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Genetics

Mutagenicity of chloroacetaldehyde, a possible metabolic product of 1,2-dichloroethane (ethylene dichloride), chloroethanol (ethylene chlorohydrin), vinyl chloride, and cyclophosphamide

(environmental carcinogens/alkyl halides)

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ABSTRACT **We have previously described a very sensitive and efficient bacterial test designed to detect chemical carcinogens as mutagens. Chloroacetaldehyde is mutagenic in this system and is of interest because it is a possible metabolite in mammals of the large volume industrial chemicals 1,2-dichloroethane (ethylene dichloride)(3.5 billion kg/yr, U.S.) and vinyl chloride (2.5 billion kg/yr, U.S.), and of the antineoplastic agent cyclophosphamide. Chloroacetaldehyde reverts a new *Salmonella* bacterial tester strain (TA100). Chloroacetaldehyde is shown to be hundreds of times more effective in reversion of TA100 than is chloroethanol (ethylene chlorohydrin), a known metabolic precursor of chloroacetaldehyde and a possible metabolite of dichloroethane and vinyl chloride, or than vinyl chloride, which is itself mutagenic for TA100. Chloroethanol is shown to be activated by rat (or human) liver homogenates to a more highly mutagenic form with reversion properties similar to chloroacetaldehyde. Reversion properties of cyclophosphamide after *in vitro* metabolic activation suggest that chloroacetaldehyde is not the active mutagenic form of this antineoplastic drug.**

We have previously described a rapid, sensitive bacterial test designed to detect chemical carcinogens as mutagens (1–7). The test utilizes a special set of histidine mutants of *Salmonella typhimurium* for reversion, and a rat (or human) microsomal system for metabolic conversion of carcinogens to their active forms. The standard bacterial tester strains have been described in detail (1, 3, 5) and contain histidine missense (TA1535) or frameshift (TA1537, TA1538) mutations. The strains also contain *uvrB* and *rfa* (deep rough) mutations which greatly increase their sensitivity to reversion by a variety of carcinogens: *uvrB* causes loss of the excision repair system and *rfa* causes loss of the lipopolysaccharide permeability barrier. The compound to be tested, the bacterial tester strain, and when required rat (or human) liver microsomal enzymes, are combined on a petri dish and after incubation at 37° for 2 days histidine revertants are scored.

Hundreds of carcinogens and noncarcinogens have been tested using the *Salmonella*/mammalian-microsome bacterial test system in this laboratory (1–13) and in many laboratories throughout the world. We (J.M. and B.N.A.) are currently preparing a compilation of these results, and so far about 85% of the known carcinogens tested have been detected as mutagens in the test, and very few (<10%) noncarcinogens (many close relatives of carcinogens have been tested) are positive. This and other evidence showing that a high percentage of carcinogens are mutagens is most easily explained if carcinogens cause cancer by somatic mutation (4). We believe there is a high probability that chemicals found to be mutagens in the *Salmonella* test will turn out to be carcinogens.

We have recently introduced two new tester strains (TA100 and TA98) which were constructed by transferring to our standard tester strains TA1535 and TA1538 respectively, a resistance transfer factor (R factor), pKM101 (7). The new tester strains are greatly enhanced in sensitivity to reversion with a variety of potent carcinogens such as the fungal toxin aflatoxin, 7,12-dimethylbenz(a)anthracene, benzo(a)pyrene, and others, and also can be used to detect a variety of carcinogens and mutagens not detected by our standard tester strains.

RESULTS AND DISCUSSION

Chloroacetaldehyde. A compound of unusual interest that was positive on the new strain TA100 was chloroacetaldehyde. Fig. 1A shows that, at the levels tested, chloroacetaldehyde is quite effective in reverting TA100, but does not revert TA1535. [We find at higher concentrations a slight activity of chloroacetaldehyde in reverting TA1535; weak activity has been found independently (14–17).] Chloroacetaldehyde is relatively specific for TA100, and does not significantly revert the other R factor tester strain, TA98 (TA1538 containing the pKM101 plasmid), or our other standard tester strains.

