent hemolysis was calculated as follows:

$$\% \text{ hemolysis} = \frac{\text{absorbance of test sample} - \text{absorbance of negative control}}{\text{absorbance of positive control}} \times 100 \text{ (Eq. 1)}$$

The data, as percent hemolysis, were plotted against the molar concentration of chloroacetaldehyde to give the dose-response curve. From this curve, the concentration required to produce 50% hemolysis was determined.

Sleeping-Time Test—Male ICR mice, weighing 25 ± 5 g., were pretreated with chloroacetaldehyde on 3 consecutive days, with the test being performed 24 hr. after the last pretreatment. In one series, mice were pretreated with intraperitoneal injections of chloroacetaldehyde; in the other series, pretreatment was by inhalation. In both instances, 24 hr. after the last exposure, each mouse was administered 50 mg./kg. of sodium pentobarbital intraperitoneally and observed for loss and then return of the righting reflex. The induction time was considered as the time interval between pentobarbital injection and loss of the righting reflex; sleeping time was the duration of the absence of the righting reflex. Ten mice per group were employed in both of these tests.

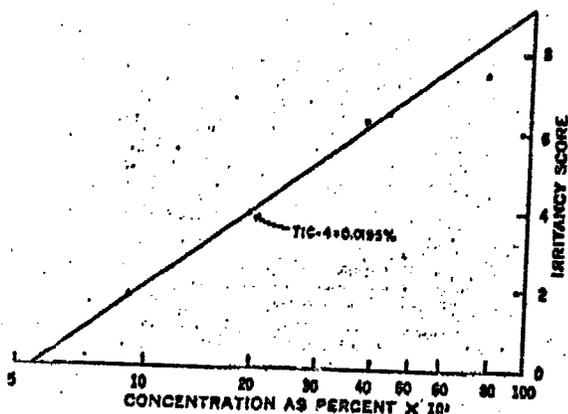


Figure 2—Intradermal irritation from chloroacetaldehyde (TIC-4 determination).

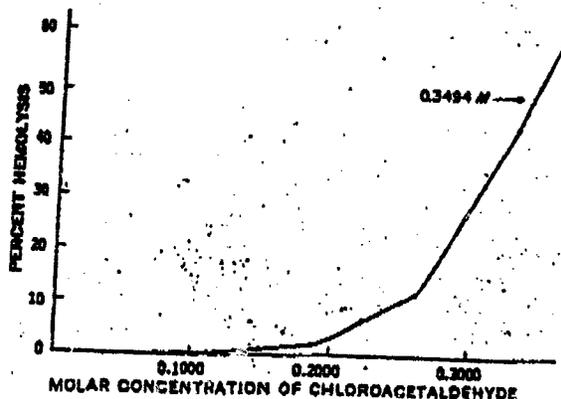


Figure 3—Hemolysis of rabbit erythrocytes by chloroacetaldehyde: 50% hemolysis = 0.3494 M (2.75%).

Sensitization Test—To determine the sensitizing potential of chloroacetaldehyde, the guinea pig maximization test of Magnusson and Kligman (9) was employed using guinea pigs of the Hartley strain as described in a previous paper (10).

Acute Effects upon Cardiovascular, Respiratory, and Neuromuscular Functions—The acute effects of chloroacetaldehyde upon cardiovascular, respiratory, and neuromuscular functions were determined in anesthetized rabbits using the methodology described in a previous publication (10).

Cumulative Toxicity—The cumulative toxic nature of chloroacetaldehyde in male Sprague-Dawley rats was determined by administering daily doses of the compound by intraperitoneal injection for 30 consecutive days. Dose levels employed represent 0.001879 and 0.003758 ml./kg. of pure chloroacetaldehyde. Details of the procedure were described in a previous publication (10).

Subacute (Subchronic) Toxicity—The toxicity of chloroacetaldehyde in male Sprague-Dawley rats was investigated when the compound was administered three times a week for 12 weeks. The dose levels utilized in this study were 0.00032, 0.0008, 0.0016, and 0.0032 ml./kg. calculated as pure chloroacetaldehyde but the doses were injected as 0.5% aqueous solutions. This procedure was described in detail in a previous article (10).

RESULTS AND DISCUSSION

The acute LD₅₀ values and their 95% confidence limits when chloroacetaldehyde was administered intraperitoneally, orally, or topically are presented in Table I. These values suggest that guinea pigs are most sensitive to intraperitoneal injection of the compound and the black rats are least sensitive, although overlap of the confi-

Table III—Effect of Chloroacetaldehyde upon Pentobarbital Sleeping Time^a

Duration of Exposure	Concentration of Compound ^b	Minutes	
		Induction Time (Mean ± SE)	Sleeping Time (Mean ± SE)
Pretreatment by Inhalation			
Control	Air	4.02 ± 0.25	78.76 ± 9.59
15 sec.	40.0 mg./l.	3.93 ± 0.21	98.25 ± 8.96
31 sec.	41.7 mg./l.	4.15 ± 0.31	127.84 ± 16.84 ^c
77 sec.	43.4 mg./l.	3.20 ± 0.14 ^d	224.57 ± 21.69 ^d
Pretreatment by Intraperitoneal Injection			
Dose of Chloroacetaldehyde Injected			
Controls		3.25 ± 0.28	61.07 ± 5.79
0.00061 ml./kg.		3.13 ± 0.31	100.31 ± 10.63 ^e
0.00122 ml./kg.		2.93 ± 0.16	137.54 ± 7.48 ^f
0.00305 ml./kg.		3.33 ± 0.23	184.52 ± 12.22 ^f

^a Mice pretreated with chloroacetaldehyde 24, 48, and 72 hr. before intraperitoneal injection of 50 mg./kg. sodium pentobarbital. ^b Calculated as weight loss of 30% chloroacetaldehyde aqueous solution per liter of air. ^c Significant at 95% level ($p = 0.05$) by Student's *t* test. ^d Significant at 99% level ($p = 0.01$) by Student's *t* test.

Table IV—Cumulative Toxicity of Chloroacetaldehyde: Hematologic Values in Rats (Mean ± SE)

Item	Saline Control	0.001879 mg./kg.	0.003758 mg./kg.
Hemoglobin, g./100 ml.	15.020 ± 0.489	15.200 ± 0.559	11.700 ± 1.073*
Hematocrit	48.080 ± 1.819	47.080 ± 1.819	38.500 ± 2.300*
Erythrocytes, mm. ³ (× 10 ⁶)	3.975 ± 0.300	6.673 ± 0.335	4.528 ± 0.355*
Total white blood cells, mm. ³ (× 10 ³)	12.050 ± 1.879	17.325 ± 2.045	19.162 ± 4.630
Platelets, mm. ³ (× 10 ⁵)	0.990 ± 0.109	1.175 ± 0.215	0.690 ± 0.087
Clotting time, sec.	66.300 ± 6.498	79.500 ± 4.817	82.200 ± 9.664
Differential white cell count, %			
Segs	12.833 ± 2.943	31.083 ± 1.680*	27.900 ± 3.852
Lymphocytes	82.083 ± 3.145	64.833 ± 1.850*	67.875 ± 6.385
Monocytes	0.417 ± 0.154	1.417 ± 0.300*	1.00 ± 0.577
Eosinophils	1.083 ± 0.473	0.667 ± 0.248	0.875 ± 0.792
Basophils	0.583 ± 0.417	0.083 ± 0.083	0.125 ± 0.125
Meta	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Juvenile	0.083 ± 0.083	0.250 ± 0.112	0.250 ± 0.250
Bands	1.333 ± 0.401	2.000 ± 0.224	1.250 ± 0.777

* Significant at the 95% level ($p = 0.05$) by Student's *t* test. † Significant at the 99% level ($p = 0.01$) by Student's *t* test.

dence limits does not permit statistical verification of the apparent trend from this data. The magnitude of difference in the LD₅₀ produced by changing the route of administration is quite obvious. Absorption of chloroacetaldehyde from the GI tract or through the skin appears to be a significant factor in influencing its toxicity. As discussed later, this is a marked departure from the pattern of response produced by the potential parent compound 2-chloroethanol, which demonstrated equivalent toxicity by each of these three routes of administration.

Chloroacetaldehyde proved to be very lethal by inhalation. Mice were placed into a chamber containing no chloroacetaldehyde, and air containing chloroacetaldehyde vapor was passed into the chamber. The time of exposure required to kill 50% of the animals, LT₅₀, was 257 min. In this time, the chamber atmosphere is calculated to have reached approximately 45% of equilibrium with the incoming air-chloroacetaldehyde vapor concentration (Fig. 1). Prior to its entering the inhalation chamber, air was bubbled through a 30% aqueous solution of chloroacetaldehyde and was found to remove 33 mg. of liquid/l. of air.

Tissue culture tests, using both the agar-overlay technique and protein assay, demonstrated the highly toxic properties of chloroacetaldehyde. Application of 0.2 ml. of a solution as dilute as 0.0078% to the agar-overlay produced a cytotoxic effect on the mouse fibroblasts (L-cells), while further dilutions, 0.0039% or less, were noncytotoxic. By using inhibition of protein synthesis in L-cell cultures as the criterion, it was found that a 3.62×10^{-5} M concentration of chloroacetaldehyde in the media was sufficient to reduce protein synthesis by 50% (ID₅₀).

