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MUTAGENIC POTENTIAL OF ALLYL AND ALLYLIC COMPOUNDS

STRUCTURE–ACTIVITY RELATIONSHIP AS DETERMINED BY ALKYLATING AND DIRECT *IN VITRO* MUTAGENIC PROPERTIES

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Abstract—A series of compounds, each containing an allylic moiety, has been tested using *Salmonella typhimurium* TA 100 in a modified Ames mutagenicity assay system. In the absence of activating enzymes (S-9) mix, those allylic compounds possessing chemically good leaving groups show direct mutagenic activity. Their activity decreases in the following order: allyl methanesulfonate > -iodide > -bromide > -chloride. This is in good agreement with the alkylating properties measured in the nitrobenzyl-pyridine (NBP) test. For all allyl and allylic compounds found to be directly mutagenic, a decrease in, and sometimes total loss, of mutagenicity is registered after addition of S-9 supernatants. Compared to the direct mutagenic activity of allyl chloride, epichlorohydrin shows a much higher mutagenicity, whereas propyl chloride has proven to be nonmutagenic. The direct mutagenic effect of this type of compound is theoretically explained by S_N-1, S_N-2 and S_N-2′ mechanisms.

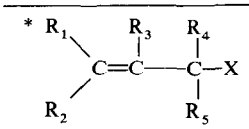
During the last few years, haloolefins have gained increasing attention as possible mutagens and carcinogens. Some of these substances have been definitively identified as carcinogens, e.g. vinyl chloride in humans [1], as well as in animal experimentation [2]. Others, like trichloroethylene, perchloroethylene, vinylidene chloride and chloroprene, to name a few, have been discussed as potentially carcinogenic on the basis of experimental or epidemiological evidence [3].

Up to now, a metabolic epoxidation of the double bond in these olefinic structures has been regarded as the common principle leading to their mutagenic and carcinogenic activity [4]. Starting with this mechanism, which is well-established in haloethylenes, the hypothesis has been brought forward that olefinic structures with more than 2 carbon atoms, such as propene and butene derivatives, may likewise be activated via epoxides [5].

In this paper, however, we describe a different mechanism with a certain group of haloolefins, the allyl and allylic* compounds: they are able to act directly as alkylating and mutagenic agents due to their high S_N-1-, S_N-2- and S_N-2′-reactivity. These compounds have found extensive use and distribution in our environment, both as synthetic and naturally occurring products e.g. as plastic monomers, pesticides, flavourings, perfumes and pharmaceuticals.

As structural parameters are very important for the degree of electrophilicity in allylic molecules, we tried to establish a close relationship between structure and reactivity for a theoretical interpretation of our experimental findings.

For this purpose we will discuss the influence of different leaving groups in allylic position to the double bond as a most important criterion for alkylating and mutagenic activities in allylic compounds.



MATERIALS AND METHODS

Chemicals

Allyl bromide, allyl chloride, allyl cyanide, allyl iodide and butyl chloride were purchased from Merck, Darmstadt, FRG.

Allyl bromide was purified by washing with an ice cold solution of $NaHCO_3$, then with water. After drying over $CaCl_2$, final purification was obtained by adsorptive filtration over silica gel/alumina at 4° (purity 100%).

Allyl iodide was washed with ice cold solutions of $NaHCO_3$ and $Na_2S_2O_3$ (to remove liberated iodine), and finally with water. After drying with $CaCl_2$, allyl iodide was passed at 4° through a column filled with silica gel/alumina. All steps were carried out in the dark in order to minimize iodine liberation. Allyl bromide and allyl iodide were purified immediately before use.

Allyl chloride was purified by redistillation (b.p. 45°) and preparative scale gas chromatography (purity 100%).

Allyl cyanide (I) was washed with water, dried over $CaCl_2$, and redistilled *in vacuo* (b.p. 20°/12 Torr, purity 100%).

Butyl chloride was purified by preparative scale gas chromatography (b.p. 78°, purity 100%).

