The Mutagenicity of Vinyl Chloride After Metabolic Activation

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Vinyl chloride has recently been shown to cause a malignant liver tumor disease in man after occupational exposure in PVC plants. This actualizes the problem of whether such hazards could be avoided or at least diminished in the future by a screening for mutagenicity of chemicals used in industries. The basis for such a screening procedure is the close correlation between carcinogenic and mutagenic effects of chemicals. Experiments with *Salmonella* bacteria showed that the carcinogenic hazard of vinyl chloride could have been traced by means of mutagenicity tests. The data indicate that vinyl chloride is not mutagenic *per se* but becomes mutagenic after a metabolic activation in the liver.

Vinyl chloride has attracted a considerable amount of public attention because of the recent discovery of its carcinogenic action in man. In several countries cases of the rare malignant disease angiosarcoma of the liver have been reported among workers who have been exposed to vinyl chloride in plastic industries (1). Maltoni has furthermore reported an increase of angiosarcoma in rats after exposure to 250 ppm vinyl chloride (2). Later results indicate carcinogenic effects at still lower doses (3). The hazard of vinyl chloride primarily concerns workers in the plastic industries, who get a particularly high exposure in different connections, but this occupational health problem may have ramifications for the general public. Vinyl chloride has, for instance, been used as a propellant for hair sprays and pesticides. Moreover, the polymerized product of vinyl chloride, PVC plastic, is used all over the world for a variety of products, among other things for the wrapping of various foodstuffs. It seems inevitable that the population is exposed to the monomer via such sources, although the doses are most likely small.

The carcinogenic effects of vinyl chloride focus attention on the general and fundamental problem of how such hazards can be avoided or at least diminished in the future—and above all how carcinogenic chemicals such as vinyl chloride can be spotted experimentally in the first place. Considering the vast number of chemicals used in industries, it is hardly realistic to rely on cancer induction tests on mice with measurement of tumor frequency; such tests take at least two years. The only feasible procedure at present seems to be a system which makes use of the close correlation between the carcinogenic and mutagenic properties of chemicals (4). This correlation, as do other data, points to the fact that changes in DNA are at least an essential cause of cancer induction. The testing for mutagenicity is much cheaper and far less time consuming, and furthermore, the results can in many cases be interpreted in molecular terms with respect to the changes occurring in DNA. This obviously is of importance for a better understanding of the biological action of suspect compounds.

One major obstacle in using mutagenicity tests with microorganisms for a preliminary screening of carcinogenic effects is the fact that many compounds behave as carcinogens only after an activation in the mammalian body. This activation, or in other cases deactivation, of a foreign compound, is principally performed by the liver and involves oxidation, reduction, hydrolysis and conjugation. A wide variety of oxidative reactions *eg* C- and N-oxygenation, O-, N-, and S-dealkylation and epoxidation are mediated by the drug metabolizing system located in the membranes of the endoplasmatic reticulum of the liver cells (5, 6, 7, 8). This mixed-function oxygenase mechanism can utilize reduced nicotinamide adenine dinucleotide phosphate (NADPH) as an electron donor (9). These reactions also take place *in vitro* after the addition of NADPH-generating system to microsomal fractions (10, 11, 12). Although microorganisms are particularly suitable from a practical genetic point of view for mutagenicity testing, they do not perform the same metabolic activation as mammals and they will therefore not pick up such indirect carcinogenic compounds. In order to overcome that problem, combined test methods have been worked out with microorganisms and mammals. The microorganisms are used for the actual mutagenicity testing, but they are exposed to those metabolites of the compound which are formed in an intact mammal (host mediated assay) (13) or in mammalian liver microsomal systems *in vitro* (12, 14).

The present investigation of the genetic effect of vinyl chloride has been performed on *Salmonella typhimurium*. The mutagenicity of the compound was analyzed by means of reverse mutations in the histidine locus. The effect of metabolic activation was studied by adding rat liver microsomal systems to the bacterial cultures according to the method worked out by Ames (14).

