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MUTAGENICITY OF CHLOROOLEFINS IN THE *SALMONELLA*/MAMMALIAN MICROSOME TEST—II

STRUCTURAL REQUIREMENTS FOR THE METABOLIC ACTIVATION OF NON-ALLYLIC CHLOROPROPENES AND METHYLATED DERIVATIVES VIA EPOXIDE FORMATION

TILMAN NEUDECKER, WOLFGANG DEKANT, MANFRED JÖRNS, ERWIN EDER and DIETRICH HENSCHLER

Institute of Toxicology, University of Wuerzburg, Versbacher Strasse 9, D-87 Wuerzburg, Federal Republic of Germany

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Abstract—Non-allylic chloropropenes and their methyl-homologues, being chloro-substituted exclusively in vinylic position, are mutagenic in the presence of metabolizing rat liver homogenate fraction (S9 mix). This can be interpreted as the result of polarizing inductive (I−) and mesomeric (M−) effects exerted by Cl− as well as by CH_3-substituents on the olefinic double bond. The extent of their mutagenic activity increases with longer preincubation time and/or a higher concentration of rat liver homogenate fraction (S9) in the S9 mix. The only exception from this rule of a qualitative correlation of C=C-bond polarization due to asymmetric substitution and mutagenic activity is 1-chloro-2-methyl-1-propene which is non-mutagenic. In this case effects of a steric hindrance of two voluminous CH_3-substituents attached to one C-atom of the C=C-bond might inhibit enzymatic attack of the double bond by microsomal oxygenase. Mutagenic activity is invariably decreased in the presence of SKF525, inhibitor of microsomal oxygenase, and increased when 1,1,1-trichloropropene-2,3-oxide (TCPO), inhibitor of epoxide hydrolase, is added to the test system. This is a strong argument for metabolic activation of these substances occurring via epoxide formation.

Epoxidation of the C=C-bond has been postulated to be the chemical mechanism of metabolic activation in several haloolefins such as vinyl chloride [1] and higher chlorinated ethylenes [2]. Chloro- and methyl-substituents can be expected to induce a more or less pronounced polarization of the olefinic C=C-bond due to inductive (I−) as well as mesomeric (M−) effects, thus leading to increased chemical reactivity and mutagenic activity. In this paper the concept of substituent-induced mutagenicity, initially proposed for the chloroethylenes by Bonse *et al.* [2] is extended to non-allylic chloropropenes and methylated homologues characterized by vinylic chloro-substitution on one or both C-atoms of the C=C-bond. As will be shown the mutagenic activity of non-allylic chloroolefins invariably depends on metabolic activation via epoxidation, the extent of which can be interpreted as the consequence of structural parameters that not only induce C=C-polarization but also might be critical due to steric hindrance of epoxide forming microsomal oxygenase activity.

MATERIALS AND METHODS

Sources, purification methods and purity of the compounds tested in this survey are listed in Table 1. 1,1,1-Trichloropropene-2,3-oxide (TCPO) was obtained from EGA-Chemie (Steinheim, F.R.G.);

Table 1. Sources, purification methods and purity of the chloroolefins tested

Compound	Source	Purification method	Purity (%)
1-Chloro-1-propene	A	Distillation (b.p. 35°)	100
2-Chloro-1-propene	A	Distillation (b.p. 24°), Prep. GC	100
1,2-Dichloro-1-propene	B	Distillation (b.p. 77°)	>99
1,1-Dichloro-1-propene	C	Distillation (b.p. 78°), Prep. GC	99.5
2-Chloro-2-butene	D	Distillation (b.p. 71°), Prep. GC	68*
1-Chloro-2-methyl-1-propene	E	—	98.5
1-Chloro-1-butene	F	—	>99
2,3-Dichloro-2-butene	G	Prep. GC	>99
1,2-Dichloroethylene	H	—	98

(A) Riedel de Haen, Seelze, F.R.G.; (B) synthesized according to Griesbaum *et al.* [9]; (C) FLUKA, Buchs, Switzerland; (D) ICN-Pharmaceuticals, New York, U.S.A.; (E) SERVA, Heidelberg, F.R.G.; (F) Aldrich-Europe, Beerse, Belgium; (G) synthesized according to Scharf and Laux [10]; (H) EGA-Chemie, Steinheim, F.R.G.

* Contains 32% 2-chloro-1-butene according to NMR-analysis. Purification methods and determination of purity are described under Materials and Methods.