Allyl phenyl ether (II) purchased from EGA-Chemie, Steinheim, FRG, was redistilled before use (b.p. 85°/15 Torr, purity > 99.5%).

After purification, all substances were characterized by their p.m.r. spectra to detect any possible changes which may have occurred during purification.

Allyl methanesulfonate was prepared by a method described by Chautemps *et al.* [6], purified by distillation *in vacuo* (b.p. 76°/3 Torr), and characterized by its p.m.r. spectrum (purity > 97.5%).

Allyl sodium sulfate (III) was prepared by a method described by Kaye [7] and characterized by its p.m.r. spectrum.

Allyl propyl disulfide (IV) was prepared by the method of Corson *et al.* [8] as a disulfide mixture consisting of 31 per cent propyl disulfide, 37 per cent allyl propyl disulfide and 32 per cent allyl disulfide (V). This mixture was used in our tests.

The following compounds were used without further purification: allylamine (VI) (b.p. 53°, purity 99%), allylbenzene (VII) (b.p. 156°, purity 97%), allyl isothiocyanate (VIII) (b.p. 150°, purity 99.8%), allyl sulfide (IX) (b.p. 139°, purity 97%), epichlorohydrin (b.p. 117°, purity 99.5%), eugenol (X) (b.p. 255°, pharmacop. grade), and isosafrole (XI) (purity 98%) were purchased from Merck, Darmstadt, FRG; allylmalonic acid diethyl ester (XII) (b.p. 222°), allyl mercaptan (XIII) (b.p. 67°, purity 70%, remainder allyl disulfide), trans-cinnamyl alcohol (XIV) (b.p. 250°, purity > 99%), 2-cyclohexen-1-ol (XV) (b.p. 164°, purity 96%), geraniol (XVI) (b.p. 229°, purity > 99%), linalool (XVII) (b.p. 194°, purity 99%) and safrole (XVIII) (b.p. 232°, purity 98%) were purchased from EGA-Chemie, Steinheim, FRG; allobarbitone (XIX) (pharmacop. grade) was obtained from Serva, Heidelberg, FRG, and propyl chloride (b.p. 46°, purity > 98%) was obtained from Fluka, Buchs, Switzerland. The chemical structures of these compounds are shown in Table 1 and Fig. 1.

Distillation was performed by a NORMAG micro spinning band column at atmospheric pressure or *in vacuo*. For preparative scale gas chromatography, a Packard model 421 with a Cycloprep model 798 was used, fitted with a 1.8 m glass column, i.d. 1/4 in., containing 30% carbowax 20 M on 80/100 mesh acid-washed chromosorb W.

The purity of liquid compounds was determined by gas–liquid chromatography (g.l.c.) with a Beckman model GC M fitted with a double flame ionization detector and connected to a HP 3385 A integrator system. Two different 2m V4A-steel columns, i.d. 1/8 in., packed with (A) 30% squalan on AW DMCS chromosorb W 80/100 mesh and (b) 30% carbowax 20M on AW chromosorb W 80/100 mesh were used to exclude accidental coincidence in retention times of parent substances and possible impurities.

The purity of solid compounds was ascertained by thin-layer chromatography (t.l.c.)

Determination of alkylating activities

Determination of alkylating activities was performed with 4-(*p*-nitrobenzyl)-pyridine (NBP-Test) [9, 10]: One millilitre of a 30 mM solution of test substance in ethyl methyl ketone or ethylene glycol and 1 ml of NBP (200 mM, in the same solvent) were refluxed in a boiling water bath for 10 min, then cooled in ice water and 1 ml of a 1 : 1 mixture of triethylamine and ethyl methyl ketone or ethylene glycol was added. Extinctions of the reaction mixtures were plotted against an appropriate blank at 560 nm with a Zeiss spectrophotometer PQM 3. Extinctions higher than 0.04 were regarded as indicative of alkylating activity. When extinctions were higher than 3.0 (the measuring limit of the photometer), 1 : 50 or 1 : 100 dilutions were made with the appropriate solvent and the extinctions remeasured. Under identical test conditions there is a quantitative correlation between extinction values and alkylating potencies.