Results of the dermal, intradermal, and ophthalmic irritation tests conducted in rabbits are presented in Table II, which illustrates the highly irritating nature of chloroacetaldehyde. The "threshold irritation concentration" (TIC-4) of Luduena and Hoppe (11) was determined for the intradermal route. The basic 0, ±, 1+, 2+, and 3+ evaluation system was converted to numerical values of 0, 2, 4, 6,

and 8, respectively, to be compatible with the system used by Luduena and Hoppe. The odd numbers were used for observed responses that were intermediate between two of the assigned even numbers. Ten observations were made at each of four concentrations for the graphic plot to determine the TIC-4 value (equivalent to 1+ response). Figure 2 shows the straight-line plot of these data and their agreement with a calculated value of TIC-4 = 0.0195%.

Chloroacetaldehyde was extremely irritating to the intact skin of the rabbit. The undiluted material (30% chloroacetaldehyde in water) produced severe and extensive tissue damage, much more intense and over a larger area than was produced by the positive control (8% sodium lauryl sulfate, w/v, in water). Similarly, it was noted in preliminary tests that the more concentrated solutions of chloroacetaldehyde produced extensive damage to the rabbit's eye, while a 0.03125% solution produced definite, but reversible, ophthalmic irritation (score of 1).

Strips of polyvinyl chloride, which had been soaked in 30% chloroacetaldehyde for 24 hr., were implanted into the paravertebral muscle of the rabbit. After 7 days, the rabbit was sacrificed and the implant sites were located in the muscle. The chloroacetaldehyde-treated strips produced an area of necrosis and purulence around the implant equal to or greater than that produced by the positive control (3+). Untreated strips of polyvinyl chloride, identical to those soaked in chloroacetaldehyde, did not produce grossly visible signs of necrosis.

Figure 3 shows the dose-response curves produced by plotting percent hemolysis versus concentration of chloroacetaldehyde. Thus, it may be seen that about 10% hemolysis should be produced by 0.2450 M and 50% hemolysis produced by 0.3494 M (or 2.74%) chloroacetaldehyde in saline.

Pretreatment of mice with chloroacetaldehyde produced a dose-related increase in pentobarbital sleeping time. This was true whether the compound was administered by intraperitoneal injection or by inhalation. Dosing of the mice was based upon 0.1, 0.2, and 0.5 of the acute LD₅₀ or LT₅₀ daily for 3 days prior to pentobarbital administration. The results are presented in Table III. The increase in sleeping time in the groups treated with chloroacetaldehyde intraperitoneally is significant at the 99% level (by *t* test) for all three dosage levels. For the animals exposed to chloroacetaldehyde vapors

Table V—Cumulative Toxicity of Chloroacetaldehyde as Shown by B.S.P. Liver Function Test in Rats*

After	Saline Control	Dose Level of Chloroacetaldehyde	
		0.001879 mg./kg.	0.003758 mg./kg.
15 min.	25.26 ± 0.81	22.88 ± 1.79	27.38 ± 3.70
30 min.	5.93 ± 0.67	7.13 ± 1.41	10.38 ± 5.71
45 min.	2.74 ± 0.55	3.97 ± 1.45	3.55 ± 1.59
	Percentage of B.S.P. Eliminated		
Between			
15-30 min.	76.04 ± 3.21	70.94 ± 8.15	69.91 ± 15.91
30-45 min.	34.72 ± 6.68	42.70 ± 13.37	45.91 ± 11.56

* Test was performed at the conclusion of the 30-day cumulative toxicity study. All rats received 75 mg./kg. of B.S.P. intravenously. Values are from six rats per group, except the high dose group in which the test was completed in only three of the four surviving animals.

Table VI—Cumulative Toxicity of Chloroacetaldehyde: Percent Organ-to-Body Weight of Rats (Mean ± SE)

Organ	Saline Control	0.001879 mg./kg.	0.003758 mg./kg.
Adrenals	0.012 ± 0.001	0.018 ± 0.003	0.025 ± 0.006
Brain	0.467 ± 0.015	0.568 ± 0.042*	0.758 ± 0.089*
Gonads	0.871 ± 0.024	0.982 ± 0.082	1.189 ± 0.083*
Heart	0.273 ± 0.008	0.263 ± 0.013	0.321 ± 0.019*
Kidneys	0.620 ± 0.014	0.701 ± 0.069	0.769 ± 0.059*
Liver	3.505 ± 0.204	3.901 ± 0.384	5.211 ± 0.858
Lungs	0.324 ± 0.011	0.409 ± 0.039*	0.565 ± 0.052*
Spleen	0.224 ± 0.011	0.321 ± 0.078	0.457 ± 0.085*

* Significant at 95% level ($p = 0.05$) by Student's *t* test. † Significant at 99% level ($p = 0.01$) by Student's *t* test.

Table VII—Subacute Toxicity of Chloroacetaldehyde: Food Consumption^a in Rats (Mean \pm SE)

Week	Saline Control	0.00032 ml./kg.	0.0008 ml./kg.	0.0016 ml./kg.	0.0032 ml./kg.
1st	109.522 \pm 8.355	100.934 \pm 12.512	92.515 \pm 7.246	39.109 \pm 5.307*	26.828 \pm 6.729*
2nd	60.008 \pm 2.466	70.251 \pm 2.832*	61.365 \pm 4.233	60.750 \pm 3.694	74.319 \pm 7.036
12th	60.058 \pm 2.026	55.141 \pm 2.656	56.035 \pm 2.195	57.555 \pm 3.443	96.376 \pm 9.671

^a Grams of food consumed per kilogram of rat per 24-hr. day. * Significant at 95% level by Student's *t* test. * Significant at 99% level by Student's *t* test.

Table VIII—Subacute Toxicity of Chloroacetaldehyde: Hematologic Values (Mean \pm SE)

Item	Saline Control	0.00032 ml./kg.	0.0008 ml./kg.	0.0016 ml./kg.	0.0032 ml./kg.
Hemoglobin, g./100 ml.	17.287 \pm 0.234	17.102 \pm 0.226	16.885 \pm 0.206	16.255 \pm 0.507	14.937 \pm 0.244*
Hematocrit	50.750 \pm 0.998	51.500 \pm 0.796	51.000 \pm 1.335	47.250 \pm 1.564	47.900 \pm 1.258
Red blood cells, mm. ³ ($\times 10^6$)	7.752 \pm 0.595	6.663 \pm 0.539	7.187 \pm 0.804	5.237 \pm 0.168*	5.227 \pm 0.424*
Total white blood cells, mm. ³ ($\times 10^6$)	14.608 \pm 1.882	14.917 \pm 1.792	16.792 \pm 2.012	12.133 \pm 1.928	16.717 \pm 0.985
Platelets, mm. ³ ($\times 10^6$)	0.637 \pm 0.109	0.669 \pm 0.079	0.661 \pm 0.063	0.769 \pm 0.057	0.607 \pm 0.119
Clotting time, sec.	80.667 \pm 8.123	94.833 \pm 4.037	67.000 \pm 6.387	119.667 \pm 17.021	134.000 \pm 6.085*
Differential white cell count, %					
Segs	11.667 \pm 2.548	11.167 \pm 3.206	14.000 \pm 2.335	33.167 \pm 4.752*	31.667 \pm 9.493*
Lymphs	79.917 \pm 2.797	85.383 \pm 1.744	77.917 \pm 3.177	60.583 \pm 5.057*	65.333 \pm 8.786
Monocytes	2.383 \pm 0.597	1.167 \pm 0.527	1.500 \pm 0.300	2.917 \pm 0.455	1.833 \pm 0.928
Eosinophils	2.250 \pm 0.443	1.083 \pm 0.352	1.500 \pm 0.465	1.417 \pm 0.375	1.000 \pm 0.289
Basophils	0.167 \pm 0.105	0.000 \pm 0.000	0.500 \pm 0.258	0.250 \pm 0.171	0.000 \pm 0.000
Metas	0.000 \pm 0.000	0.333 \pm 0.167	0.250 \pm 0.171	0.000 \pm 0.000	0.000 \pm 0.000
Juvenile	0.667 \pm 0.279	0.333 \pm 0.167	0.750 \pm 0.359	0.667 \pm 0.247	0.167 \pm 0.167
Bands	2.917 \pm 0.851	2.917 \pm 1.179	3.750 \pm 1.167	1.083 \pm 0.300	0.167 \pm 0.167

* Significant at 99% level ($p = 0.01$) by Student's *t* test. * Significant at 95% level ($p = 0.05$) by Student's *t* test.

(by inhalation), the increase was not significant for the shortest exposure time (15 sec.), but it was significant at the 95% level for those exposed for 31 sec. and it was significant at the 99% level for those exposed 77 sec./day. Such a response could be explained on the basis of hepatic necrosis or other damage; however, in another study in which chloroacetaldehyde was administered daily for 30 days, hepatic damage was not observed in hematoxylin-eosin-stained specimens from those animals surviving to the end of the experiment.

The sensitizing potential of chloroacetaldehyde was tested in five guinea pigs, using the "guinea pig maximization test" of Magnusson and Kilgman (9). Due to the highly toxic and extremely irritating nature of the compound, it was necessary to use a very dilute solution, 0.002%. The test produced negative results, indicating that this concentration of chloroacetaldehyde was not sensitizing by this test.