The objective of the present investigation has primarily been to use vinyl chloride as an example of a compound carcinogenic to man, in order to study the applicability of mutagenicity testing for the detection of carcinogens in the human environment. We are likely to be confronted with this kind of problem at an accelerated rate in the future and the necessity of finding a sensible solution to the screening of carcinogenic hazards of chemicals is imminent. Considering the vinyl chloride problem, one could raise the following question: Had this genetic test method been available and been used for screening of vinyl chloride when it was introduced for industrial production, would such a screening have given a warning of the carcinogenicity of this compound?

MATERIALS AND METHODS

Four histidine-requiring strains of *Salmonella typhimurium* (TA 1535, TA 1536, TA 1537, and TA 1538) were used for this investigation. These strains have been described in detail by Ames *et al* (15). They are used in a back-mutation system, where reversion to histidine independence occurs either as a result of base-pair substitution (TA 1535) or base-pair addition or deletion (TA 1536—TA 1538). Cultures were grown overnight in com-

plete medium (Difco, Antibiotic medium 3). Platings with the soft agar technique were made on complete medium (CA plates) and on minimal medium (MA plates) described by Vogel and Bonner (16) with supplements according to Ames (17). The soft agar consisted of 0.9 percent NaCl and 0.6 percent agar.

Undiluted bacteria (0.1 ml) were added to soft agar (2 ml) and poured onto MA plates and 0.1 ml of appropriate dilutions correspondingly onto CA plates. Dilutions were made in 0.9 percent NaCl. Colonies on MA plates were counted after two days' incubation at 37° C, giving the number of mutants. The number of viable cells (surviving cells) were counted on CA plates after one day's incubation.

Male rats (Sprague-Dawley, Anticimex) maintained on normal diet were starved 16—20 hours before they were sacrificed by decapitation and the liver was homogenized. The homogenate was centrifuged at 9000×g for ten minutes. The supernatant containing the microsomes was mixed with a NADPH-generating system consisting of NADP, glucose-6-phosphate, phosphate buffer (pH 7.4), $MgCl_2$ and KCl in the proportions given by Ames *et al* (14). When microsomal systems are mentioned in the text below or in the tables it includes the 9000×g supernatant containing the microsomes and the NADPH-generating system. The negative control without NADP is therefore designated "microsomal systems −NADP". 2-aminoanthracene was used as a positive control. This compound induces mutations after microsomal activation in all the strains used (14).

The vinyl chloride used (AB Aerosol Packing Co, Vallentuna, Sweden) has been analyzed with gas chromatography and mass spectrometry for impurities by S Jensen (18). The purity was very high and only trace amounts of isopropanol were found. When nothing else is stated the procedure of Ames *et al* (14) was followed in the experiments.

Three different types of treatment with vinyl chloride were used in preliminary experiments with TA 1535. A water solution of vinyl chloride was prepared by bubbling vinyl chloride gas through water in a test tube for a couple of minutes. This solution was added to the soft agar together with the microsomal system and bacteria before plating. An attempt was also made to dissolve vinyl chloride directly in the microsomal suspension by bubbling it through for half a minute prior to the addition to soft agar. In the third procedure the bacteria were first plated together with the microsomal system and then treated 75 minutes in a vinyl chloride-containing atmosphere (11 percent v/v) at room temperature (23° C). This last procedure was adopted in the following main experiments and will therefore be described in some detail. After plating of the bacteria with and whithout microsomal systems the petri dishes were placed in a closed vessel (standard vacuum desiccator, volume 10 l) equipped with two teflon tubes for inlet and outlet of gas. The heavy vinyl chloride gas was pressed into the glass vessel via one of the tubes which ended near the bottom. This addition caused an expulsion through the other tube (at the top) of a corresponding volume of air, which could be measured. The atmospheric concentration of vinyl chloride measured in this way was during the main experiments 20 percent. When all vinyl chloride had been added, the tubes were closed and a magnetic stirrer was started to ensure homogeneous distribution of the gas. Three different times of exposure to the gas were used, 30, 60 and 90 minutes. Two experiments with TA 1535 were made in this way. An experiment with all four strains TA 1535—TA 1538 was finally performed with a treatment time of 90 minutes.