Table 1. Mutagenicity and alkylating activity of allyl compounds

Compounds	Mutagenicity revertants/μmole +S-9 mix‖	−S-9 mix	Alkylating activity $\Delta E_{560\ nm}$
$CH_2{=}CH{-}CH_2{-}OSO_2CH_3$ Allyl methanesulfonate	0	2600	1.174*
$CH_2{=}CH{-}CH_2{-}J$ Allyl iodide	0	1000	1.828*
$CH_2{=}CH{-}CH_2{-}Br$ Allyl bromide	0	700	1.208†
$CH_2{=}CH{-}CH_2{-}Cl$ Allyl chloride	1	9	0.285
$CH_2{=}CH{-}CH_2{-}NCS$ Allyl isothiocyanate	0	< 1	0(0.182)‡
Epichlorohydrin	70	275	1.237
$CH_3CH_2CH_2{-}Cl$§ Propyl chloride	0	0	0.003

* Δ E after dilution of the reaction mixture 1 : 100.
† Δ E after dilution of the reaction mixture 1 : 50.
‡ Second value obtained with ethylene glycol as solvent.
§ Similar results were obtained with butyl chloride.
‖ From Aroclor 1254 pretreated rats.

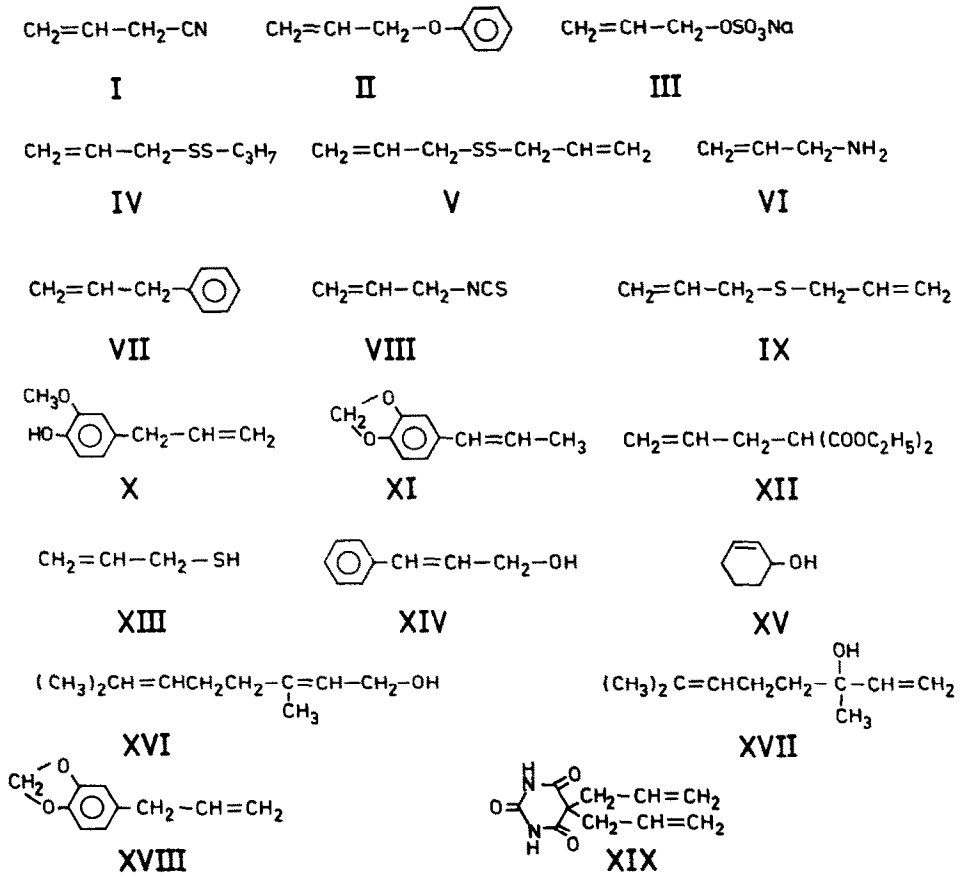


Fig. 1. Chemical structures of tested compounds.