SKF525 (SKF) from Smith, Kline and French Laboratories (England); NAD, NADP and pyruvate from Boehringer, Mannheim, (F.R.G.); glucose-6-phosphate, L-histidine and D-(+)-biotine from SERVA (Heidelberg, F.R.G); Oxoid CM67 Nutrient Broth No. 2 (25 g/l) from Oxoid GmbH, (Wesel, F.R.G). All other chemicals (analytical grade) were purchased from Merck, Darmstadt (F.R.G.).

Distillation was performed in a NORMAG micro spinning band column at atmospheric pressure. A Packard Model 421 with a Cycloprep model 798 was used for preparative scale gas chromatography. It was fitted with a 1.8 m glass column, i.d. $\frac{1}{4}$ in., containing 30% carbowax 20M on 80/100 mesh acid-washed chromosorb W. The purity of the compounds was checked by gas liquid GC with a Beckman model GC M fitted with a double flame ionization detector and connected to a HP3385 A integrator system. Two different 2 m V4A-steel columns, i.d. $\frac{1}{8}$ in., packed with (a) 30% squalan on AW DCMS chromosorb W 80/100 and (b) 30% carbowax 20M on AW chromosorb W 80/100 mesh were used in order to exclude accidental coincidence in retention times of the substances and possible impurities.

The sensitivity of the *Salmonella typhimurium* tester strain TA100, kindly provided by Dr. B. Ames, Berkeley, U.S.A., was routinely checked with each experiment by using NaN_3 and 2-aminoanthracene as positive controls in the absence or in the presence of S9 mix, respectively. S9 mix with a protein content of either 4 or 12 mg/ml mix was prepared with S9 from Aroclor-1254 induced male Wister rats according to Ames *et al.* [3]. For mutagenicity testing a preincubation method similar to that described by Yahagi *et al.* [4] was applied. 0.1 ml of bacterial cell suspension, grown for 16 hr in Oxoid medium, and 10 μl dimethylsulfoxide (DMSO) containing test substance in the given amounts or undiluted test substance in case of volumes exceeding 10 μl (up to 50 μl) were added to 0.5 ml each of either phosphate buffer (0.1 M, pH 7.4), S9 (containing all components of S9 mix except NADP and glucose-6-phosphate), S9 mix (with or without TCPO or SKF added) or S9 with the cofactors NADP and glucose-6-phosphate substituted by equimolar concentrations of NAD and pyruvate (all other components of S9 mix unchanged) ("S9 + NAD"). The plastic vials (7 cm × 1 cm) were then closed with air-tight caps and put into a shaker water bath at 37°. After incubation times of either 20 or 120 min 2 ml each of molten (45°) top agar [3] were added to each vial (see legends) and the mixtures poured onto Petri plates containing Vogel-Bonner medium E [5] with 1.5% Bacto-Difco agar and 2% glucose (minimal agar). Revertant colonies were counted after two days of incubation at 37°. All determinations were made in duplicate and all experiments performed at least twice. Differences in colony counts of analogous plates never exceeded 20% within one experiment. Specific mutagenicity was determined as the number of revertant colonies per μmole of test compound from the linear part of the dose–response curves. As described and documented elsewhere [6] control experiments proved that the number of living bacteria did not significantly change during preincubation at 37° for up to 2 hr.

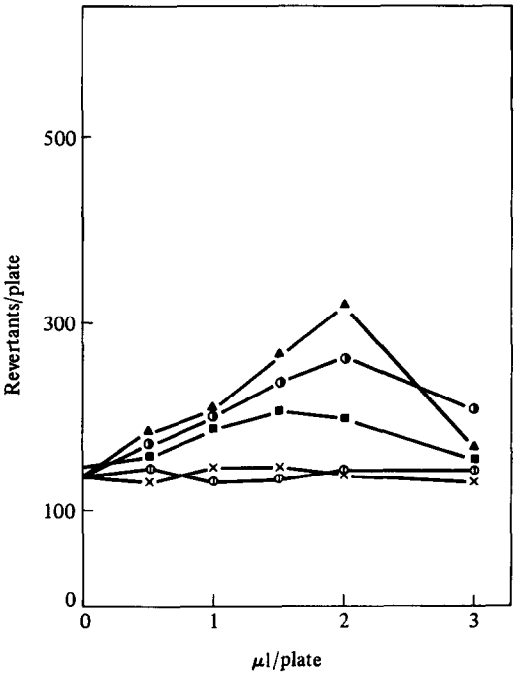


Fig. 1.

