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MUTATION INDUCTION IN CHINESE HAMSTER V79 CELLS BY TWO VINYL CHLORIDE METABOLITES, CHLOROETHYLENE OXIDE AND 2-CHLOROACETALDEHYDE

by

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Chloroethylene oxide and 2-chloroacetaldehyde, two possibly carcinogenic metabolites of vinyl chloride in mammals, caused a dose-dependent induction of 8-azaguanine- and ouabain-resistant mutants in Chinese hamster V79 cells in vitro. Up to one-hundredfold higher concentrations of 2-chloroethanol or monochloroacetic acid, a urinary vinyl chloride metabolite in rats and man, were inactive.

The various adverse biological effects of vinyl chloride (I, Fig. 1), its toxicity (Patty *et al.*, 1963; Tribukh *et al.*, 1949) and carcinogenicity in experimental animals and in man (Creech and Johnson, 1974; IARC, 1974*a*, 1975; Lee and Harry, 1974; Maltoni and Lefemine, 1974; Viola *et al.*, 1971), its mutagenic action in microbial systems (Bartsch *et al.*, 1975; Bartsch and Montesano, 1975; Malaveille *et al.*, 1975; Loprieno *et al.*, 1975; Rannug *et al.*, 1974) and perhaps its chromosomal breaking activity in human cells (Ducatman *et al.*, 1975; Funes-Cravioto *et al.*, 1975) appear to be largely dependent upon its metabolic conversion into chemically reactive metabolites. *In vitro* studies have shown that microsomal hepatic mixed-function oxidases of rats, mice and humans are equally effective in metabolizing vinyl chloride into reactive metabolites (Bartsch *et al.*, 1975; Bartsch and Montesano, 1975).

2-Chloroethanol has been suggested as a possible intermediate in vinyl chloride metabolism in rats (Hefner *et al.*, 1975) and exhibits a weak mutagenic activity in bacteria (Bartsch *et al.*, 1975; Malaveille *et al.*, 1975; Rosenkranz *et al.*, 1974). 2-Chloroacetaldehyde (III) and chloroethylene oxide (II) have been identified as vinyl chloride metabolites by *in vitro* techniques (Bartsch and Montesano, 1975; Guthe *et al.*, 1974); monochloroacetic acid (IV) is a urinary metabolite in rats and man exposed to vinyl chloride (Grigorescu and Toba, 1966; Hefner *et al.*, 1975). Because the relationship between carcinogenicity and mutagenicity is becoming more formally established (Miller and Miller, 1971), we have studied the mutagenic effect of these compounds in mammalian cells. We have used Chinese hamster V79 cells, a mammalian cell system that has already been successfully used for the detection of other chemical carcinogens and/or their metabolites as mutagens (Huberman *et al.*, 1971; Huberman *et al.*, 1972; Huberman and Sachs, 1974).

MATERIAL AND METHODS

Chemicals

Chloroethylene oxide (b.p., 65-67 C) was prepared by chlorination of ethylene oxide in the gas phase (Gross and Freiberg, 1969) by

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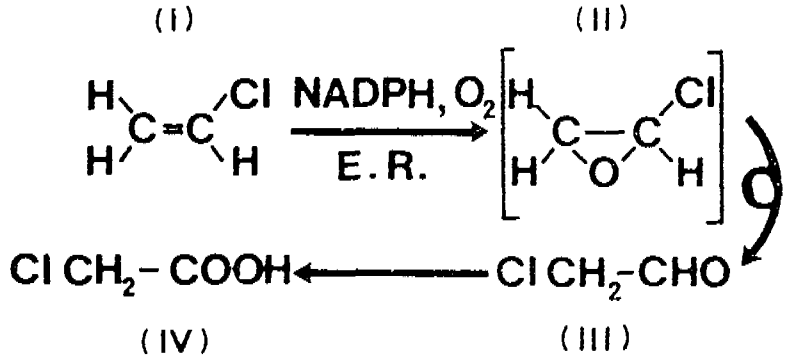

FIGURE 1

Bio-transformation of vinyl chloride (I) to chloroethylene oxide (II), which rearranges to 2-chloroacetaldehyde (III). The latter is further oxidized to monochloroacetic acid (IV), a urinary metabolite in humans and rats following their exposure to vinyl chloride.