We originally tested chloroacetaldehyde because of its known unique reactions with nucleotides, which have been extensively studied in connection with its use as a fluorescent label (18–21). It is known to react specifically with the N-1 and N^6 nitrogens of adenosine and the N-3 and N^4 nitrogens of cytidine to form a highly fluorescent cyclic derivative. It has been used extensively for fluorescent labeling of a variety of adenine and cytosine containing compounds, including single-stranded DNA. The mutagenic activity of chloroacetaldehyde reported here strongly suggests it can also react with chromosomal DNA, possibly at exposed single-stranded regions such as the replication fork, or in regions of the DNA undergoing recombinational repair.

Chloroacetaldehyde has also become of interest recently as a possible metabolic product of the large volume industrial chemical, and human carcinogen vinyl chloride (15–17, 22–25). It is also a possible active metabolite of several widely used chemicals and we discuss this in relation to its mutagenicity.

1,2-Dichloroethane (ethylene dichloride) is metabolized in mammals to chloroacetic acid, probably through chloroethanol and chloroacetaldehyde (26, 27). It is an extremely toxic chemical produced industrially in enormous quantities [about 3.5×10^9 kg in 1974 in the U.S. (28) and about 20 ×

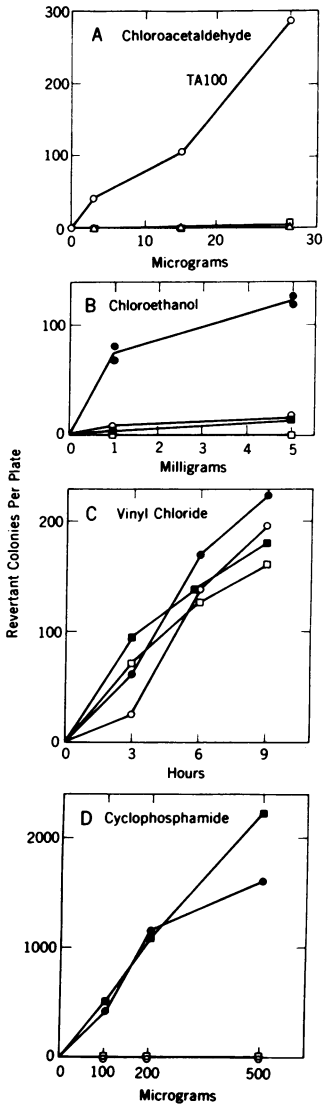

FIG. 1. Reversion of TA100 and TA1535 with (A) chloroacetaldehyde, (B) chloroethanol, (C) vinyl chloride, and (D) cyclophosphamide. Procedures were as described previously (ref. 3 and Table 1). S-9 (150 μl per plate) obtained from phenobarbital-induced rats (4) was added for vinyl chloride activation; S-9 obtained from Aroclor-induced rats (12) was added for chloroethanol (150 μl S-9 per plate) and cyclophosphamide (300 μl S-9 per plate) activation. □, TA1535; △, TA1538; ○, TA100; ●, TA100 + S-9; ■, TA1535 + S-9.

10^6 tons worldwide (29)]. It is used primarily in the manufacture of vinyl chloride and other industrial chemicals, but is also used in large amounts as a lead scavenging agent in gasoline (over 100 million kg in 1970), in various solvent applications, and as a component of fumigants for grain, upholstery, and carpets (30).

In Table 1 the reversion of TA100 with chloroacetaldehyde is compared directly to reversion with two other metabolic products of dichloroethane, i.e., chloroethanol and chloroacetic acid. Although dichloroethane and chloroethanol are weakly mutagenic in bacteria without metabolic activation as previously shown (31–35)†, on a molar basis chloroacetaldehyde is hundreds of times more effective in reverting TA100. In similar assays chloroacetic acid was inactive. The metabolites were also tested on the complete set of tester strains (TA1535, TA1537, TA1538, TA98) and except for the expected weak reversion of TA1535 with chloroethanol and chloroacetaldehyde, the other strains were not reverted. Attempts to activate dichloroethane to a more mutagenic form using the standard rat liver *S-9 Mix* (4, 12) for activation were unsuccessful. This could be related to the relatively inefficient conversion of chloroethanol to chloroacetaldehyde in the *in vitro* system (Fig. 1B), or to other undetermined factors. We are currently optimizing our rat liver activation system for the metabolic conversion of this and a variety of other alkyl halides such as carbon tetrachloride and the results of these experiments will be published elsewhere.

