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MUTAGENICITY OF WASTE PRODUCTS FROM VINYL CHLORIDE INDUSTRIES

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The by-product from vinyl chloride production, EDC-tar, is a complex mixture of mainly short-chained chlorinated aliphatic hydrocarbons. This mixture has been tested for mutagenicity by means of Ames' Salmonella/mammalian microsome method.

Since most of the components in the tar are poorly soluble in water, three agents were used as solvents or emulsifier: ethanol, DMSO, and Tween 80. The results with all these agents showed that EDC-tar contains direct as well as indirect mutagenic constituents. It could be concluded that the mutagenic effect observed in the test could not be due to any significant extent to one of the main components, ethylene dichloride (1,2-dichloroethane). This substance showed a weak mutagenic effect, but only at higher concentrations than could be available in the highest concentration tested of the tar. Although the microsomal system enhanced the mutagenicity both of the EDC-tar and of 1,2-dichloroethane, this enhancement was dependent on NADPH in the case of EDC-tar but independent of NADPH with 1,2-dichloroethane.

The Salmonella/mammalian microsome method seems to be a suitable tool for both mutagenicity screening of complex chemical mixtures and identification of mutagenic constituents in such mixtures.

INTRODUCTION

The carcinogenic effects of vinyl chloride on humans and on laboratory animals have recently been reviewed (Haley, 1975). The mutagenic effect of this substance is also well documented (Rannug et al., 1974, 1976; Bartsch et al., 1975; Malaveille et al., 1975; Loprieno et al., 1976) and has been reviewed by Bartsch and Montesano (1975). These harmful properties

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together with its enormous annual production worldwide has made vinyl chloride a serious environmental problem. The situation has been most obvious at the level of occupational health, since the predominant risk has been exposures in vinyl chloride and PVC plants.

The circumstances mentioned above therefore make the manufacturing processes of vinyl chloride of interest. Since the final product is mutagenic as well as carcinogenic, it is of importance to know whether other products formed in the processes could have the same properties.

When vinyl chloride is synthesized either from acetylene or ethylene or from a mixture of these substances, a tarlike by-product is formed, called EDC-tar. This name for the by-product originates from one of the main components, ethylene dichloride or 1,2-dichloroethane (Jensen et al., 1970, 1975).

EDC-tar is, however, a complex mixture, mainly consisting of chlorinated, aliphatic hydrocarbons; a detailed chemical analysis of the constituents has recently been done (Jensen et al., 1975).

Another factor that renders EDC-tar important from an environmental point of view is the fact that until recently it has been dumped in large quantities in the North Sea (Jensen et al., 1970).

Reports dealing with toxicity, accumulation, and excretion of EDC-tar among marine invertebrates and fishes have been published (Jernelöv et al., 1972; Braaten et al., 1972; Rosenberg et al., 1975; Jensen et al., 1975). Toxic effects on the bacterium *Escherichia coli* have also been studied (Hagström and Normark, 1974). So far, however, no mutagenicity testing of EDC-tar has been reported. The mutagenicity test adopted in the present study is the *Salmonella*/mammalian microsome method (Ames et al., 1973, 1975).

This method has proved to be a very useful tool in mutagenicity testing and in carcinogenicity screening. A recent study of 300 carcinogens and noncarcinogens of a wide variety of chemical types has revealed a high correlation between carcinogenicity and mutagenicity. It was shown that 90% of the carcinogens were mutagenic in the test (McCann et al., 1975a; McCann and Ames, 1976).

The screening of chemicals for mutagenicity and carcinogenicity has mostly been performed with defined chemicals. When dealing with chemicals in the evironment, however, one often has to consider complex mixtures rather than identified substances. The *Salmonella* microsomal test system has been used successfully for detection of the mutagenic activity of cigarette smoke condensates (Kier et al., 1974). One of the purposes of the present study was to use EDC-tar as a model for the procedure of mutagenicity screening of complex industrial waste products. EDC-tar is suitable in this respect because it exhibits some of the characteristics and problems often encountered in this connection—it is highly complex, with many constituents poorly soluble in water. Because of the latter problem, a comparative investigation was performed with different solvents.