Drs. A. Croisy and P. Jacquignon, CNRS, Gif-sur-Yvette, France. The compound, which contained traces of 2-chloroacetaldehyde (< 1%) and ethylene oxide (< 5%), was stored at below −60° C. Under these conditions no change in the NMR spectrum (in $CDCl_3$) was noted after 12 days. 2-Chloroacetaldehyde (50% aqueous solution) and monochloroacetic acid (m.p., 61-63° C) were obtained from Fluka Co., Buchs, Switzerland and 2-chloroethanol (b.p., 120-130° C) from Merck-Schuchardt, Darmstadt, Federal Republic of Germany. 8-Azaguanine and ouabain were purchased from Sigma Chemical Co., St. Louis, Mo., USA.

Cell cultures

The V79 cells derived from a clone isolated from the male Chinese hamster cell line V79-4, kindly supplied by Dr. L. M. Y. Chu, University of Michigan, Ann Arbor, Mich., USA. Cells were grown in Eagle's medium containing a four-fold concentration of amino acids and vitamins and 10% foetal calf serum; 5 ml medium were used per 50 mm plastic Petri dish (Nunclon, Nunc-A/S, Roskilde, Denmark), and the cultures were incubated at 37 ± 0.5° C in a humidified incubator supplied with a constant flow of 10% CO_2 in air. No detectable mycoplasmic contamination was found when the cultures were tested on mycoplasma agar (Chanock *et al.*, 1962). Stock solutions of 200-fold concentrations of 8-azaguanine in dimethylsulphoxide (DMSO) were prepared and stored for up to 4 weeks at −20° C. The final concentration of DMSO in the medium was 0.5%; it did not affect the cloning efficiency of V79 cells.

Cytotoxicity assay

Dishes were seeded with 200 cells per dish. After 16-18 h, various concentrations of the compounds to be tested were added for 3 h, after which fresh medium was added. After incubation for 6-8 days, the dishes were fixed and the colonies counted. Each point of cloning efficiency is based on an average value taken from 5-6 dishes. Of 200 cells plated, about 90% were still single cells 18 hours later.

Mutagenicity assay

Selection of 8-azaguanine- or ouabain-resistant mutants was performed in a similar manner to that described above: 16-18 h after seeding, $2 \cdot 10^4$ cells/dish for 8-azaguanine resistance and $1 \cdot 10^5$ cells/dish for ouabain resistance, the cells were treated for 3 h with different concentrations of the compounds to be tested. Fresh medium was then added and the cells were incubated for an additional 48 h. For selection of 8-azaguanine-resistant colonies, the cultures were re-fed every 2 days with medium containing 0.2 mM 8-azaguanine, usually for 10-12 days; for selection of ouabain-resistant colonies, the cultures were treated once with 1 mM ouabain for 14-16 days. At the end of treatment, the colonies were fixed and stained; the number

of cells was determined from 10-12 dishes per value for 8-azaguanine resistance and from 32-40 dishes per value for ouabain resistance. The heritability of this resistance was shown from experiments with nine isolated ouabain-resistant colonies (Huberman and Sachs, unpublished) and 17 isolated 8-azaguanine-resistant colonies (Huberman *et al.*, 1971, Huberman and Sachs, 1974), all of which remained resistant to the drug used for selection after growth for 1 month in the absence of 8-azaguanine. Mutation frequency was calculated for 10^5 or 10^6 survivors, taking into account the number of cells seeded and the cytotoxic effect of the mutagen.