Mutagenicity testing

Bacterial strain. Of the five strains tested (TA 1535, TA 1537, TA 1538, TA 98, TA 100), TA 100 of *Salmonella typhimurium,* kindly provided by Dr. B. N. Ames, was shown in a screening test to be the most sensitive for detecting mutagenic activity of allyl chloride under our test conditions. It was used in all of our investigations dealing with allyl and allylic compounds.

Preparation of liver homogenate fraction (S-9). The S-9 mix, prepared according to Ames *et al.* [11], contained per ml: 8 mM $MgCl_2$, 33 mM KCl, 5 mM glucose-6-phosphate, 4 mM NADP, 100 mM sodium phosphate (pH 7.4) and 0.1 ml of liver homogenate (S-9) (9000 *g* supernatant) (protein contents 40 mg/ml) from male Wistar rats weighing approximately 250 g each. The rats were treated for enzyme induction by a single i.p. injection of Aroclor 1254 diluted with corn oil to a concentration of 200 mg/ml. A dose of 500 mg/kg was given to each rat five days before killing. In one case (see Fig. 2) we also used S-9 supernatants from phenobarbitone induced Wistar rats (0.1% phenobarbitone in the drinking water for one week) and from uninduced NMRI mice (20–25 g each) for comparative studies.

Test procedure. Because of the rather high volatility of many allyl and allylic compounds, a modified liquid suspension test system as described by Rannug *et al.* [12] was used instead of the plate assay. The bacteria were grown overnight in nutrient broth (DIFCO), washed and resuspended in 0.1 M phosphate buffer (pH 7.4) to half of the original density. The cell suspension (containing approximately 1×10^9 cells/ml) was then divided into aliquots of 1.5 ml in centrifuge tubes, and another 0.5 ml of either 0.1 M phosphate buffer (pH 7.4) or 'S-9 mix' and the test compound [diluted in 10 µl dimethylsulfoxide (DMSO)]* added. Then the tubes were tightly closed with screw caps and incubated for 90 min at 37° in a shaker water bath.

The treatment was terminated by centrifugation and resuspending in 1.1 ml fresh buffer. Volumes of 0.5 ml of the cell suspensions were then added to molten top agar (2 ml each) and duplicate petri plates containing Vogel–Bonner medium E (= minimal agar) [13] overlaid. For the determination of survival rates, an aliquot of the cell suspension was diluted by a factor of 10^4 in 0.9% NaCl, and 10 µl of this dilution added to 2 ml top agar containing a 100-fold

* Because of their instability, allyl bromide and allyl iodide were diluted in ice cold acetonitrile instead of DMSO, which rapidly reacts with these compounds and turns solid at lower temperatures. The mutagenicity test system itself is not affected by this change of solvents.