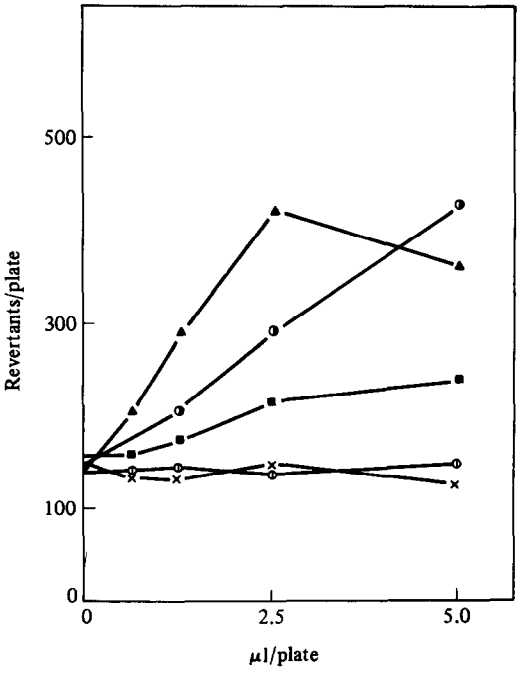


Fig. 2.

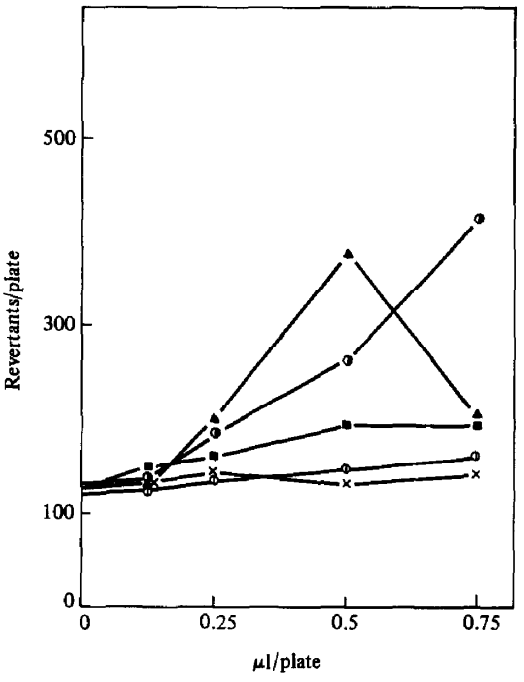


Fig. 3.

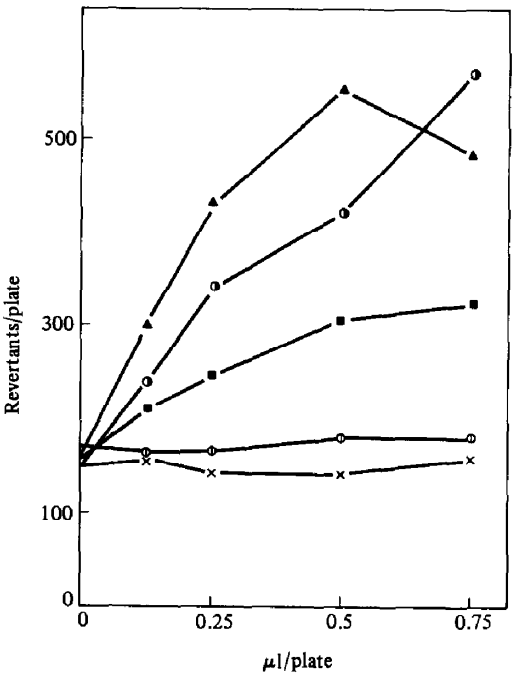


Fig. 5.

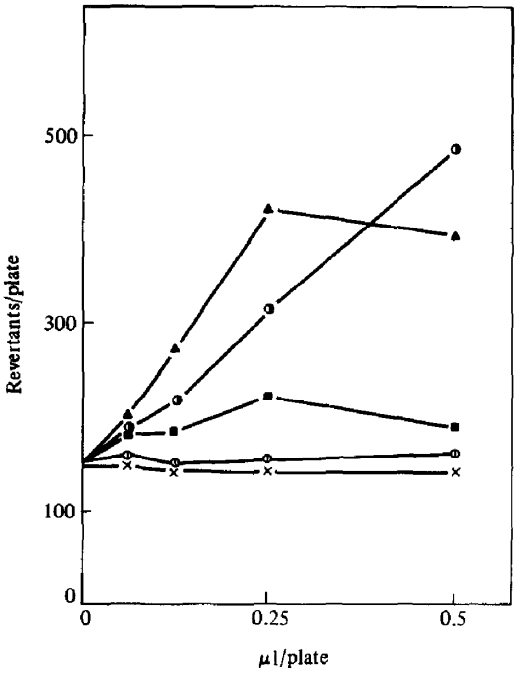


Fig. 4.