Experiments were conducted to assess the acute effects of intravenous chloroacetaldehyde on blood pressure, respiration, and neuromuscular function in anesthetized rabbits. The various dose levels employed produced three generalized patterns of response upon blood pressure (Fig. 4). There was no uniform or dramatic effect upon respiration. Doses of 0.02–0.10 ml./kg. of 30% chloroacetaldehyde produced little or no effect upon contraction of the anterior tibialis and gastrocnemius muscles from stimulation of the sciatic nerve; however, 0.2 ml./kg. or more of 30% chloroacetaldehyde inhibited or blocked this response.

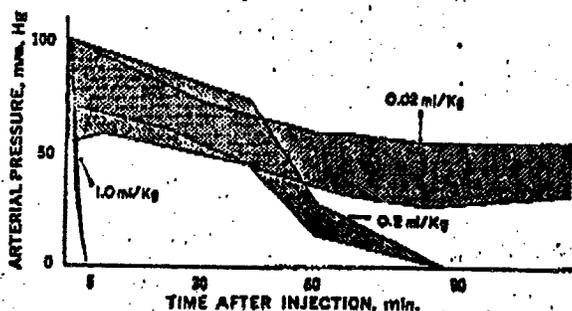


Figure 4—Acute effects of graded doses of 30% chloroacetaldehyde solution, intravenously, upon blood pressure in the anesthetized rabbit.

An investigation into the cumulative toxicity of chloroacetaldehyde in which groups of rats received 0.001879 and 0.003758 ml./kg. of chloroacetaldehyde (representing 0.3 and 0.6 of the acute LD₅₀ dose, respectively) daily for 30 consecutive days resulted in a mortality of 25 and 66.7%, respectively. During this time, the chloroacetaldehyde-treated rats gained weight more slowly than the controls (Fig. 5). These differences were significant (at 95 or 99% level) for both treated groups prior to the conclusion of the test period. Hematologic tests (Table IV) at the end of 30 days showed that there was a significant (at 95% level or better) decrease in hemoglobin, hematocrit, and erythrocytes in the high dose group; for the low dose group, there was an increase in segs and monocytes with a decrease in lymphocytes. At the end of the experiment, a liver function test, B.S.P. disappearance from the plasma, was conducted on the three groups of animals. The data are presented in Table V; however, there were no significant differences between the controls and the chloroacetaldehyde-treated groups.

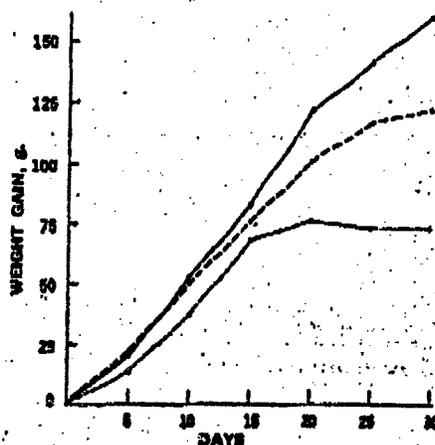


Figure 5—Cumulative toxicity of chloroacetaldehyde (mean body weight gain of rats). Key: —, saline control; ---, 0.001879 ml./kg.; and ····, 0.003758 ml./kg.

Table IX—Subacute Toxicity of Chloroacetaldehyde as Shown by B.S.P. Liver Function Test in Rats*

After	B.S.P. Concentration (mg. %) in Plasma of Rats (Mean \pm SD)				
	Saline Control	0.00032 ml./kg.	0.0008 ml./kg.	0.0016 ml./kg.	0.0032 ml./kg.
15 min.	29.16 \pm 1.80	28.32 \pm 1.65	31.19 \pm 3.31	30.31 \pm 2.66	20.99 \pm 4.39
30 min.	15.38 \pm 3.19	15.94 \pm 2.75	17.97 \pm 2.28	10.39 \pm 1.87	6.53 \pm 2.30
45 min.	8.21 \pm 2.03	10.63 \pm 3.05	11.38 \pm 2.57	2.03 \pm 0.27	3.52 \pm 1.24
Percentage of B.S.P. Eliminated					
Between					
15-30 min.	49.00 \pm 8.52	42.85 \pm 10.84	41.34 \pm 5.80	65.39 \pm 6.32	62.39 \pm 19.35
30-45 min.	49.10 \pm 5.89	40.41 \pm 10.23	39.42 \pm 11.07	78.97 \pm 2.79 ^b	46.16 \pm 7.11

* Test was performed at the conclusion of the 12-week subacute toxicity study. All rats received 75 mg./kg. of B.S.P. intravenously. Values are from six rats per group, except for 0.0032 ml./kg. in which there were only three surviving animals. ^b Significant at 99% level ($p = 0.01$) by t test.

Table X—Subacute Toxicity of Chloroacetaldehyde: Percent Organ-to-Body Weight of Rats (Mean \pm SE)

Organ	Saline Control	0.00032 ml./kg.	0.0008 ml./kg.	0.0016 ml./kg.	0.0032 ml./kg.
Adrenals	0.013 \pm 0.001	0.009 \pm 0.001 ^a	0.014 \pm 0.001	0.010 \pm 0.000 ^a	0.016 \pm 0.002
Brain	0.454 \pm 0.023	0.363 \pm 0.021 ^a	0.386 \pm 0.012 ^a	0.461 \pm 0.023	0.804 \pm 0.172 ^b
Gonads	0.768 \pm 0.042	0.728 \pm 0.019	0.799 \pm 0.034	0.824 \pm 0.041	0.808 \pm 0.171
Heart	0.283 \pm 0.012	0.311 \pm 0.022	0.261 \pm 0.014	0.245 \pm 0.008 ^a	0.290 \pm 0.023
Kidneys	0.673 \pm 0.022	0.637 \pm 0.028	0.614 \pm 0.017	0.543 \pm 0.027 ^a	0.663 \pm 0.043
Liver	3.086 \pm 0.314	3.254 \pm 0.194	3.423 \pm 0.185	3.355 \pm 0.191	4.946 \pm 0.198 ^b
Lungs	0.387 \pm 0.016	0.432 \pm 0.112	0.373 \pm 0.029	0.340 \pm 0.019	0.380 \pm 0.056
Spleen	0.212 \pm 0.018	0.237 \pm 0.009	0.228 \pm 0.22	0.244 \pm 0.026	0.265 \pm 0.022

^a Significantly different from controls at $p = 0.05$. ^b Significantly different from controls at $p = 0.01$.

After these tests, the animals were sacrificed and certain organs were removed, weighed (for calculation of organ-to-body weight ratio), and fixed in 10% buffered formalin for histopathologic evaluation. The organ-to-body weight ratios, expressed as percentages, are presented in Table VI. The ratios for both the brain and lungs were significantly greater in the low dose group, while the high dose group showed a significant increase in the brain, gonads, heart, kidneys, liver, lungs, and spleen. Hematoxylin-eosin-stained sections of these organs did not reveal any abnormalities which could be attributed to chloroacetaldehyde administration, except for the lungs which showed more severe bronchitis, bronchiolitis, and bronchopneumonia than were seen in the controls.

A series of doses, starting at a lower level, of chloroacetaldehyde was employed in a subacute (subchronic) study. A 0.5% aqueous solution was injected, with the doses being 0.00032, 0.00080, 0.00160, and 0.00320 ml./kg. of pure chloroacetaldehyde administered three times a week (Monday, Wednesday, and Friday) for 12 weeks. Each group consisted of 12 rats, except the 0.00320 ml./kg. group which contained eight rats. With the exception of the high dose group, only

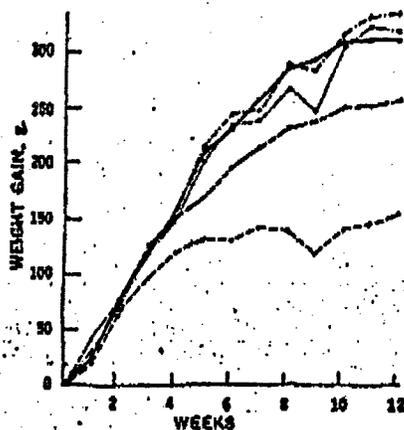


Figure 6—Subacute toxicity of chloroacetaldehyde (mean body weight gain of rats). Key: ····, saline controls; ---, 0.00032 ml./kg.; —, 0.0008 ml./kg.; - - - -, 0.0016 ml./kg.; and - · - ·, 0.0032 ml./kg.

two mortalities were produced: one in the low dose group during the 1st week and the other in the 0.00160 ml./kg. group during the 12th week. The 0.00320 ml./kg. dose produced five deaths in the eight animals (62.5%); although an LD₅₀ cannot be calculated from these data, it would appear that the subacute LD₅₀ would be in the range of 0.0030 ml./kg. or about one-half of the acute LD₅₀.

The weight gain of the rats treated with the two lower doses did not differ significantly from the controls; however, there was a significant decrease in weight gain for the two higher dose levels which was dose related (Fig. 6). Food consumption was determined during the 1st, 7th, and 12th weeks. There was a significant decrease in consumption by the two higher dosed groups during the 1st week, but little effect was noted otherwise (Table VII).

Hematologic determinations and B.S.P. liver function tests were performed at the conclusion of the 12th week; these data are presented in Tables VIII and IX, respectively. None of these values was significantly different from the controls (at $p = 0.05$ or less) for the two lowest doses, while 0.0016 ml./kg. showed a decrease in red cell count and lymphocytes, an increase in segmented neutrophils, and an increased rate of B.S.P. disappearance between 30 and 45 min.; the highest dose (0.0032 ml./kg.) showed a significant decrease in red blood cells and hemoglobin with an increase in clotting time and segmented neutrophils.