In order to identify separate components responsible for a mutagenic effect of such complex mixtures, the results with the whole mixture should be followed up by successive testing of fractions of the complex down to single compounds. This is being done at this laboratory in cooperation with S. Jensen in the division of analytical chemistry. The aim of investigation of EDC-tar at this stage, however, has been to gain some experience in using the *Salmonella*/mammalian microsome test for screening complex mixtures rather than identifying separate mutagenic constituents that may occur. As one of the main components is 1,2-dichloroethane (approximately 35%) this compound has been studied in some detail separately. This chemical is in itself a very important industrial product also used as an additive in gasoline and has shown weak mutagenic effect in other investigations (Ehrenberg et al., 1974; Brem et al., 1974; McCann et al., 1975b).

MATERIAL AND METHODS

Test Compounds

The EDC-tar used was obtained from Kema Nord, Stenungsund, Sweden. The content of vinyl chloride monomer in the tar was approximately 0.06% according to GC-analysis carried out by the division of analytical chemistry at Wallenberg Laboratory. Ethylene dichloride (1,2-dichloroethane) was obtained from BDH Chemicals Ltd. Methylmethane sulfonate (MMS) was from Ega-Chemie KG and 2-aminoanthracene from Aldrich.

Solvents

Ethanol, 95%; dimethylsulfoxide (DMSO), spectrophotometric grade (Merck); and polyoxyethylene sorbitan mono-oleate (Tween 80) (Hopkin & Williams) were used for EDC-tar, but only DMSO was used for 1,2-dichloroethane.

Bacterial Strain and Metabolizing System

Salmonella typhimurium strain TA 1535 has been used earlier by our group (Rannug et al., 1974, 1976) and was originally kindly provided by B. Ames. For a detailed description of the strain, see Ames et al. (1973, 1975).

The 9,000 x *g* liver fraction was prepared from male rats (strain R bred at our laboratory) maintained on normal diet. The animals were starved 16–20 hr before they were sacrificed. The livers were washed and minced in 0.15 *M* KCl and then homogenized in three volumes of 0.15 *M* KCl (3 ml/g wet liver). No drug was used to induce liver enzymes for these experiments. The microsomal system consisted of three-tenths of the 9,000 x *g* supernatant and seven-tenths of an NADPH generating system

[NADP, glucose 6-phosphate, $MgCl_2$, KCl, and phosphate buffer (pH 7.4)]. In the series without NADP a corresponding volume of phosphate buffer was used (see Rannug et al., 1974).

Test Procedure

EDC-tar was added to ethanol, DMSO, or Tween 80 to give the concentrations indicated in figures and tables per 0.1 ml. In the case of Tween 80 the tar was emulsified in 10% Tween 80 (v/v in 0.9% saline). It should be mentioned, however, that with ethanol the highest concentration of the tar did not dissolve completely. Portions (0.1 ml) of the solutions or emulsions were added together with 0.5 ml of the microsomal system or phosphate buffer and 0.1 ml of bacteria to 2 ml soft agar (0.6% agar in 0.9% NaCl) and poured onto the plates. The bacterial suspension was either an undiluted overnight culture in complete medium (Antibiotic medium 3) or an appropriate dilution in 0.9% NaCl of the same culture. In the first case the soft agar was poured onto minimal medium (Vogel and Bonner, 1956) with supplements according to Ames (1971). Five plates were used per concentration and control. When a diluted bacterial suspension was added, three plates with complete medium were used instead. The latter plates give the number of surviving cells and the plates with minimal medium the number of mutants after 24 and 48 hr incubation, respectively, in the dark at 37°C.

The numbers given in figures and tables are thus mean values of three plates for survival measures and five plates for mutations.

For positive controls MMS was used in experiments without a microsomal system and 2-aminoanthracene was used when the metabolizing system was included.

RESULTS

The toxic and mutagenic effects of EDC-tar on *S. typhimurium* TA 1535 are summarized in Table 1. Tested directly without a microsomal system, EDC-tar exhibits both a toxic and a mutagenic effect in all three solvents. With ethanol and DMSO survival is reduced to about 40% with the highest concentration of EDC-tar tested (900 μg per plate). The number of mutants per plate rises to about five times the control value. When the tar is emulsified in 10% Tween 80 it does not cause the same reduction in survival. Only 30% of the bacteria were killed with the highest concentration in this case. The corresponding increase in number of mutants per plate was more than tenfold.