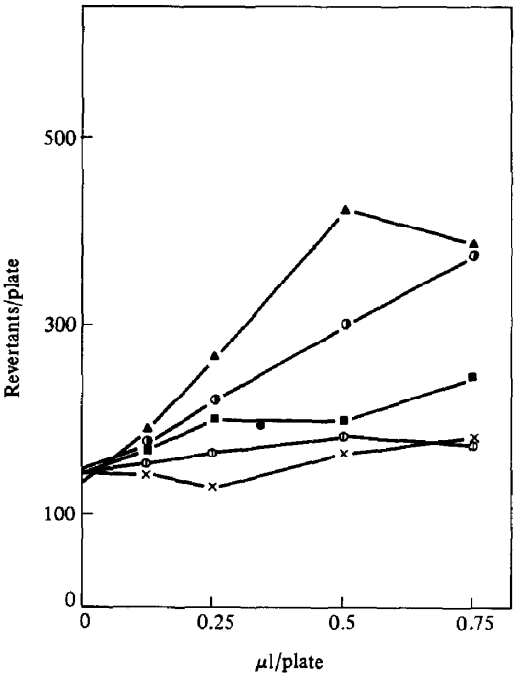
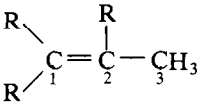


Fig. 6.

Figs. 1–6. Effect of rat liver homogenate fraction (S9) on the expression of mutagenic activity of (1) 1-chloro-1-propene, (2) 2-chloro-1-propene, (3) 1,2-dichloro-1-propene, (4) 1,1-dichloro-1-propene, (5) 2-chloro-2-butene and (6) 1-chloro-1-butene in the presence or absence of an NADH- or NADPH-regenerating system and the influence of the enzyme inhibitors SKF and TCPO. Testing procedure as described under Materials and Methods, preincubation time 120 min, concentration of rat liver protein always 4 mg/ml. SKF or TCPO were added to concentrations of 20 µg and 0.02 µl per ml S9 mix, respectively. Mutagenicity testing was performed in the presence of: S9 mix (◑), S9 mix + TCPO (▲), S9 mix + SKF (■), S9 plus a NADH-regenerating system ("S9 + NAD") (Ⓞ) and S9 alone (×).

RESULTS

The chloropropenes tested in this survey are characterized by vinylic, i.e. non-allylic chloro-substitution(s):



As can be seen from Table 2 all are mutagenic and their mutagenicity is invariably an indirect one, requiring S9 mix for metabolic activation. The efficiency of metabolic activation obviously depends on both incubation time and concentration of rat liver protein (S9, i.e. 9000 G supernatant of liver homogenate) in the S9 mix. Increased incubation time as well as higher S9 concentration lead to an increase in mutagenicity. In no case is there a direct (– S9 mix) mutagenicity to be observed (Table 2)

Modifications of the metabolizing rat liver homogenate system and addition of inhibitors of enzymes supposed to be involved in the metabolism of these compounds can considerably influence the efficiency of metabolic activation. Such influences can be found with all activable chloropropenes listed in Table 2 (Figs. 1–6). Presence of S9 mix, i.e. rat liver homogenate fraction (S9) plus a NADPH-regenerating system, obviously is a *conditio sine qua non* for metabolic activation.

Replacement of the NADPH-regenerating system for a NADH-regenerating one by substituting the cofactors of S9 mix, NADP and glucose-6-phosphate, by NAD and pyruvate ("S9 + NAD") leads to ineffectivity of the rat liver homogenate metabolizing system. The same is true for the presence of S9 alone, without the cofactors of a NADPH- or NADH-regenerating system added.

Metabolic activation of all chloropropenes listed in Table 1 is clearly influenced by both SKF525 (SKF), inhibitor of microsomal oxygenase(s) [7], and by 1,1,1-trichloropropene-2,3-oxide (TCPO), inhibitor of epoxide hydrolase [8]. Whereas SKF inhibits metabolic activation by S9 mix, TCPO increases its efficiency in the lower dose range (Figs. 1–6).