At autopsy, organ-to-body weight ratios were determined for several organs (Table X). Eight of the 32 values were statistically significant (at $p \leq 0.05$), although there was no apparent dose-related response with the quantities of chloroacetaldehyde employed. However, the ratio for the brain and liver in the high dose group (0.0032 ml./kg.) was considerably higher; this may have been due to a selective effect of the drug or a reflection of smaller body weights of these animals.

Histological examination of hematoxylin-eosin-stained sections of these organs revealed significant pathological changes in the lungs of animals receiving the two highest dose levels of chloroacetaldehyde. These changes included focal, chronic bronchopneumonia and certain changes of respiratory epithelium suggestive of a pre-malignant condition. Similar changes, to a lesser degree, were seen in the two low dose groups and controls, except there was no evidence of atypia of the respiratory epithelium. None of the other organs revealed any changes that could be attributed to administration of chloroacetaldehyde.

In concluding the discussion of chloroacetaldehyde, it might be appropriate to examine it in relation to its biological parent compound 2-chloroethanol. Some comparative data for these two compounds are presented in Table XI.

Table XI—Comparison of Acute Toxicity for Chloroacetaldehyde and 2-Chloroethanol

Test	Chloroacetaldehyde ^a	2-Chloroethanol ^b
Acute toxicity, oral LD ₅₀		
Mice, male	0.0692 ml./kg.	0.0671 ml./kg.
Rats, male	0.0731 ml./kg.	0.0388 ml./kg.
Acute toxicity, intraperitoneal LD ₅₀		
Mice, male	0.00398 ml./kg.	0.0810 ml./kg.
Rats, male	0.00602 ml./kg.	0.0328 ml./kg.
Guinea pigs, male	0.00212 ml./kg.	0.0707 ml./kg.
Rabbits, male	0.00464 ml./kg.	0.0671 ml./kg.
Acute toxicity, dermal LD ₅₀		
Rabbits	0.2243 ml./kg.	0.0339 ml./kg.
Acute toxicity, inhalation LT ₅₀		
Mice, male	2.57 min.	6.47 min.
Tissue culture, L-cells		
Agar-overlay, cytotoxic/noncytotoxic	0.0078% 0.0039%	10.0%/5.0%
Protein assay, ID ₅₀	0.0000563 M	0.03193 M
Irritation tests		
Rabbits, intradermal	0.0193% = 1+	5.0% = 1+
Rabbits, dermal	7.5% = 3+	
	0.488% = 0	100% = 0
Rabbits, ophthalmic	0.03125% = 1+	1.25% = 1+
7-Day muscle implant ^b	3+	0

^a All LD₅₀ values expressed in terms of pure compound. ^b Strips of a nonreactive polyvinyl chloride were soaked in pure 2-chloroethanol or 30% chloroacetaldehyde for 24 hr. prior to implantation.

Note the very close agreement between the LD₅₀ values for chloroacetaldehyde and 2-chloroethanol when they were administered orally to mice and the reasonably good agreement for rats. When the two compounds were administered intraperitoneally, however, chloroacetaldehyde appeared to be about 10-30 times as toxic as 2-chloroethanol. On the other hand, when the compounds were applied topically, 2-chloroethanol was about four times more toxic than chloroacetaldehyde. While the toxic quantity of 2-chloroethanol remained relatively constant between species of animals and routes of administration (approximately 53-81 μl./kg.), a similar comparison for chloroacetaldehyde shows more than a 100-fold variation in its LD₅₀ values.

When tested on L-cells in culture, using the agar-overlay technique, chloroacetaldehyde was more than 1200 times as toxic as 2-chloroethanol. ID₅₀ values obtained from L-cells indicate chloroacetaldehyde to be 368 times as potent in inhibiting protein synthesis as 2-chloroethanol.

A comparison of the two compounds for their irritant properties also shows chloroacetaldehyde to be more active. In the case of intradermal irritation, it required approximately 250 times as much 2-chloroethanol to produce a 1+ irritant response as was needed for chloroacetaldehyde, while 40 times as much 2-chloroethanol was required to produce a comparable degree of ophthalmic irritation (1+). Dermal irritant tests found solutions of 7.5% or greater of chloroacetaldehyde sufficient to produce a 3+ response, while undiluted (100%) 2-chloroethanol did not produce significant irrita-

tion. Seven-day implantation tests in the rabbit muscle, conducted by soaking strips of a nonreactive polyvinyl chloride material in 2-chloroethanol or 30% chloroacetaldehyde for 24 hr. prior to implantation, revealed a marked reaction (3+) to chloroacetaldehyde but no observable response to 2-chloroethanol.

It is apparent that chloroacetaldehyde has a much greater irritant activity and is inherently more toxic than 2-chloroethanol; however, 2-chloroethanol appears to have the greater penetrant capacity. This is particularly noticeable in the dermal toxicity of the two compounds.

In considering the potential danger of accidental exposure to toxic quantities of the two compounds, 2-chloroethanol probably presents the most hazard. Although it is quantitatively less toxic, it produces little or no irritation to the intact skin but penetrates quite readily; thus, contamination and absorption may occur without the individual being aware of them. The pronounced irritation produced by chloroacetaldehyde, coupled with its lesser ability to penetrate, alerts the individual to its presence and encourages him to remove it. Similarly, the more pronounced irritation of chloroacetaldehyde vapors serves as a deterrent to prevent the individual from remaining in an area of exposure to toxic quantities of the vapors, while the less irritating vapors of 2-chloroethanol may not provide such early warning.

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▲ To whom inquiries should be directed.

Dose-response relationships for mutations induced in *E. coli* by some model compounds

S. HUSSAIN

Department of Radiobiology, Wallenberg Laboratory, University of Stockholm, Sweden

With an addendum:

Reaction kinetics in water of chloroethylene oxide, chloroacetaldehyde, and chloroacetone

SIV OSTERMAN-GOLKAR

Department of Radiobiology, Wallenberg Laboratory, University of Stockholm, S-106 91 Stockholm, Sweden

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The dose-response relationships for mutation to streptomycin nondependence in *E. coli* Sd-4 were studied for three chemical agents (ethylene oxide, methyl methanesulfonate and ethyl methanesulfonate) with large scale experiments, and for ten agents (propylene oxide, 1,2-butene oxide, glycidol, epichlorohydrin, chloroethylene oxide, chloroacetone, bromoacetone, chloroacetaldehyde, glycolaldehyde and glyceraldehyde) with normal scale experiments. The data for ethylene oxide could be fitted to a single straight line. The mutation curves for methyl and ethyl methanesulfonates were resolved into two components. The dose-response relationships were indicated to be linear for six out of the remaining ten agents tested. Marked contributions from the quadratic components were reflected in the curves obtained with chloroethylene oxide, chloroacetaldehyde, chloroacetone and bromoacetone.

Saeed Hussain, Wallenberg Laboratory, Department of Radiobiology, University of Stockholm, S-106 91 Stockholm, Sweden

Information on the shape of dose-response curves for a number of agents in the same system is important both for comparisons of their genotoxic effectiveness and for a preliminary opinion on their reaction mechanisms. With regard to environmental genotoxicology, the very low-dose region is of special interest, but its investigation needs large-scale experiments and, hence, is time consuming. Although the intermediate region of the dose-response curve is easy to investigate, data in one mutation test system with a sufficient number of agents are usually not available for utilization for quantitative purpose.

Some data are available for the low-dose region of ionizing radiation in a number of systems, and a literature survey by BROWN (1976) shows that the dose-response curves for the mutagenic effects for a number of end-points (such as pink mutations in

Tradescantia stamen hairs, ad-3 mutations in *Neurospora*, deletion mutations in *E. coli*, sex-linked lethal mutations in *Drosophila*, and specific locus mutations in mouse) are compatible with linearity up to 1 Gy. The dose-response for gene conversion in *Saccharomyces cerevisiae* has been reported to be linear down to the region of 0.01 Gy (UNRAU et al. 1981). In the case of chemicals, data are available only for a few agents in a few systems. LINDGREN'S studies (1971) on the waxy system in barley indicated a hump in the initial region after treatment with ethylene oxide. In a recent study on Chinese hamster V79 cells (JENSEN and RAMEL 1980), the dose response curves for mutation to 6-thioguanine resistance induced by ethyl methanesulfonate, *N*-ethyl-*N*-nitrosourea, methyl methanesulfonate and *N*-methyl-*N*-nitrosourea, were reported to be linear for ethylating agents and

nonlinear for methylating agents. For the induction of micronuclei, nonlinear dose-response curves were obtained for both ethylating and methylating agents (JENSEN and RAMEL 1976, 1980). In the intermediate region, a number of *N*-nitroso compounds and alkyl methanesulfonates were investigated by COUCH and HSIE (1978) and COUCH et al. (1978) with the HGPRT mutation test system in Chinese hamster ovary cells, and the dose-response curves were found to be linear in all cases, including methylating agents.