When EDC-tar is tested in the presence of the microsomal system the results qualitatively resemble those obtained without a metabolizing system; that is, a toxic as well as a mutagenic effect is produced. Also, in this series the toxic effect with Tween 80 differs from that with the other two solvents. Under these conditions, however, a stronger toxic action

TABLE 1. Effect of EDC-Tar in Different Solvents on *Salmonella typhimurium* TA1535 (Base pair Substitution) with and without Microsomal System

Solvent	Dose (μg/plate)	Without microsomal system		With microsomal system	
		Survival (%)	No. of mutants per plate ± SE	Survival (%)	No. of mutants per plate ± SE
Ethanol	0	100	10.4 ± 1.33	100	15.0 ± 1.41
	100	85	16.2 ± 1.98	104	29.6 ± 2.36
	300	95	27.0 ± 3.22	101	76.4 ± 2.50
	600	69	46.8 ± 2.08	77	124.2 ± 4.48
	900	42	55.4 ± 4.65	46	112.4 ± 14.98
DMSO	0	100	12.6 ± 1.63	100	17.2 ± 1.69
	100	94	19.2 ± 2.89	98	31.6 ± 3.31
	300	80	38.6 ± 1.69	95	78.0 ± 6.46
	600	58	52.0 ± 4.83	69	119.6 ± 5.21
	900	40	63.8 ± 1.65	52	137.0 ± 3.81
Tween 80	0	100	7.4 ± 0.93	95	15.2 ± 1.02
	100	94	14.6 ± 1.08	89	90.2 ± 6.22
	300	87	29.6 ± 1.86	74	112.4 ± 4.38
	600	79	53.0 ± 2.47	53	116.6 ± 6.38
	900	69	83.2 ± 3.76	20	116.8 ± 4.84

occurs with Tween 80. Only 20% of the bacteria survive 900 μg EDC-tar with Tween 80, while ethanol and DMSO both give approximately 50% survival. The effect of the liver microsomes on the survival with EDC-tar is not the same with all the solvents. With ethanol and DMSO the addition of liver microsomes seems to decrease the toxic effect by the tar, while the opposite is true with Tween 80.

From Table 1 it can be seen that the mutagenic effect of EDC-tar is greatly enhanced by the microsomal system. This holds true for all three solvents. The lowest concentration of EDC-tar tested (100 μg per plate) differs in all cases significantly from the corresponding controls ($p < 0.01$). The enhancement of the mutagenic effect of EDC-tar caused by the microsomal system is also illustrated in Fig. 1, where the effect of EDC-tar in a normal metabolizing system with all necessary cofactors is compared with the corresponding effects in a microsomal system where NADP has been omitted. EDC-tar causes little or no killing in the incomplete metabolizing system, but if the system is made complete by adding NADP the situation changes. In the latter case the number of surviving cells decreases strongly with increasing EDC-tar concentration, resulting in approximately 30% survival for the highest concentration in the experiment shown in Fig. 1. Furthermore it is evident that the NADPH-dependent functions in the metabolizing system are necessary for the high mutagenic effect. In this

particular experiment the maximum number of mutants, within the concentration range, was approximately 200 per plate.

The main component, 1,2-dichloroethane, has been tested in the concentration range 5–45 μmol per plate (Fig. 2). This substance, however, does not show the same pattern as the entire EDC-tar in this mutagenicity test. Irrespective of whether 1,2-dichloroethane is tested in the presence of a complete or an incomplete microsomal system—that is, with or without NADP or in a system where the liver fraction has been replaced by a KCl solution—the survival curves largely have the same slope (Fig. 2). The highest concentration, 45 μmol per plate, reduces the survival approximately 30% compared with the corresponding control. To attain a high mutagenic effect, on the other hand, the presence of the liver fraction is necessary, although the NADP addition has no influence on

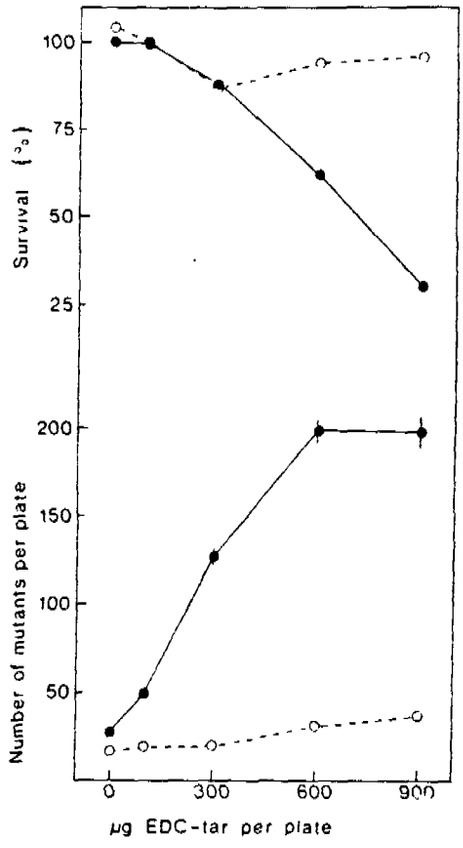

FIGURE 1 Toxic and mutagenic effects of EDC tar on *Salmonella typhimurium* TA 1535 (base-pair substitution) in the presence of a microsomal system with (●) or without (○) NADP. The solvent used in this experiment was DMSO.