RESULTS

The results of the preliminary experiments with TA 1535 and liver microsomes is presented in Table 1. As can be seen, no mutagenic effect could be traced after the treatment with vinyl chloride dissolved in water or in the microsomal suspension. After an exposure to 11 percent vinyl chloride gas, on the other hand, there was a significant increase of mutants. It should be pointed out that the survival of the bacteria was not affected in any detectable way by the treatment and therefore the results cannot be due to a selective survival. The fact that only the treatment with vinyl chloride gas had any effect on the mutation rate indicates that a continuous treatment given directly to the bacteria with gaseous vinyl chloride brings about a higher exposure dose than a treatment with vinyl chloride previously dissolved in water or in the liver microsomal suspension.

In another set of experiments (Table 2 and Figure 1), the treatment was given with 20 percent gaseous vinyl chloride. The results were in good accordance with the previous ones, that is, a significant increase of mutants was obtained with liver microsomal systems. Without liver

Figure 1. TA 1535 (base substitution) treated in an atmosphere of air containing 20 percent (v/v) vinyl chloride (VC). Experiment I in Table 2.

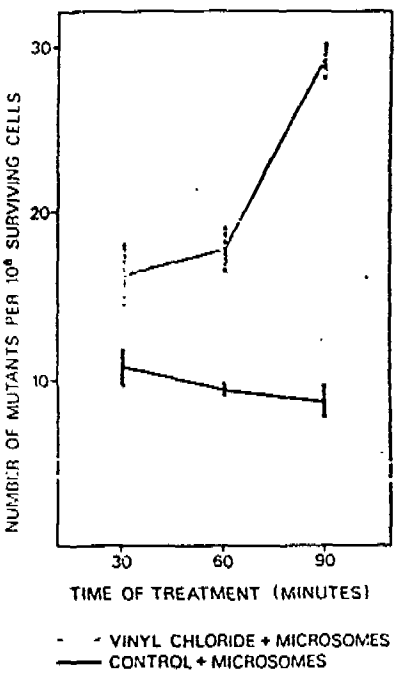

Table 1. The effect on TA 1535 (base substitution) after different types of treatment with vinyl chloride (VC) at the presence of liver microsomes. Series 1–2 and 3–5 respectively were performed simultaneously. Statistical analyses with t-test. Significance levels: * $0.01<p<0.05$; ** $0.001<p<0.01$; *** $p<0.001$.

Series	Type of treatment	Number of viable cells per plate $\times 10^{-8}$ Mean value of 2 plates	Number of mutants per plate Mean value of 5 plates	Number of mutants per 10^8 surviving cells ±S E
1	VC dissolved in water		19.2	
2	Control		18.0	
3	VC dissolved in microsomal suspension	4.1	27.2	6.6±0.4
4	11% VC in atmosphere	4.5	78.2	17.6±2.6**
5	Control	4.3	23.0	5.3±0.2

Table 2. TA 1535 (base substitution) treated in an atmosphere of air containing 20 percent (v/v) gaseous vinyl chloride (VC). The effect measured as number of mutants per 10^8 surviving cells. Statistical analyses, see Table 1.