In bacteria, a quantitative study was undertaken on the mutagenicity of alkyl methanesulfonic esters (TURTOCZKY and EHRENBERG 1969), and linear-exponential and linear dose-response curves were reported for ethyl methanesulfonate and isopropyl methanesulfonate, respectively. HINCE and NEALE (1977) carried out detailed studies on the biological parameters of importance to the mutagenic effectiveness of different nitroso compounds and some alkylating agents. The response of the test system in this study conformed to linearity and was noted to be markedly affected by the phase of growth in which the treatment was done. In contrast, in other bacterial mutation test system (KONDO et al. 1970; ISHII and KONDO 1975), linearity was indicated only for X-rays and mitomycin C; quadratic relationships were reported for the other chemicals tested—methyl methanesulfonate, ethyl methanesulfonate, 4-nitroquinoline oxide, hydroxylamine, and *N*-methyl-*N*-nitrosoguanidine.

In the present study, it has been tried to examine the dose-response curves for mutation to streptomycin nondependence for a number of agents in *E. coli* Sd-4. For three agents—ethylene oxide, methyl methanesulfonate and ethyl methanesulfonate—a greater amount of work, in terms of experimentation, was allocated than in the case of other agents.

Materials and methods

Bacteria. — *Escherichia coli* Sd-4, a streptomycin dependent strain, obtained from Prof. G. Bertani, Stockholm was employed; a resistant derivative of this strain, selected inadvertently, was also used; mutation to streptomycin nondependence was studied.

Chemicals. — Chloroethylene oxide and chloroacetaldehyde were prepared in the laboratory by Dr. R. Göthe; the other chemicals were purchased as follows; ethylene oxide and epichlorohydrin from Fluka, methyl methanesulfo-

nate from Koch and Light, ethyl methanesulfonate, glycolaldehyde and glyceraldehyde from Sigma, propylene oxide, glycidol, 1,2-butene oxide, chloroacetone and bromoacetone from Merck.

Media. — The media were described previously (HUSSAIN and EHRENBERG 1979).

Treatment. — The bacteria, cultured overnight, were washed twice with phosphate buffer (0.1 M, pH 7) and resuspended in the same buffer (cell density about 10^8 cells/ml) for treatment at 37°C for 1 h. Cold buffer was added to stop the treatment. The treated bacteria were washed twice with buffer and resuspended for the determination of surviving and mutant cells. Epichlorohydrin, chloroacetaldehyde and chloroethylene oxide were dissolved in ethanol (final concentration of ethanol in the treatment mixture was 0.2 M); other solutions were prepared in buffer.

Analysis of data. — In the case of ethylene oxide the results from experiments performed on different occasions were pooled: The net mutants per 10^8 bacteria for the respective doses from each experiment were weighted with respect to the number of mutant colonies counted. The weighted mean frequency was then plotted with its confidence limits (as calculated by HUSSAIN and EHRENBERG 1979; cf. EHRENBERG et al. 1983).

In the case of other agents the net mutation frequency, with its confidence limits, was plotted for each dose. Only in some cases, when the treatments were repeated at the same dose, do the points represent the weighted means of net mutation frequencies.

Results

The obtained data are presented in Fig. 1–11 with log-log coordinates. In this representation, the linear and quadratic relationships are indicated by straight lines with slopes equal to 1 and 2, respectively. The mutation frequencies refer to the number of surviving bacteria in each case, and the survival of bacteria is indicated only when affected.

After pooling of the data, all the points on the mutagenicity of ethylene oxide can be fitted to a straight line with slope equal to 0.85 ± 0.04 (Fig. 1).

The dose-response curves for mutations with methyl methanesulfonate (obtained with *E. coli* Sd-4 original strain and its resistant derivative; Fig. 2 and 3) and ethyl methanesulfonate (Fig. 4 and 5) appear to be bipartite with two straight line segments in each case. The composite nature of these curves

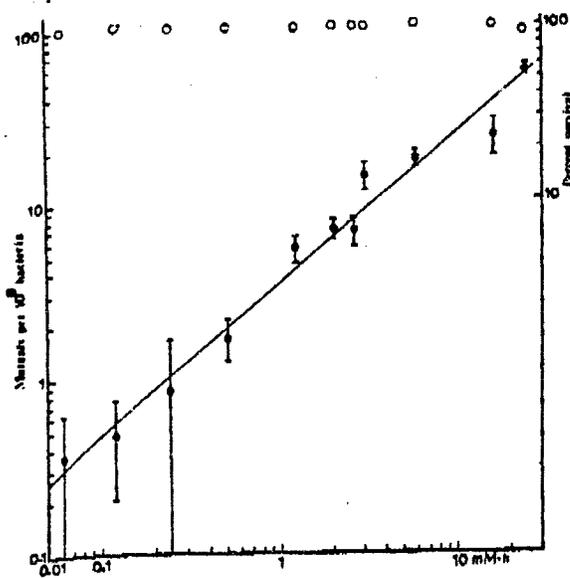


Fig. 1. Mutagenicity of ethylene oxide in *E. coli* Sd-4, plotted as a function of dose. The data represent 15 pooled experiments, and the points bear 95 % confidence limits. The slope with standard error is 0.85 ± 0.04 . The survival is indicated by open circles.

was evaluated statistically. First, the hypothesis (H_0) was formulated that the curve had the same slope over the whole dose range investigated; then the following tests were done:

a) Slope coefficient 'k' and intercept 'a' were calculated in the expression

$$\log y = a + k \log x$$

A theoretical line, calculated according to this expression, was then drawn through the data points (see the inset of Fig. 2 with the same coordinates). This line was divided into segments with equal number of points. The distribution of the points above and below the line was checked by the χ^2 test, as explained below with the actual data for methyl methanesulfonate with the resistant derivative of *E. coli* Sd-4.

	Number of points above the line	Number of points below the line	Sum
Segments at extremities	7	3	10
Segments in the middle	3	7	10
Sum	10	10	20

$$\chi^2 = 1.8, P > 0.05$$

When the probability 'P' of random distribution was calculated directly, a P equal to 0.088 was obtained.

b) The same data were tested against the hypothesis

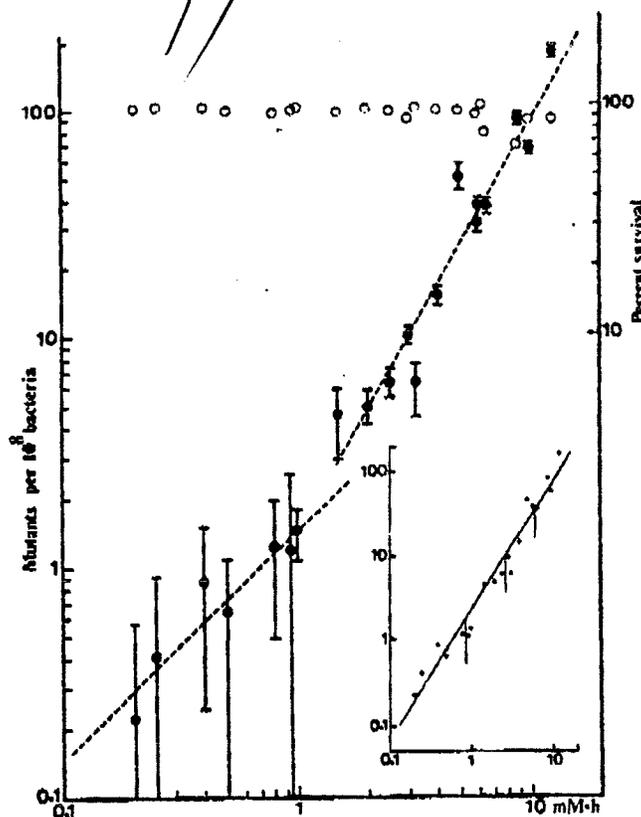


Fig. 2. Mutagenicity of methyl methanesulfonate in *E. coli* Sd-4 (resistant type), plotted as a function of dose. The data represent 10 experiments, and the points bear 80 % confidence limits. The slope with the standard errors in the first and second regions are 1.00 ± 0.16 and 2.00 ± 0.20 , respectively. The inset shows the theoretical line, drawn according to the analysis method 'a' (see Results), divided into segments with equal number of points plotted on the same coordinates. The survival is indicated by open circles.

that the line through the points was continuous and had the same slope as a quadratic curve. The quotient between the mutation frequency 'Y' and the square of the dose ' χ^2 ' was calculated for every point. The numbers of points above and below the median value of the quotient were determined for doses above and below the median dose. The probability 'P' of random distribution was then calculated directly from the following values:

	Points above median quotient	Points below median quotient	Sum
Below median dose	9	1	10
Above median dose	2	8	10
Sum	11	9	20

$$P = 0.003$$

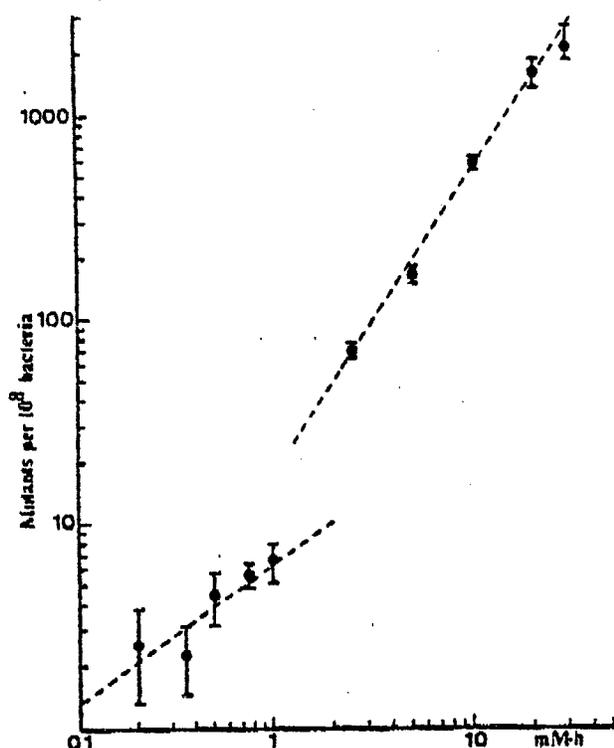


Fig. 3. Mutagenicity of methyl methanesulfonate in *E. coli* Sd-4, plotted as a function of dose. The data represent 4 experiments, and the points bear 95 % confidence limits. The slopes with standard errors in the first and the second regions are 0.67 ± 0.18 and 1.46 ± 0.07 , respectively. The survival was not affected.