Table 1. Reversion of TA100 with 1,2-dichloroethane, vinyl chloride, and several possible metabolites

		Revertant colonies	
	μg	per plate	per μmol
Dichloroethane*	1.3×10^4	25	0.19
Chloroacetaldehyde	30	285	746
Chloroethanol	2.1×10^4	159	0.6
Chloroacetic acid	1.0×10^3	0	—
Vinyl chloride	7.4×10^3	128	1.0

Results are from linear dose-response curves after subtracting spontaneous background revertants (approximately 150 per plate). Reversion was determined on petri plates by incorporating mutagen and bacteria directly into an agar overlay as previously described (3); no microsomes were used. For vinyl chloride, TA100 was exposed on petri plates in desiccators for various times to a vinyl chloride atmosphere (20% v/v) (15). Revertant colonies were scored after incubation of the petri plates for 48 hr at 37°. All chemicals were of the highest purity available: vinyl chloride (99.9% pure) was from Union Carbide; vacuum distilled chloroacetaldehyde was a gift of Nelson Leonard; 1,2-dichloroethane and chloroethanol were from Aldrich.

* Dichloroethane is an extremely weak mutagen, and reproducible dose-response curves were not obtained; results reported are averages from several experiments.

Chloroethanol, itself an industrial chemical, is a likely metabolic product of dichloroethane and precursor of chloroacetaldehyde. It is known to be metabolized *in vivo* (38) and *in vitro* to chloroacetaldehyde by rat (38) or human (39) liver alcohol dehydrogenase. We have examined the reversion of TA100 and TA1535 with chloroethanol, and Fig. 1B shows that in the presence of rat liver microsomes, chloroethanol is activated to a form which shows clear activity on TA100 and a very slight activity for TA1535‡. Similar results were obtained for TA100 using human liver extracts. Fig. 1B also shows that chloroethanol, at higher concentrations, is weakly mutagenic directly (without microsomes) for TA100 and shows a trace of activity for TA1535, as previously reported (31, 33, 34). In contrast to the microsomal activation, we have not been able to show any clear mutagenic effect on incubating these amounts of chloroethanol with horse liver alcohol dehydrogenase and NAD, and other enzymes in our S-9 homogenate could be involved (40–42). Exogenously added NADP, normally present in our *S-9 Mix*, was not necessary for the S-9 activation. The enhanced reversion of TA100 relative to TA1535 after metabolic activation could suggest that chloroacetaldehyde, which has simi-

§ Dichloroethane has also been reported quite mutagenic in Drosophila (36, 37).

‡ Malaveille, *et al.* (16) have independently shown the weak (approximately 2-fold) enhancement in reversion of TA1530 (one of our *Salmonella* tester strains similar to TA1535) with chloroethanol after *in vitro* metabolic activation.

lar specificity in reversion of TA100 (Fig. 1A), is the active metabolite.

Vinyl Chloride, a major industrial chemical and a carcinogen in humans and rodents (43–45), has been recently reported mutagenic in yeast (46), and *Salmonella* (15–17, 22), and chromosome breaks have been observed in humans exposed to high doses of vinyl chloride (47, 48). Vinyl chloride is metabolized in rats, and it appears likely that a metabolite is the active mutagenic or carcinogenic form (15–17, 22–25).

Two of the metabolites of dichloroethane (chloroacetaldehyde and chloroethanol) are also suggested metabolites of vinyl chloride (15, 16, 22–25). Another proposed active metabolite of vinyl chloride, chloroethylene oxide (22, 23), also has been recently shown to be quite mutagenic in the *Salmonella* test (16, 17) and in a recent study both chloroacetaldehyde and chloroethylene oxide have been implicated as likely *in vitro* metabolites of vinyl chloride (23).