RESULTS

The mutagenic activity of chloroethylene oxide and its rearrangement product 2-chloroacetaldehyde (Gross and Freiberg, 1969; Ziel and Schramm, 1964) was assayed in Chinese hamster V79 cells. Mutation frequency was scored with two different genetic markers either by resistance to 8-azaguanine (Chu and Malling, 1968) or by resistance to the steroidal compound ouabain (Baker *et al.*, 1974). The mutation frequency has been expressed as the number of 8-azaguanine- or ouabain-resistant mutants per 10^5 and 10^6 survivors, respectively.

The mutagenic response increased as a function of the concentration of chloroethylene oxide and 2-chloroacetaldehyde in the medium (Table 1). At a concentration of 6 μM, chloroethylene oxide or 2-chloroacetaldehyde mutation frequency of 8-azaguanine resistance reached 4 or 13 times that of the control. At the same concentration, the number of ouabain-resistant mutants per 10^6 survivors was 10 times higher than in the control with chloroethylene oxide but only twice as high as in the control with 2-chloroacetaldehyde. At a concentration of 13 μM chloroethylene oxide, the mutation frequency, determined with 8-azaguanine or ouabain resistance, reached 8 or 23 times, respectively, the spontaneous mutation frequency, while the cloning efficiency was 45%. 2-Chloroacetaldehyde, however, at 13 μM, had strong cytotoxicity, and cloning efficiency was less than 0.1%; thus, no mutagenic effect was detectable. Below a concentration of 2.5 mM, 2-chloroethanol or monochloroacetic acid had no activity in inducing 8-azaguanine- and ouabain-resistant mutants.

DISCUSSION

The data, with mammalian cells, confirm previous results concerning the mutagenicity of 2-chloroethylene oxide and 2-chloroacetal-

TABLE 1

MUTAGENIC EFFECTS OF CHLOROETHYLENE OXIDE AND 2-CHLOROACETALDEHYDE

Compound	Conc (μM)	Cloning efficiency of treated cells %	Number of 8-azaguanine-resistant mutants 10^5 survivors	Number of ouabain-resistant mutants 10^6 survivors
Control (DMSO)		93	10	3
Chloroethylene oxide	3	89	8	2
	6	77	37	28
	13	45	81	68
	19	21	369	29
	25	3.3	1066	ND [1]
2-Chloroacetaldehyde	1.6	91	9	2
	3.2	77	12	3
	6.4	28	131	7
	12.8	0.1	ND [1]	ND [1]
2-Chloroethanol	2,500	94	14	3
Monochloroacetic acid	2,100	94	9	4

[1] ND = not detectable

dehyde in *S. typhimurium* strain TA1530 (Bartsch *et al.*, 1975; Malaveille *et al.*, 1975). As is evident from Table I, chloroethylene oxide caused a mutagenic response with a much lower toxicity as compared to that of 2-chloroacetaldehyde at equimolar concentration; the same result was obtained with microbial indicators (Malaveille *et al.*, 1975). Thus, 2-chloroethylene oxide acts as a mutagen *per se* and not exclusively *via* its spontaneous rearrangement product 2-chloroacetaldehyde (Gross and Freiberg, 1969; Zieff and Schramm, 1964). The strong mutagenicity of these two compounds, as compared to that of 2-chloroethanol and monochloroacetic acid, points to a possible role for chloroethylene oxide and 2-chloroacetaldehyde in vinyl chloride carcinogenesis, since it is now recognized that the majority of chemical carcinogens, some of which cause cancer in man, have been found to be mutagens when tested as their ultimate reactive forms or when the parent compounds were combined with an *in vitro* or *in vivo* metabolic activation system (Miller and Miller, 1971; Huberman and Sachs, 1974).

The genetic activity of chloroethylene oxide can be ascribed to its strong alkylating potency which can be shown in assays with 4-(*p*-nitrobenzyl) pyridine (Bartsch and Montesano, 1975, Malaveille *et al.*, 1975). This property, as well as its short half-life in aqueous solution at neutrality ($t_{1/2} \simeq 1.6$ min), links this compound to the group of highly reactive α-chlorinated aliphatic ethers, among which *bis* (chloromethyl) ether ($t_{1/2}$ 2 min [Van Duuren *et al.*, 1972]) is carcinogenic in animals and in man (IARC, 1974*b*).