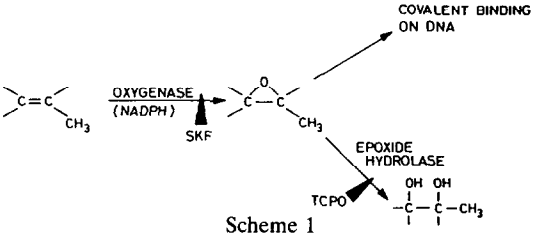
Methyl substitution in either vinylic or vicinal position (i.e. methylation of the C-atoms of the double bond or of those next to it) strongly affects the mutagenic potency as can be seen from the case of 1-chloro-1-propene. Its 1- and 3-methyl-derivatives, 2-chloro-2-butene and 1-chloro-1-butene, are stronger mutagens; the 2-methyl-derivative, however, 1-chloro-2-methyl-1-propene, is non-mutagenic (Table 2). Methyl groups also considerably influence the mutagenic behaviour of 1,2-dichloroethylene, a substance which is non-mutagenic both in the absence and in the presence of a metabolizing system [2]. Its mono-methyl-derivative, 1,2-dichloro-1-propene, is clearly mutagenic in the presence of S9 mix whereas the bi-methyl-derivative, 2,3-dichloro-2-butene, is non-mutagenic under the same testing conditions (Table 2).

DISCUSSION

All the non-allylic chloropropenes tested in this survey are non-mutagenic in S. t. TA100 in the absence of a metabolizing rat liver homogenate (S9 mix). However, they all can be activated by S9 mix, obviously due to metabolic reactions giving rise to mutagenic intermediates. According to the concept of Bonse *et al.* [2] which correlates the (indirect) mutagenicity of chloroethylenes with asymmetric chloro-substitution—inducing asymmetric charge distribution with the C = C-bond—a corresponding model can be derived for chloropropenes.

Considering substituent-induced polarization of the C = C-bond as a consequence of vinylic substitution mutagenic activity should be expected for the asymmetrically chloro-substituted compounds 1-chloro-1-propene, 2-chloro-1-propene and 1,1-dichloro-1-propene; not necessarily, however, for 1,2-dichloro-1-propene. The latter substance is symmetrically substituted with regard to —Cl. Yet a certain polarization of the C = C-bond can be expected from the influence of the methyl-group in C2-position. The importance of such a polarisation of the C = C-bond is stressed by the fact that the methyl-homologue of 1,2-dichloro-1-propene, the symmetrically structured 2,3-dichloro-2-butene, is non-mutagenic (see Table 2).

Activation of non-allylic chloropropenes by S9 mix can be expected to be provided by epoxidizing microsomal oxygenase attacking the polarized C = C-bond according to Scheme 1:



Scheme 1

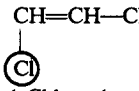
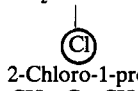
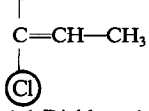
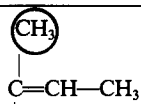
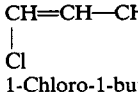
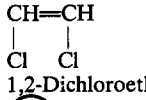
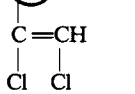
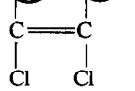
This hypothesis is supported by the following experimental results (see Figs. 1–6):

Metabolic activation is suppressed by SKF, inhibitor of microsomal oxygenase.
Activation is possible only in the presence of NADPH, coenzyme of microsomal oxygenase. It is impossible if this coenzyme is not added to the test system or if it is substituted by NADH.
Addition of TCPO, inhibitor of epoxide hydrolase, distinctly increases the indirect mutagenic activity.

According to Scheme 1 the formation of highly reactive epoxides as mutagenic intermediates of metabolic activation could be blocked by SKF due to inhibition of microsomal oxygenase. Conversely, inhibition of epoxide hydrolase by TCPO should increase the steady-state concentrations of such reactive epoxides, thus leading to a corresponding increase in mutagenic activity.