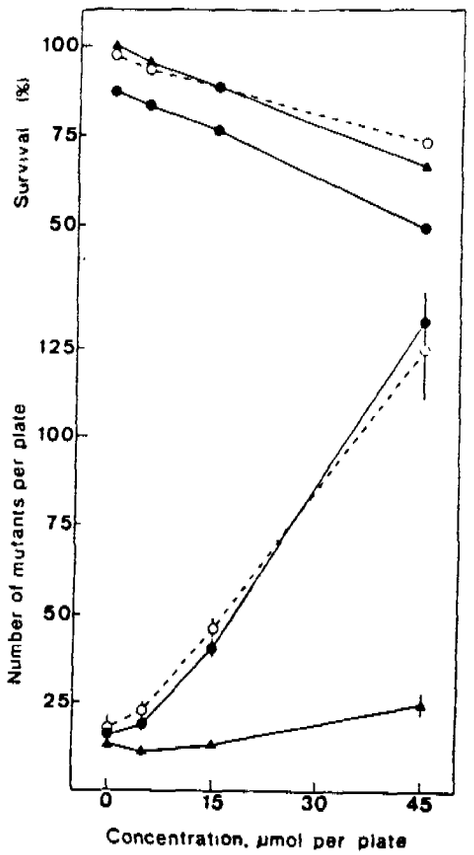

FIGURE 2. Toxic and mutagenic effects of 1,2-dichloroethane (ethylene dichloride) on *Salmonella typhimurium* TA 1535 (base-pair substitution) in the presence of a microsomal system with (●) or without (○) NADP or in a system with NADP but without the 9,000 x *g* liver fraction (▲).

the mutagenicity. Under these circumstances the highest concentration resulted in approximately 125 mutants per plate. In the absence of liver fraction the corresponding number was 25 mutants per plate.

DISCUSSION

The by-product from vinyl chloride production, EDC-tar, is a complex mixture of mainly short-chained chlorinated aliphatic hydrocarbons (Jensen et al., 1970). From the present results it is evident that this mixture includes direct as well as indirect mutagenic constituents. Since most of the components are poorly soluble in water, three agents were used as solvents or emulsifier: ethanol, DMSO, and Tween 80. Qualitatively

no difference was found between the solvents used; that is, the mutagenic effect was apparent in all series. Quantitatively, however, some differences in both survival and mutagenicity, were obtained. These differences between the solvents can have many causes, for instance different effects on the permeability of the bacterial cell wall or various interactions with the microsomal system (see Rannug et al., 1975).

Concerning the effects of the individual solvents on the mutagenicity of EDC-tar, it is evident from the results both with and without liver microsomes that ethanol and DMSO are quite similar. Tween 80, on the other hand, deviates from the other two agents. This is particularly true with the microsomal system present. Ethanol and DMSO give an expected dose-effect relation, while with Tween 80 the mutagenicity varies only slightly (between 90 and 117 mutants per plate) from the lowest to the highest dose of the tar, indicating that a plateau has been reached around the lowest dose. This plateau may occur because Tween 80 causes more severe damage to the metabolizing system, resulting in stronger inhibition, at least with the concentrations used in these experiments (Rannug et al., 1975). Tween 80 deviates from the other solvents in another respect also. The effect of the lowest dose with liver microsomes is clearly higher than with ethanol or DMSO. Apparently Tween 80 affects the availability of the test compound to the action of the microsomal enzyme system. It should, however, be emphasized that independently of the solvents used, an induction of mutations could be detected.