2-Chloroacetaldehyde is also a chemically reactive compound, although its rate of hydrolysis in aqueous solution differs from that of chloroethylene oxide by a factor of 1,000 ($t_{1/2}$ 24 h [Van Duuren *et al.*, 1972]), and under conditions whereby chloroethylene oxide reacts with 4-(*p*-nitrobenzyl) pyridine, 2-chloroacetaldehyde shows no detectable rate of reaction (Bartsch and Montesano, 1975; Malaveille *et al.*, 1975). At acidic pH, it reacts slowly with adenosine or cytidine to give cyclic fluorescent derivatives which have been characterized as 3 β-D-ribofuranosyl-imidazo-(2,1-i)purine or 5,5-dihydro-5-oxo-5-β-D-ribofuranosyl-imidazo (1,2-c) pyrimidine (Barrio *et al.*, 1972). Alterations of this kind in DNA activity may explain the effect of 2-chloroacetaldehyde as a mutagen in bacterial (Malaveille *et al.*, 1975) and mammalian cells (Table I). The role of 2-chloroacetaldehyde in carcinogenesis still needs to be studied.

Although we have obtained evidence that chloroethylene oxide is formed as a primary metabolite of vinyl chloride by microsomal enzymes *in vitro* (Bartsch and Montesano, 1975), the significance of this compound in vinyl chloride carcinogenesis can be indicated only by circumstantial evidence: chloroethylene oxide is a strong mutagenic and alkylating agent, and epoxides are now recognized as obligatory intermediates in the metabolism of many olefins by hepatic microsomal mixed-function oxidases (Daly *et al.*, 1972). Compounds structurally related to chloroethylene oxide, such as 1,2-epoxybutene-3 or epichlorhydrine, are known to be carcinogenic, while 2-chloroacetaldehyde (assayed as diethylacetal) and monochloroacetic acid have been reported to induce no tumours (Van Duuren *et al.*, 1972, 1974).

Alternative pathways of metabolic activation of vynil chloride to ultimate carcinogenic forms cannot be extended, *i.e.* an enzymic dechlorination (Gandolfi and Van Dyk, 1973) of vinyl chloride to produce ethylene which may be further oxidized to ethylene oxide, a mutagenic compound (Ehrenberg and Gustafsson, 1957). Studies on the nature of the binding products of vinyl chloride and/or its metabolites to cellular macromolecules are in progress.

NOTE ADDED IN PRESS

Since completion of this work, McCann *et al.* (1975) have reported a pronounced mutagenic effect of vinyl chloride and 2-chloroacetaldehyde in *S. typhimurium* TA100, while the concept of involvement of chloroethylene oxide in the covalent binding of ^{14}C-labelled vinyl chloride to cellular macromolecules mediated by a rat liver microsomal system has been strongly supported by the results of Kappus *et al.* (1975).

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MUTAGÉNICITÉ DE DEUX MÉTABOLITES DU CHLORURE DE VINYLE, LE CHLOROÉTHYLÈNE OXYDE ET LA 2-CHLOROACÉTALDÉHYDE, DANS DES CELLULES V79 DE HAMSTERS CHINOIS

Les cellules V79 de hamster chinois peuvent être mutées efficacement et devenir résistantes à la 8-azaguanine ou la ouabaïne, en fonction de la dose utilisée, par le chloroéthylène oxyde et la 2-chloroacétaldéhyde, deux métabolites du chlorure de vinyle chez les mammifères. Avec des concentrations cent fois plus fortes, le 2-chloroéthanol ou l'acide monochloroacétique, un métabolite urinaire du chlorure de vinyle chez le rat et l'homme, sont inactifs.

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