The analysis 'a' indicated that one line fitted all the points whereas the analysis 'b' led to a rejection of the hypothesis (H_0) that this line (Fig. 2) had the slope of a quadratic curve.

The data presented for the original strain with methyl methanesulfonate consist of only 10 points (cf. Fig. 3). A line with slope 1.53 ± 0.08 can be fitted to these points. This slope is significantly different from the slopes equal to 1 and 2. An arbitrary calculation of the separate lines in the regions of 0.2 to 1 and 2.5 to 30 mM h showed that the slope for the first region was not significantly different from 1 (0.67 ± 0.18) and the slope for the second region (1.46 ± 0.075) was significantly different from both 1 and 2.

The data for ethyl methanesulfonate, obtained with both the original and the resistant types of *E. coli* Sd-4 (Fig. 4 and 5), respectively, were combined and tested for quadratic relationship as done for methyl methanesulfonate. The distribution of points above and below the median quotient, in the regions above and below the median dose, is noted

quotient was counted twice on both sides of the median dose):

	Points above median quotient	Points below median quotient	Sum
Below median dose	4+3	0+2	9
Above median dose	0+2	4+3	9
Sum	9	9	18

The probability 'P', calculated directly, was found to be equal to 0.02, which indicates that the dose-response relationship for the mutational endpoint with this agent is not quadratic. The slopes of the mutation curves with the original and the resistant derivative (being 1.45 ± 0.39 and 1.66 ± 0.12 , respectively) do not differ significantly from one another. Hence, a mean slope, 1.56 ± 0.28 was calculated to see whether this was different from 1 and 2; it was not different from either of these, which indicates again that the mutation curve obtained with ethyl methanesulfonate is a composite one.

From these analyses it is concluded that the dose-response curves for methyl and ethyl methanesulfonates are composed of two components in each case.

The results obtained with the other electrophilic reagents are presented in Fig. 6-11, with slopes and standard errors given in the figure texts. The dose-response relationships are indicated to be linear for six out of the ten agents tested. In the case of 1,2-butene oxide, a slope lower than 1 was obtained indicating the possibility of a hump in the initial region, but these data are not enough for a definitive conclusion. The slopes of the curves for four carbonyl compounds—chloroethylene oxide, chloroacetaldehyde, chloroacetone and bromoacetone—appear to have marked contributions from the quadratic components.

Discussion

In a previous report (LINDGREN 1971) a higher mutagenic effectiveness of ethylene oxide in the low-dose region than that in the high-dose region was indicated. In the present study a slope lower than 1 (0.85 ± 0.04) was obtained; this increases the probability for the existence of a hump in the low-dose region. It is difficult to speculate on the bacterial population heterogeneity as being the basis of this possible hump, as the survival curves with radi-

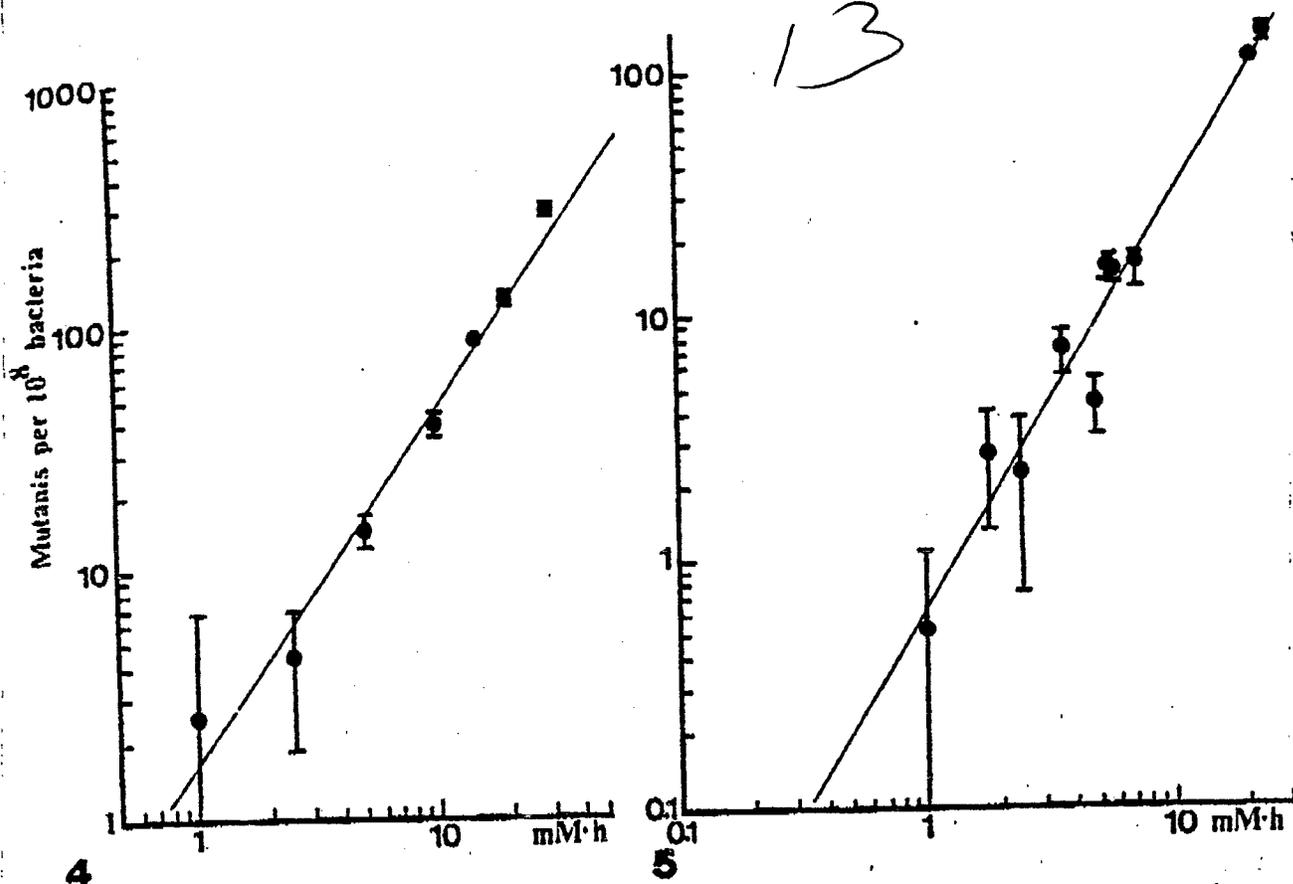


Fig. 4 and 5. Fig. 4. Mutagenicity of ethyl methanesulfonate in *E. coli* Sd-4, plotted as a function of dose. The data represent two experiments, and the points bear 95 % confidence limits. The slope with standard error is 1.66 ± 0.12 . The survival was not affected. Fig. 5. Mutagenicity of ethyl methanesulfonate in *E. coli* Sd-4 (resistant type), plotted as a function of dose. The data represent three experiments, and the points bear 80 % confidence limits. The slope with standard error is 1.45 ± 0.39 . The survival was not affected.

ation (data not reported) did not appear to be bimodal; heterogeneity has been considered to be the basis of the hump following irradiation of *Drosophila* spermatogonia (cf. OFTEDAL 1968). The extent of participation of different repair processes, after exposure of bacteria to ethylene oxide, is underway at our laboratory and it appears that the pattern of mutagenicity of this chemical agent is more like γ -radiation than the typical alkylating agents such as ethyl methanesulfonate and methyl methanesulfonate (KOLMAN, unpublished; cf. also KOLMAN and NASLUND 1983).

The dose-response relationships for mutations induced by methyl and ethyl methanesulfonates have been reported to be quadratic in previous studies in bacteria (KONDO et al. 1970; ISHII and KONDO 1975). A scrutiny of these data reveals that these investigators have been operating in the intermediate-high dose region. The present study also demonstrates that the dose region in the previous

studies has lain outside the linear component region. The bipartite appearance of the curves is possibly a consequence of a compromise between two types of events occurring in the low and high-dose regions: in the low-dose region, besides single base alterations, deletions are occurring, too, as reported for *Neurospora* by WEBBER and DE SERRES (1965; cf. also ABRAHAMSON 1976). This shape of the curve may also be a consequence of the gradual saturation of the cellular repair systems, with increasing damage. Methyl methanesulfonate depends for its action on the error-prone repair system (BRIDGES et al. 1973), whereas ethyl methanesulfonate does not (cf. LAWLEY 1974). The introduction of ethyl groups by the latter results in miscoding errors, while the introduction of methyl groups by the former probably results in misrepair of single-strand breaks (GARNER et al. 1979).