Vinyl chloride itself has been shown to be mutagenic in the presence of the S-9 microsomal fraction by Rannug *et al.* (22) and later by Bartsch *et al.* (15). Bartsch *et al.* also reported considerable mutagenic activity in the absence of microsomes. We find that most of the mutagenic activity of vinyl chloride (Table 1, Fig. 1C) is direct activity, not requiring activation with rat liver homogenates (*S-9 Mix*)[§]. So far the cause of the reported differences in direct and microsomally activated mutagenesis with vinyl chloride is not clear. It is also not clear whether the mutagenic activity of vinyl chloride in the absence of microsomes is due to a possible bacterial activation. One argument against chloroacetaldehyde being involved in the mutagenic activity of vinyl chloride is the equal activity of vinyl chloride against tester strains TA100 and TA1535 (compare Fig. 1C with Fig. 1A). On the other hand we see so little metabolic activation of vinyl chloride with the *S-9 Mix* that this argument may not be pertinent. We are also uncertain about the involvement of chloroacetaldehyde as an active mutagenic form of vinyl chloride because of the relatively inefficient metabolic activation of chloroethanol (Fig. 1B) in our system. An alternative metabolic intermediate between vinyl chloride and chloroacetaldehyde is chloroethylene oxide, which is known to rearrange spontaneously to chloroacetaldehyde (49). The mutagenicity of both chloroethylene oxide and chloroacetaldehyde suggest that one or both of these forms may be the true active carcinogenic form of vinyl chloride.

Cyclophosphamide (also called Endoxan, or Cytoxan) is used extensively as an antineoplastic agent, and is also a known carcinogen (50); its mutagenic activity (51–53) requires metabolic conversion to an active form. Several metabolic products of cyclophosphamide are known, but the exact nature of the active form is still uncertain. It has recently been suggested, largely on hypothetical grounds, that chloroacetaldehyde could be the active product (54). We have compared directly the reversion of TA100 and TA1535 with cyclophosphamide before and after activation with rat liver *S-9 Mix* and the results are shown in Fig. 1D. Cyclophosphamide is clearly mutagenic for both tester strains after metabolic conversion, but there is no preference for TA100. We conclude that the primary mutagenic metabolite of cyclophosphamide *in vitro* is not chloroacetaldehyde. Other possibilities are the known metabolite, aldophosphamide (55), or nitrogen mustard itself. We have previously shown that a close relative of nitrogen mustard, *bis*-2-chloroethylamine, reverts TA1535 and TA100 to a similar degree (7), and thus has reversion properties similar to activated cyclophosphamide.

In view of the accumulating evidence of the correlation between mutagenicity and carcinogenicity, we believe that the mutagenic activity of chloroacetaldehyde indicates it has a high probability of being a carcinogen. We are not aware of any studies on the carcinogenicity of chloroacetaldehyde. However, in an extensive study dealing with its toxicity, Lawrence *et al.* (56) report changes of respiratory epithelium in lungs of rats exposed to chloroacetaldehyde that are suggestive of a pre-malignant condition.

The likely involvement of chloroacetaldehyde in the metabolism of dichloroethane and chloroethanol indicates the carcinogenicity of these industrial chemicals should be thoroughly examined. Though dichloroethane is produced in billions of kg/yr it is not unique among large industrial chemicals in never having been thoroughly tested for carcinogenicity[¶]. Carcinogenicity studies with chloroethanol (59, 60) are somewhat conflicting and are by no means definitive.

Angiosarcoma, the rare form of liver cancer associated with human exposure to vinyl chloride might also be found in workers exposed to dichloroethane or chloroethanol if chloroacetaldehyde is actually the active carcinogenic form of all three chemicals.

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[§] We have used liver microsomes from both phenobarbital- and Aroclor-induced rats. Attempts to obtain more significant activation of vinyl chloride as a mutagen in liquid suspensions in sealed vials using the standard *S-9 Mix*, purified rat liver microsomes, or using *S-9 Mix* from which small molecules (i.e., glutathione) were removed by passage through a Sephadex G-50 column were all unsuccessful, as were attempts to add liver alcohol dehydrogenase and NAD to the standard *S-9 Mix*.

Note Added in Proof. In more recent experiments on the mutagenicity of vinyl chloride we have been able to obtain a 2-fold increase over the direct activity adding 40 μl of *S-9* (Aroclor-induced rat liver) to the 0.5 ml of *S-9 Mix*. This microsomal dependent activity is about the same for both TA1535 and TA100 and thus does not appear to be due to chloroacetaldehyde.

[¶] A carcinogenicity study with dichloroethane in rodents is currently in progress at the National Cancer Institute (E. Weisburger, personal communication). The close relative of dichloroethane, 1,2-dibromoethane, which was detected over 4 years ago as a mutagen in *Salmonella* (1) and in other systems, was recently reported carcinogenic (57, 58).

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