From a quantitative point of view the mutagenicity of 1-chloro-1-propene and 2-chloro-1-propene is relatively weak as both an increased concentration of rat liver homogenate fraction (S9) in the S9 mix and a prolonged incubation time of 120 min are necessary for clearly positive results (see Table 2). In contrast, the mutagenic potency of 1,1-dichloro-1-propene and 1,2-dichloro-1-propene is considerably stronger. It is conceivable that at least in the case of

Table 2. Mutagenic activity of chloroolefins substituted in vinylic position, with and without S9 mix, in dependence from its S9 contents and from preincubation time. Testing procedure as described under Materials and Methods

No.	Compound	Mutagenicity (revert./μmole) 20′ Incubation −S9mix	20′ Incubation +S9mix*	20′ Incubation +S9mix†	120′ Incubation −S9mix	120′ Incubation +S9mix*	120′ Incubation +S9mix†
1	 1-Chloro-1-propene(*cis/trans*)	0	0	1	0	3	4
2	 2-Chloro-1-propene	0	0	1	0	5	11
3	 1,2-Dichloro-1-propene(*cis/trans*)	0	10	35	0	45	80
4	 1,1-Dichloro-1-propene	0	60	60	0	90	100
5	 2-Chloro-2-butene(*cis/trans*)	0	20	38	0	65	90
6	 1-Chloro-2-methyl-1-propene	0	0	0	0	0	0
7	 1-Chloro-1-butene(*cis/trans*)	0	12	25	0	30	70
8	 1,2-Dichloroethylene(*cis/trans*)	0	0	0	0	0	0
3	 1,2-Dichloro-1-propene(*cis/trans*)			see above			
9	 2,3-Dichloro-2-butene(*cis/trans*)	0	0	0	0	0	0

* 4 mg protein/ml.
† 12 mg protein/ml.

the former compound an increased polarization of the C=C-bond due to the additive effect of two chloro-substituents on one of the C-atoms favours the attack of microsomal oxygenase, i.e. the formation of the epoxide.

Introduction of additional methyl-groups into 1-chloro-1-propene considerably affects its mutagenic potency (see Table 2). There is a loss of mutagenic activity after introduction of a methyl group in C2-position (1-chloro-2-methyl-1-propene). The fact

that additional methyl groups in C1- and C3-position (2-chloro-2-butene and 1-chloro-1-butene) do not lower (but even increase) mutagenic potency whereas methyl substitution in C2-position is followed by a loss in mutagenicity cannot be explained by the +I− and +M− effects of methyl-substituents on the polarization of the C═C-bond. These effects should even lead to a decrease in polarization, most obviously in the case of 2-chloro-2-butene. Here the −I− effect of the chloro-substituent is counteracted by both the +I− and the +M− effect of the methyl group attached to the same C-atom:

$$CH_3 \rightarrow \underset{\displaystyle \underset{\displaystyle Cl}{\downarrow}}{C} = C \leftarrow CH_3$$

It is conceivable that steric parameters, too, influence the effectivity of haloolefins as substrates of microsomal oxygenase. Both characteristics, substituent-caused steric hindrance as well as substituent-induced polarization, may eventually determine the rate and extent of metabolic activation via epoxide formation.

In the case of the non-mutagenic compound 1-chloro-2-methyl-1-propene the steric effect of two voluminous methyl groups bound to one of the double bond C-atoms might render enzymatic epoxidation impossible. On the other hand, the importance of a substituent-induced polarization of the C═C-bond is stressed by the results shown in Table 2. Although all these dichloro-olefins are symmetrically chloro-substituted in vinylic position—and do not exert direct mutagenicity—only in the case of 1,2-dichloro-1-propene which is "asymmetric" *per se* due to its molecular composition of three C-atoms a metabolic activation can be observed in the presence of S9 mix. 1,2-Dichloroethylene and 2,3-dichloro-2-butene, both substances showing a symmetrical structure and consequently no polarization of the C═C-bond, are non-mutagenic.

Non-mutagenicity, however, cannot reliably be taken as a proof for chemical non-reactivity or metabolic inactivity. Moreover, due to different mutagenic activities of different metabolites, there is not necessarily a quantitative correlation between the extent of mutagenicity and the rate of metabolic transformation in different bioactivable compounds to be compared. Such principal interpretative problems of establishing and quantifying a structure–mutagenicity relationship have to be taken into account for all indirect mutagens.

Summarizing the results of this survey it has to be stated:

None of the chloroolefins being substituted in vinylic position is directly mutagenic. All require metabolic activation.

Polarization of the C═C-bond due to asymmetric chlorosubstitution is a precondition of metabolic activation via epoxide formation.

1-Chloro-2-methyl-1-propene is the only exception from this rule. It is non-mutagenic; conceivably due to an effect of steric hindrance of enzymatic epoxidation exerted by two methyl groups attached to one of the C-atoms of the olefinic double bond.

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