Calculations were done on the number of guanines alkylated per bacterium at the lowest dose

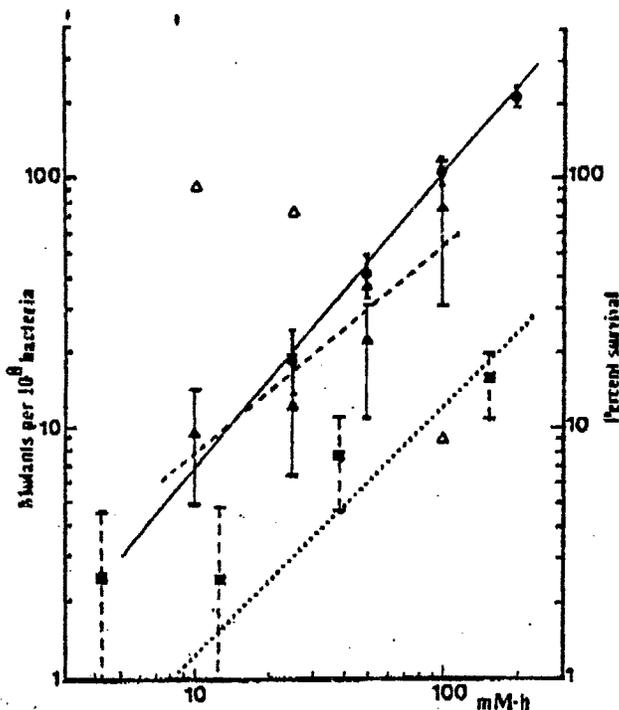


Fig. 6. Mutagenicity of propylene oxide, glycidol and 1,2-butene oxide in *E. coli* Sd-4, plotted as a function of dose. The points bear 95 % confidence limits. The slopes with standard errors are 1.24 ± 0.27 and 0.92 ± 0.19 for propylene oxide and glycidol, respectively; for 1,2-butene oxide, the slope 1 is fitted to the points except the lowest one: only data with newly received preparations are presented. ● —, propylene oxide; ▲ ---, glycidol; ■, 1,2-butene oxide; survival not affected in the case of propylene oxide and 1,2-butene oxide; The survival is indicated by open triangles.

of ethylene oxide: at 0.06 mM h only 0.1* Gua-⁰ per bacterium was hydroxyethylated. This implies that there is no threshold for ethylene oxide, because the

* This figure for ethylene oxide was obtained as noted below:

$$\begin{aligned} \text{Degree of alkylation of Gua-N-7} &= k_{\text{Gua-N-7}} \times D \\ (k_{\text{Gua-N-7}}: \text{rate constant for reaction of ethylene oxide with} \\ &\text{Gua-N-7 (EHRENBERG and HUSSAIN 1981); D, dose)} \\ &= 13 \cdot 10^{-3} \text{ M}^{-1} \text{ h}^{-1} \times 0.06 \cdot 10^{-3} \text{ Mh} \\ &= 7.8 \cdot 10^{-6} \end{aligned}$$

$$\text{DNA per haploid bacterial genome} = 10^{-16} \text{ g (DAVIDSON 1972)}$$

$$\text{Mol. Gua per bacterium (25 \% Gua)} = \frac{10^{-16}}{1280} = 7.6 \cdot 10^{-19}$$

$$\begin{aligned} \text{Number of Gua per bacterium} &= 7.6 \cdot 10^{-19} \times 6.02 \cdot 10^{23} \\ &= 4.55 \cdot 10^5 \end{aligned}$$

$$\begin{aligned} \text{N-7-HOEtGua per haploid genome} &= 7.8 \cdot 10^{-6} \times 4.55 \cdot 10^5 \\ &= 35 \end{aligned}$$

Ratio ⁰-MeGua: N-7-MeGua for methyl methanesulfonate (cf. LAWLEY 1980) as assumed also for ethylene oxide = 0.004

$$\text{⁰-HOEtGua per bacterial genome} = 35 \times 0.004$$

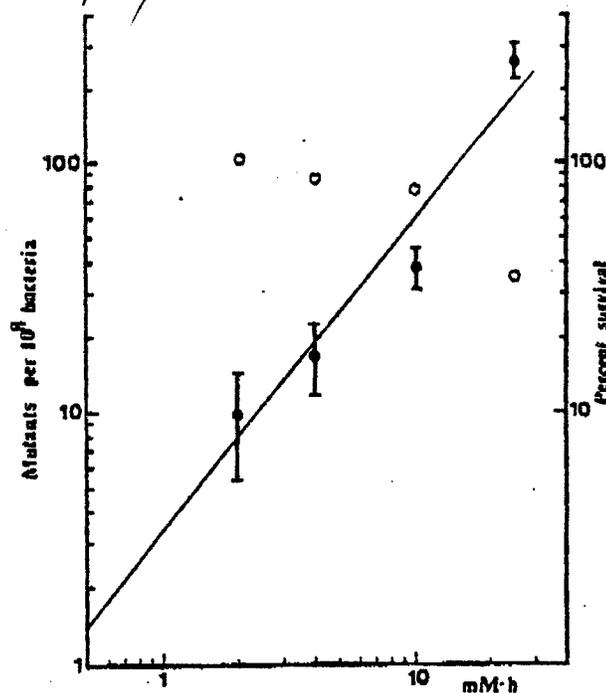


Fig. 7. Mutagenicity of epichlorohydrin in *E. coli* Sd-4; plotted as a function of dose. One test performed. The points bear 95 % confidence limits, and the slope with standard error is 1.27 ± 0.19 . The survival is indicated by open circles.

lowest dose is effective even when less than one alkylation at Gua-⁰ per bacterium has occurred. The scale and the number of experiments in the case of methyl methanesulfonate was not as large as in the case of ethylene oxide, hence the region where less than multiple alkylations would occur has not been investigated.

The observation of linear dose-response relationships with six out of the ten agents tested points to a prevalence of miscoding errors in the low as well in the intermediate regions (LAWLEY 1974). (The data on propylene oxide, butene oxide, glycidol, epichlorohydrin, glyceraldehyde and glycolaldehyde should be considered very preliminary at this stage.) The agents with the slope coefficients greater than 1.4, all have substrate constants (substrate constant is a parameter describing the dependence of chemical reactivity on nucleophilicity) s , greater than or about 1.3 (see Addendum). A high substrate constant indicates a greater proportion of reaction at centres with a high nucleophilicity, such as sulphhydryl groups of proteins (which may imply an inactivation of repair enzymes) than at centres with a low nucleophilicity. A high reactivity at centres with a low nucleophilicity is related to a high reactivity. For comparison, a mean value, i.e.

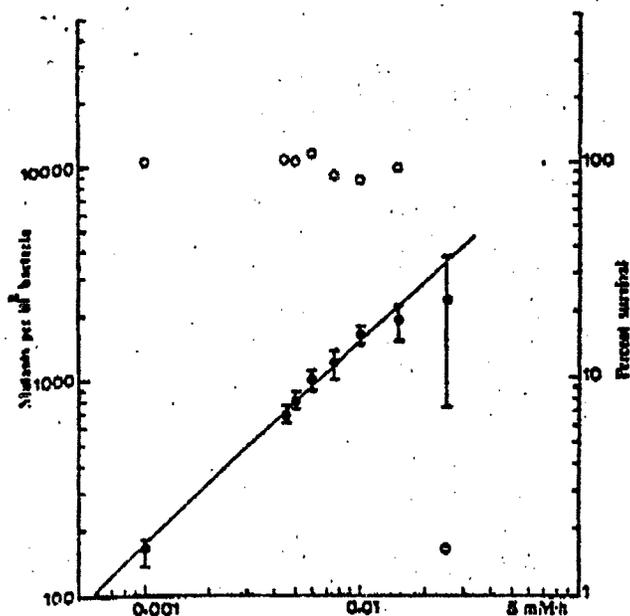


Fig. 8. Mutagenicity of chloroethylene oxide in *E. coli* Sd-4, plotted as a function of dose. The data represent two experiments, and the points bear 95 % confidence limits. The slope with standard error is 0.98 ± 0.04 . The survival is indicated by open circles.

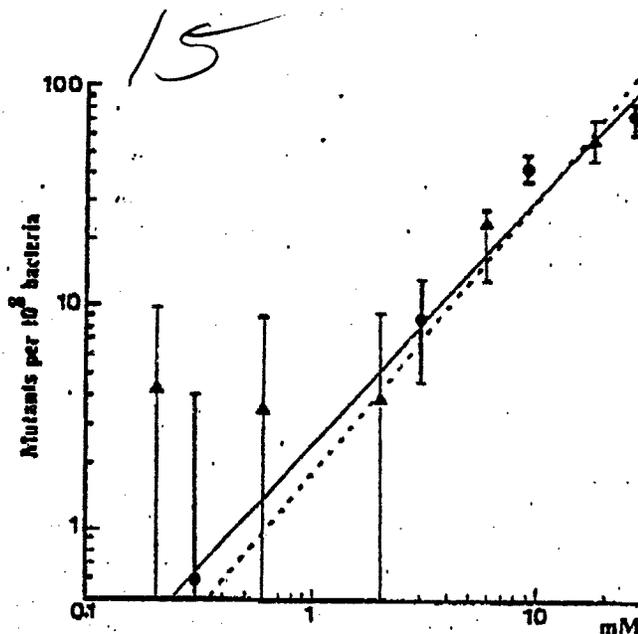


Fig. 9. Mutagenicity of glycolaldehyde and glyceraldehyde in *E. coli* Sd-4, plotted as a function of concentration. Only one test carried out with each compound. The points bear 95 % confidence limits. The slopes (in the case of glyceraldehyde, first two points excluded for this calculation) with standard errors are 1.12 ± 0.11 and 1.23 ± 0.16 , respectively. • —, glycolaldehyde; ▲ ---, glyceraldehyde; survival was not affected, in both cases.