	EXPERIMENT I			EXPERIMENT II			
Type of treatment	Number of viable cells per plate $\times 10^{-8}$ Mean value of 3 plates.	Number of mutants. Mean value of 5 plates	Number of mutants per 10^{-8} surviving cells ± S E	Number of viable cells per plate $\times 10^{-8}$ Mean value of 3 plates.	Number of mutants. Mean value of 5 plates	Number of mutants per 10^8 surviving cells ± S E	Time of treatment (minutes)
VC+microsomal system	4.2	69.2	16.3±2.0	3.8	49.8	13.2±1.0**	30
VC	3.1	19.2	6.2±0.7	2.0	10.6	5.4±0.9	
Control+microsomal system	4.1	45.0	10.9±1.1	3.7	25.0	6.7±0.9	
VC+microsomal system	4.1	72.2	17.8±1.3***	3.9	51.6	13.4±1.0***	60
VC	3.4	18.4	5.4±0.8	2.3	18.8	8.1±0.6	
Control+microsomal system	4.1	39.4	9.6±0.4	3.9	20.4	5.2±0.7	
VC+microsomal system	4.0	115.8	29.0±1.1***	4.2	77.2	18.4±0.9***[1]	90
VC	3.7	22.8	7.1±1.0	3.6	22.6	6.2±0.9	
Control+microsomal system	4.1	37.4	9.0±1.0	3.9	—[1]	—	

[1] The number of mutants from "Control+microsomal system" in this series had to be excluded because of infection. However, there is a good agreement between the control series within both experiments I and II, and therefore the comparison of the series with 90 minutes treatment has been made versus the pooled controls for 30 and 60 minutes of experiment II.

microsomes, vinyl chloride did not exhibit any mutagenic effect. Concerning the survival of the cells, as in the previous experiment there was no sign of a toxic effect by vinyl chloride together with liver microsomal systems. It may be pointed out, however, that the data indicate some toxic effect on the bacteria by vinyl chloride itself without liver microsomes.

In order to elucidate the activation of vinyl chloride by the liver microsomes, a series without NADP was included in this experiment. The results, presented separately in Table 3 together with the relevant data from Table 2, show that a mutagenic effect was only obtained with the liver microsomal system and NADP, but not without NADP. It should be mentioned that because of lack of space in the container used for the treatment, the series measuring the survival of the bacteria had to be omitted for the treatment with the liver microsomal system without NADP. However, on the basis of the survival rate measured in the other experimental series, it can be concluded that the number of mutants per plate should give a sufficiently accurate estimation of the mutation rate. In particular, the similarity of mutation frequencies between the series with liver microsomal systems without NADP as compared to the series with only vinyl chloride is indicative for the interpretation of the mechanism of activation of vinyl chloride. As mentioned above, this lack of mutagenic effect without NADP indicates that in the test system used, an active metabolism by the microsomes is required for the mutagenic effect.

An experiment with all four strains TA 1535—TA 1538 was finally performed, in order to throw some light on the molecular mechanism behind the mutagenic effect of vinyl chloride. The results are shown in Table 4. As in the previous experiments a mutagenic effect was obtained with TA 1535, responding to base pair substitutions, but no increase of mutations was caused in TA 1536—TA 1538, responding to frame shift mutations.

DISCUSSION

From the experiments reported above, it can be concluded that vinyl chloride induces point mutations, but only after a metabolic activation. It can furthermore be established that vinyl chloride causes base pair substitution, but apparently no insertion or deletion of base pairs in DNA. The latter kind of effect is hardly to be expected with a compound like vinyl chloride.

It is evident that vinyl chloride resembles many other carcinogens in the respect that it acts as a mutagen via a metabolite, which seems to be formed in the liver. It is reasonable to assume that the carcinogenic action is also dependent on the same metabolite.

Alkenes, cycloalkenes as well as a variety of aromatic compounds are known to be metabolized, *eg* in the rat liver, via intermediate epoxides, formed in the NADPH-dependent oxygenation by microsomes (5, 19). Furthermore, trichloroethylene is metabolized in the rat to trichloroacetic acid and trichloroethanol, which compounds are assumed to be formed via an intramolecular rearrangement to trichloroacetaldehyde of a primary metabolite, trichloroethylene oxide (5). The most plausible primary metabolite from vinyl chloride (I) in the present system hence would be chloroethylene oxide (II). This compound has been synthesized by several methods from ethylene oxide (20, 21) and is described as a liquid, bp 65—67° C (21), which is rapidly hydrolysed by water and even at room temperature slowly rearranges to chloroacetaldehyde (III).