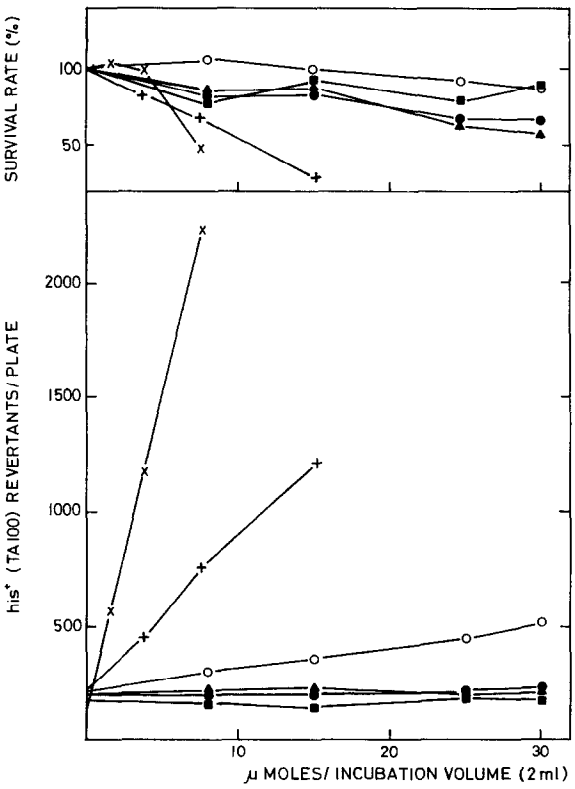


Fig. 2. Mutagenicity of epichlorohydrin with (+) and without S-9 mix (×), allyl chloride without S-9 mix (○), allyl chloride with S-9 mix from Aroclor induced rats (●), allyl chloride with S-9 mix from phenobarbitone induced rats (▲), allyl chloride with S-9 mix from uninduced mice (■).

concentration (5 mM) of histidine, compared to top agar for counting revertant colonies and poured on minimal agar medium plates. After 48 hr of incubation at 37°, the colonies of revertants and survivors were counted.

From the resulting linear dose–response curves, the mutation frequencies were determined as the number of revertants per μmole of test compound. All values represent an average of at least two independent experiments.

With each experiment we run sterility control plates for the solvent and for the S-9 mix. The sensitivity of the TA 100 tester strain was routinely checked by using sodium azide as a positive control in the absence of S-9 mix (6.5 μg NaN_3 per 2 ml incubation volume led to a three- to four-fold increase of the number of revertants as compared with the spontaneous back mutation rate) and by using 2-aminoanthracene in its presence (25 μg per 2 ml incubation volume increased the number of revertants by a factor of 15–20). The spontaneous back mutation rate for TA 100 was between 165 and 240 revertants per plate.

* Solid substance, dose given in mg. With III the highest dose was obtained by adding 50 μl of a nearly saturated DMSO-solution; with XIX a standard solution of 1 mg/ml DMSO was used, adding up to 100 μl to the incubation mix. Both substances did not show any toxic effect. Survival rates were higher than 90 per cent even at the highest concentrations.

RESULTS AND DISCUSSION

Four out of the 22 allyl (CH_2=CH−CH_2−X) and allylic compounds, tested for the influence of the leaving group X on mutagenicity and alkylating activity, clearly proved to be mutagenic even without addition of S-9 mix to the mutagenicity test system: allyl methanesulfonate, allyl iodide, allyl bromide and allyl chloride. These compounds were also clearly positive in the NBP test. Therefore, they can be regarded as directly acting mutagens similar to other mutagenic and carcinogenic alkylating compounds, e.g. mustard gas-like substances, epoxides and dialkylsulfates.

The degree of their alkylating and mutagenic activity, however, depends to a great extent on the chemical nature of the leaving group in this special kind of haloolefins. Good leaving groups like methanesulfonate and iodide, as expected, have the highest alkylating and mutagenic potency, the mutagenicity of allyl iodide probably being even greater than the value given because of a permanent splitting-off of iodine under test conditions, which is highly toxic to the bacteria. Between allyl bromide and allyl chloride there is a large drop in alkylating as well as in mutagenic activity, and allyl isothiocyanate seems to exhibit only borderline effectiveness in both qualities. It is a poor alkylating substance in the NBP test, being slightly positive only if ethylene glycol is used instead of the standard solvent ethyl methyl ketone. In the mutation test system the number of revertants induced by this compound hardly exceeds the spontaneous back mutation rate (tested both with and without S-9 mix in a dose range between 0.001 and 0.05 μl per 2 ml incubation volume, the survival rate at the highest concentration being less than 10 per cent).

All other allyl compounds with chemically poor leaving groups, (e.g. −SH, −SR, $-NH_2$, −CN), listed in Fig. 1, have been clearly negative both in the NBP test and in the mutagenicity test system, regardless of the presence of S-9 mix.