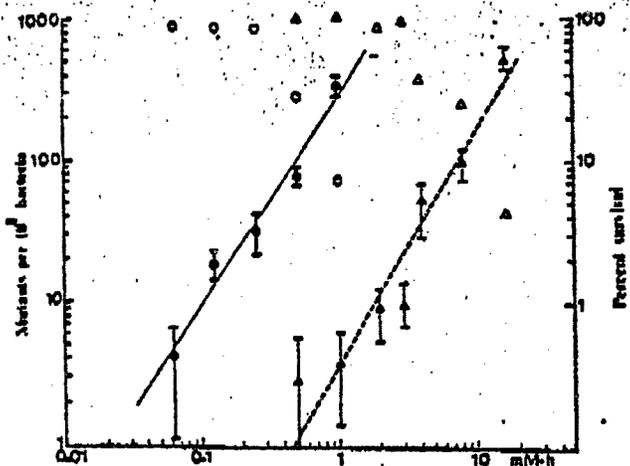


Fig. 10. Mutagenicity of chloroacetone and bromoacetone in *E. coli* Sd-4, plotted as a function of dose. The data represent two tests, and the points bear 95 % confidence limits. The slopes with standard errors are 1.7 ± 0.36 and 1.44 ± 0.18 , in the order mentioned. ▲, chloroacetone; •, bromoacetone. The survival is indicated by open circles and triangles for bromoacetone and chloroacetone, respectively.

$n=2$, of nucleophilicities of carbonyl groups (which include oxygens) with low reactivities has been considered. Except for chloroethylene oxide, chloroacetaldehyde, chloroacetone and bromoacetone (and excluding glycolaldehyde and glyceraldehyde, since they react as carbonyl compounds not as alkylating

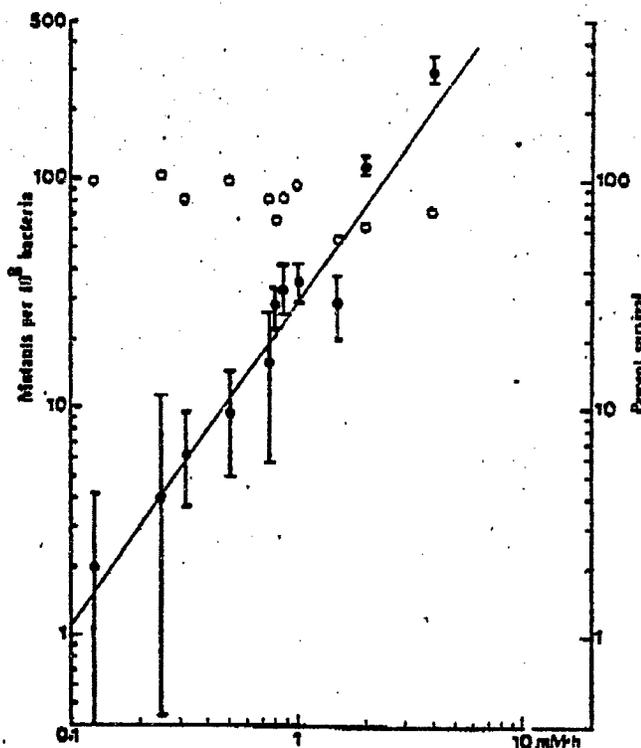


Fig. 11 Mutagenicity of chloroacetaldehyde in *E. coli* Sd-4, plotted as a function of dose. The data represent 4 experiments, and the points bear 95 % confidence limits. The slope with standard error is 1.42 ± 0.11 . The survival is indicated by open circles.

Table 1. Data on chemical reactivity and mutagenic effectiveness of some chemical agents. After 1 h treatment of *E. coli* Sd-4 at 37°C, the cells were washed twice with buffer and resuspended, after which plating was done for the determination of surviving and mutant cells

Compounds	$k_{n=2}$ M ⁻¹ h ⁻¹	s	Mutants per 10 ⁷ survivors per mM·h	Mutants per 10 ⁷ survivors divided by $k_{n=2}$
Methyl methanesulfonate	7.8 10 ⁻²	0.88	7	90
Ethyl methanesulfonate ^a	2.6 10 ⁻²	0.69	2	77
Ethylene oxide ^c	1.3 10 ⁻¹	0.96	2.7	200
Propylene oxide ^c	1.2 10 ⁻¹	0.96	0.95	80
Butene oxide ^c	1.0 10 ⁻²		0.6	60
Glycidol ^c	1.0 10 ⁻¹	0.96	1	100
Epichlorohydrin ^c	3.3 10 ⁻¹	1	3.4	100
Chloroethylene oxide ^b	4.3	0.83	1.6 10 ³	3.7 10 ³
Chloroacetaldehyde ^b	3.0 10 ⁻²	1.3	30	1·10 ³
Chloroacetone ^c	4 10 ⁻²	1.3	4	1.0 10 ³

^a Rate constants from EHRENBERG and HUSSAIN 1981

^b Rate constants from HUSSAIN and OSTERMAN-GOLKAR 1976

^c Rate constant from OSTERMAN-GOLKAR, see Addendum

^d Expected to be 0.8 of that of ethylene oxide (cf. EHRENBERG and HUSSAIN 1981)

agents) the mutagenic effectiveness in the linear regions of the dose-response curves is proportional to the rate constants for alkylation at the nucleophilicity, $n=2$. A high mutagenic effectiveness of these four chemicals is probably a consequence of their ability to form cyclic derivatives with nucleic acid bases (cf. HUSSAIN and OSTERMAN-GOLKAR 1976). The formation of any extra ring would be expected to reduce the fidelity in replication. The demonstrated rough proportionality between mutagenic effectiveness and the reaction rates (see Table 1) can be utilized in a preliminary assessment of the genetic toxicity of the alkylating agents with known reaction kinetics (cf. EHRENBERG 1979; EHRENBERG and OSTERMAN-GOLKAR 1980).

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Addendum

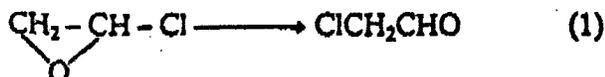
Reaction kinetics in water of chloroethylene oxide, chloroacetaldehyde, and chloroacetone

SIV OSTERMAN-GOLKAR

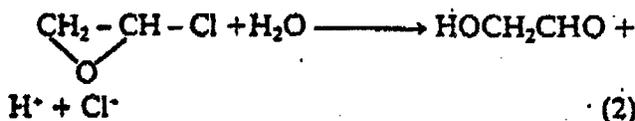
Department of Radiobiology, Wallenberg Laboratory, University of Stockholm, 106 91 Stockholm, Sweden

Information on the reactivity of chemical agents is useful in quantitative evaluation of their genotoxicity. This has been demonstrated previously in a series of papers from this laboratory (TURTOCZKY and EHRENBERG 1969; OSTERMAN-GOLKAR et al. 1970; EHRENBERG et al. 1974; OSTERMAN-GOLKAR and WACHTMEISTER 1976; EHRENBERG and HUSSAIN 1981; HUSSAIN 1981). In an early paper (HUSSAIN and OSTERMAN-GOLKAR 1976) reaction rate constants for chloroacetaldehyde and chloroethylene oxide have been given without any details on methodology. It is considered to be of value to report such details for the investigation of these agents and of chloroacetone as an addendum to the paper on dose-response relationships by Hussain (this journal) in the above perspective (see EHRENBERG 1980; EHRENBERG and OSTERMAN-GOLKAR 1980).

Chloroethylene oxide is a highly reactive and unstable electrophile. It rearranges, especially on heating (GROSS and FREIBERG 1969), to chloroacetaldehyde



The half-life ($t_{1/2}$) in dry acetonitrile has been estimated as about 5 h at room temperature, due to this reaction (RANNUG et al. 1976). The half-life in d_2 -H₂O/ d_6 -dimethyl sulfoxide (80/20, v/v) at 4°C has been determined to be 46.2 min by NMR technique (ELMORE et al. 1976). These authors claim that the reaction is a rearrangement to chloroacetaldehyde. The stability has further been determined in water at 37°C ($t_{1/2}$ = 1.6 min) by analysis of remaining alkylating agent as a function of time (BARBIN et al. 1975). According to GROSS and FREIBERG (1969) chloroethylene oxide forms glycolaldehyde during hydrolysis in water



(Glycolaldehyde was identified as glyoxal-bisdinitrophenylhydrazone after precipitation with 2,4-dinitrophenylhydrazine in sulfuric acid. Chloroacetaldehyde under the same conditions gives chloroacetaldehyde-dinitrophenylhydrazone.)

Chloroethylene oxide reacts analogously with