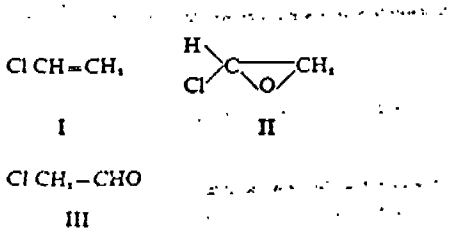

Table 3. TA 1535 (base substitution) treated in an atmosphere of air containing 20 percent (v/v) gaseous vinyl chloride (VC). Number of mutants per plate, mean value of 5 plates ± S E

Type of treatment	Time of treatment		
	30 minutes	60 minutes	90 minutes
Experiment I			
VC+microsomal system	69.2±8.6	72.2±5.3	115.8±4.2
VC+microsomal system—NADP	20.6±3.5	18.6±0.7	30.8±1.3
VC	19.2±2.1	18.4±2.6	22.8±3.1
Control+microsomal system	45.0±4.7	39.4±1.7	37.4±4.3
Experiment II			
VC+microsomal system	49.8±3.9	51.6±3.9	77.2±3.6
VC+microsomal system—NADP	12.4±1.9	16.4±0.6	20.2±2.2
VC	10.6±1.8	18.8±1.5	22.6±3.4
Control+microsomal system	25.0±3.5	20.4±2.7	—

Chloroethylene oxide can be expected to react as a bifunctional alkylating agent. Moreover, it belongs to the group of highly reactive α-chlorinated aliphatic ethers, among which, *eg*, chloromethyl methyl ether (CMME) and bis(chloromethyl) ether are recognized as strong carcinogens (22).

Mutagenicity tests of the metabolites discussed are under way.

The fact that vinyl chloride needs a metabolic activation before it acquires a mutagenic property focuses attention on the necessity of using a test system which takes into consideration and as far as possible mimics the metabolism performed in the mammalian body. The liver microsomal system with *Salmonella* is a very useful tool in this respect. It should, however, be pointed out that the way of distributing the compound is essential. False negative results can easily be brought about by a low water solubility and a high volatility as in the present case with vinyl chloride. The mutagenic property of this compound would have been undetected if only water solutions had been tested.

There is an increasing wealth of data which indicate that potential carcinogens can at least be pointed out by means of mutagenicity tests (4, 14) and the present findings with vinyl chloride are in good accordance with such a view. The question posed above, whether the carcinogenic effect of vinyl chloride could have been indicated earlier by means of mutagenicity tests, can therefore be answered with "yes". The actual sequence of events with this compound was, however, the reverse—the carcinogenic effect was discovered before any mutagenicity tests had been performed. This is clearly unsatisfactory. If a realistic testing procedure for chemicals with respect to mutagenic and carcinogenic effects is to be built up, it seems that cooperation between governmental authorities, scientists and industry is essential.

Table 4. Strains TA 1535 (base substitution) and TA 1536–1538 (frame shift) treated for 90 minutes in an atmosphere of air containing 20 percent (v/v) gaseous vinyl chloride (VC). Statistical analyses, see Table 1.

Type of treatment	Strain	Number of viable cells per plate $\times 10^{-8}$ Mean value of 3 plates	Number of mutants per plate Mean value of 5 plates	Number of mutants per 10^8 surviving cells. ± S E
VC+microsomal system	TA 1535	4.1	41.2	10.1±0.4***
	TA 1536	1.2	0.8	0.7±0.3
	TA 1537	1.7	13.6	7.9±1.1
	TA 1538	3.7	20.4	5.5±0.6
Control+microsomal system	TA 1535	4.3	15.2	3.5±0.7
	TA 1536	1.1	0.8	0.8±0.4
	TA 1537	1.9	14.4	7.7±0.9
	TA 1538	4.0	22.2	5.6±0.1

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