They were tested both with and without S-9 mix using at least five different concentrations, the highest ones causing a survival rate of less than 10 per cent in our test system. The doses given are μl per 2 ml incubation volume. I: 0.1 – 50; II: 0.01 – 0.3; III: 4.2 – 83*; IV, V: 0.003 – 0.3; VI: 0.05 – 5; VII: 0.025 – 0.5; VIII: 0.0003 – 0.1; IX: 0.01 – 1; X: 0.01 – 3; XI: 0.01 – 1; XII: 0.01 – 30; XIII: 0.01 – 3; XIV: 0.01 – 10; XV: 0.01 – 10; XVI: 0.01 – 1; XVII 0.01 – 3; XVIII: 0.003 – 0.3; XIX: 0.1 – 100*; propyl chloride: 0.02 – 10; butyl chloride: 0.1 – 10.

In no case and at no concentration tested was an increase in the number of revertants found which exceeded the spontaneous back mutation rate by a factor of two. Similar results were obtained in control experiments with TA 1535 and TA 98.

Considering the consequences of the addition of S-9 mix to the mutagenicity assay for allyl and allylic compounds, the following general rules have been found to be valid under our test conditions:

(1) allyl and allylic compounds which are not mutagenic *per se* do not gain mutagenicity by the addition of rat S-9 mix;

(2) the presence of rat S-9 mix does not lead to any significant increase in mutagenicity of those com-

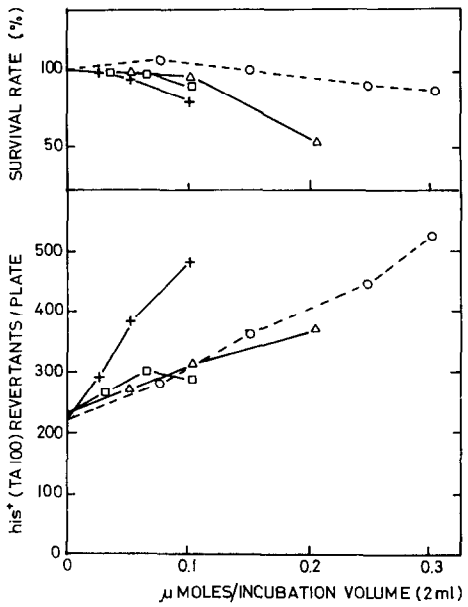


Fig. 3. Mutagenicity of allyl methanesulfonate (+), allyl iodide (□), allyl bromide (△) and allyl chloride (○) without S-9 mix. The amount of allyl chloride was 100 times that indicated on the scale.

pounds which are directly mutagenic. In most cases addition of S-9 mix is followed by a marked, sometimes total, decrease in mutagenicity.

For some allylic compounds like 1,3-dichloropropene, allyl chloride, 1-chloro-2-butene, 1-chloro-2-methyl-2-propene and allyl alcohol, the validity of the latter rule has already been confirmed and published by our group [14–16]. Similar observations have been made by other authors [17, 18]. This phenomenon could be explained by the high reactivity of alkylating allylic substances. Most probably such compounds can also easily react with S-9 proteins or conjugate with glutathione, loosing their mutagenic activity by this and similar processes.

An exception from the second rule stated above has recently been reported by Bartsch *et al.* [2] who found an increase in mutagenicity of an allylic compound e.g. 3,4-dichloro-1-butene in the presence of phenobarbitone-treated mouse liver S-9. Whether this discrepancy is due to the use of mouse S-9 instead of rat S-9 or the result of other factors, e.g. the double chlorine substitution in this compound, has still to be evaluated. This problem will be dealt with in more detail in a forthcoming paper [19].

We could not find a significant difference in the effectiveness of S-9 supernatants of different origin. In the case of allyl chloride, addition of three kinds of S-9 mix — from Aroclor induced rats, phenobarbitone induced rats and uninduced mice — to the test system is always followed by practically a total loss in mutagenicity (Fig. 2). The same is true for allyl bromide, allyl iodide and allyl methanesulfonate (see Table 1).