When it comes to interpreting the mutagenic constituents it is clear that one or several directly acting mutagens are present. The enhancement of mutagenicity with liver microsomes furthermore points to the fact that one or several constituents are converted into mutagens by the liver microsomal fraction. This biotransformation can occur either through the microsomal enzymes of the mixed function oxygenase category or by other enzymatic or nonenzymatic processes. Biotransformation through the mixed function oxygenase enzymes can be inferred from the fact that it is NADPH-dependent. To analyze the process through which chemicals are activated into mutagens by the liver microsomal system, tests should therefore be performed both with and without NADP in the NADPH-generating system. The usefulness of this NADP control was evident in the present investigation. The enhancement in mutagenicity found with total EDC-tar was shown to be NADPH-dependent (Fig. 1). The corresponding enhancement of the mutagenicity of 1,2-dichloroethane by the microsomal system, on the other hand, turned out to be independent of NADPH. Further conclusions can also be drawn from these results. Although 1,2-dichloroethane is one of the main components of EDC-tar, constituting approximately 35% of the total (Jensen et al., 1975), its contribution to the mutagenicity of the tar must be rather limited. Approximately 3 μmol of 1,2-dichloroethane is present in the highest concentration of EDC-tar,

but the lowest concentration of 1,2-dichloroethane tested, 5 mmol per plate, is not significantly different from the corresponding control value under any of the conditions shown in Fig. 2. From the data presented in Table 1 and Fig. 1 it is evident that the mutagenic effect of EDC-tar without NADP is as low as, or even lower than, the corresponding value without a microsomal system. This is a further indication that the contribution of 1,2-dichloroethane to the mutagenicity of total EDC-tar is negligible. The behavior of 1,2-dichloroethane in the mutagenicity test will, however, be further discussed in another context (Rannug and Ramel, in preparation).

Of other compounds that could be responsible for the mutagenicity of EDC-tar, vinyl chloride itself should also be considered. As mentioned above, EDC-tar contains about 0.06% of the vinyl chloride monomer. Now, according to Bartsch et al. (1975) the lowest dose of vinyl chloride tested in *Salmonella* was 0.2% in air, which corresponded to 4×10^{-5} *M* in the substrate. This is a ten times higher concentration of vinyl chloride than in our experiment with the highest dose of EDC-tar, and judging from the data presented by Bartsch et al. vinyl chloride in this concentration can not be expected to give a measurable increase of mutations. It can therefore be concluded that the contribution of vinyl chloride to the mutagenicity of EDC-tar is negligible.

The mutagenic effects discussed in this paper were detected in the concentration range 100–900 μg per plate. In terms of parts per 10^6 (ppm) it would equal 40–300 in the top agar layer or 4–40 if calculated on the whole agar medium content of a plate. Concentrations of EDC-tar at which other biological effects arise have been reported by several authors. Jernelöv et al. (1972) found an increased frequency of c-mitotic cells in *Allium cepa* at concentrations from 1–50 ppm. Acute toxicity in some marine animals gave LC_{50} values ranging from 2–20 ppm (see Jensen et al., 1975). Effects on ^{14}C fixation of phytoplankton were found at even lower concentrations. Rosenberg et al. (1975) have shown, however, that there can be differences in acute toxicity between Swedish and Norwegian EDC-tar. In their experiments the Swedish tar was approximately nine times more toxic (48 hr LC_{50}) to shrimps (*Crangon crangon*). They also point out that the composition of EDC-tar varies not only from factory to factory but also from time to time from the same factory. These circumstances together with different ways of exposing the test organisms to the EDC-tar—that is, momentary or successive exposures—give rise to discrepant results concerning LC_{50} as well as other biological parameters.

Considering the cause of the toxicity of EDC-tar, Hagstrom and Normark (1974) conclude from their work on *E. coli* that the killing effect is associated with a decreased stability of the cytoplasmic membrane, and they therefore suggest an interaction with the membrane. These findings seem to be in good agreement with the strong tendency of EDC-tar to adhere to particles noted by Jernelöv et al. (1972).

Another conclusion drawn by Hagstrom and Normark (1974) as well as Rosenberg et al. (1975) is that neither of the two main components 1,2-dichloroethane or 1,1,2-trichloroethane could be responsible for the biological effects caused by EDC-tar. This is in good agreement with the results reported here, which show that 1,2-dichloroethane gives a different mutagenicity pattern when tested as a pure chemical than does total EDC-tar. The appearance of both 1,2-dichloroethane and 1,1,2-trichloroethane in the mutagenicity test with *Salmonella* and microsomal system is, however, different from that of EDC-tar (Rannug and Ramel, in preparation).

In conclusion, it should be emphasized that the *Salmonella*/mammalian microsome method seems to be a suitable tool for both mutagenicity screening of complex chemical mixtures and identification of mutagenic constituents in such mixtures. It may be pointed out that the limited detoxification action in the indicator organism, *Salmonella*, is an advantage in this connection. It makes it possible to manipulate experimentally with the liver microsomal system in order to elucidate the process of metabolic conversion of the chemicals to mutagens.

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