These findings do not support the hypothesis of an enzymatic epoxidation as a prerequisite for mutagenicity in these compounds nor as a relevant contribution to the effect. Another argument against this hypothesis results from a comparison of the mutagenicity of allyl chloride with its epoxide, epichlorohydrin. According to the epoxidation theory, addition of S-9 mix to the weakly mutagenic allyl chloride should lead to the formation of the strong mutagen epichlorohydrin, and hence to an increase in mutagenicity.

However, the opposite is found to be true in our experiments, although they cannot definitely exclude the possibility that in the presence of S-9 mix there is to a certain extent a formation of epichlorohydrin from allyl chloride. Epichlorohydrin may be a substrate for hydrase activity, thus loosing its mutagenic potential (Fig. 2). It also remains open as to what extent such a hypothetical epoxide formation depends on the kind and concentration of the S-9 mix used, or what occurs under *in vivo* conditions, respectively. Such considerations, however, do not invalidate this discussion of a direct mutagenic activity of certain allylic compounds.

These observations contrast remarkably to results with other, non-allylic haloolefins, which exhibit mutagenicity only after metabolic activation or are at least increased in their mutagenic activity by enzymatic action [2, 20]. An allylic moiety combined with an appropriate leaving group, however, is an absolute condition for mutagenicity and alkylating activity in these substances. Haloaliphatics like propyl chloride or butyl chloride, as expected, do not show any such activity in our tests.

The exceptional status of allylic compounds among halogenated hydrocarbons can be explained by their high electrophilicity. There is a very good correlation between theoretically expected electrophilicity, experimentally measured alkylating activity and direct mutagenic potency. The high electrophilicity, due to both S_N-1- and S_N-2′-reactivity, can be explained on molecular theoretical grounds as follows:

$$CH_2{=}CH{-}CH_2X \rightarrow$$

$$\rightarrow [CH_2{=}CH{-}\overset{\oplus}{C}H_2 \leftrightarrow \overset{\oplus}{C}H_2{-}CH{=}CH_2]X^{\ominus}.$$

S_N-1-reactivity

The allyl cation generated by the loss of the leaving group X^- can be stabilized by resonance in the ¶-electron system [21].

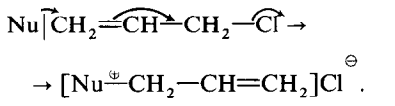
The nonallylic compound vinyl chloride, conversely, cannot react by such a mechanism, because resonance produces a stabilization of the C—Cl bond:

$$CH_2{=}CH{-}\overline{Cl}| \leftrightarrow \overset{\ominus}{\overline{C}}H_2{-}CH{=}\overset{\oplus}{Cl}.$$

So vinyl chloride is not mutagenic *per se*, but only after enzymatic activation via epoxide formation [2].

Due to the relatively low energy requirement for the allyl cation formation, the S_N-1-reactivity of allyl chloride is supposed to be considerably higher than in the case of vinyl chloride or propyl chloride, needing much more energy for cation formation.

Another type of reaction with nucleophiles (Nu), the S_N-2′-mechanism [22, 23], leads to a considerable increase in bimolecular reactivity:



This mechanism, however, is possible only in allyl- and allylic compounds.

CONCLUSIONS

The results presented clearly demonstrate a different mechanism of action, other than epoxidation, in the mutagenic and carcinogenic activity of haloolefins: in the special case of allyl and allylic structures a direct alkylating mechanism has been detected and can be theoretically explained by S_N-1 and S_N-2′ reactivities. From *in vitro* experiments, no indication can be derived for the involvement of metabolic activation through epoxidation. Whether this exclusiveness of direct action is also true for *in vivo* conditions remains to be shown by experiments with intact animals.

At present, the structure–activity relationship outlined above allows for the prediction of the mutagenic potency of the allylic compounds by determining the suitability of different substituents as leaving groups. The influence of further substituents on the alkylating and mutagenic properties of allylic molecules will be dealt with in a forthcoming paper.

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